The effects of diet-induced obesity on metabolic and vascular functions: role of insulin signalling and insulin resistance

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Sameer Hasan M. Fatani

July 2007

Declaration

I declare that the work presented in this thesis is all my own and has not been previously submitted for any other degree.

Sameer Fatani

Acknowledgements

I would like to dedicate the whole of this work and thesis to my great mother and honest wife "Amal Bafageeh", the two persons whom encouraged me by all means to finish this work successfully.

Also, I would like to express my great thanks to Dr. Ebrahim K. Nederali for his unique supervision, support, and encouragement throughout this work. And great thanks as well to Professor Paul Trayhurn for his supervision and facilitating the laboratories and equipments.

Special thanks to Dr. Lucy C Pickavance, Mr. Leif Hunter, and Dr. Wafea Jumaa for their valuable help, technically and practically. Thanks as well to all friends and colleagues in the School of Clinical Sciences, Obesity Biology Unit.

Abstract

The effects of diet-induced obesity on metabolic and vascular functions: role of insulin signalling and insulin resistance

Sameer H. Fatani

High energy diets and diet-induced obesity are known to induce detrimental effects on various physiological functions. However, insulin resistance is postulated to play a central role in mediating and linking these detrimental effects with related clinical symptoms which include cardiovascular disorders. Therefore, the present study aimed to investigate the relationship between insulin resistance and "different negative effects and pathological signs" in diet-induced obese rats.

Long term feeding of a highly-palatable diet induced obesity, with concomitant alterations in metabolic, vascular, and molecular functions, namely insulin resistance and attenuated endothelial-dependent vasorelaxation. At the molecular level, several abnormalities were detected in protein expression levels of both PI 3-kinase and MAP-kinase insulin signalling pathways in vascular, hepatic and skeletal muscular tissues. In all tissues the greater changes were in the level of PI 3-kinase. These defects in PI 3-kinase levels may play a crucial role in inducing local and systemic insulin resistance, and subsequent pathological symptoms.

Consumption of all types of high energy diets in the present study (such as biscuit, chocolate or high-fat diets) induced vascular dysfunctions mainly the attenuation of endothelium-dependent vasorelaxation. Moreover, such diets did not induce obesity or metabolic changes, but were associated with a changed lipid profile, in particular hypertriglyceridaemia, suggesting a role for triglyceride in dietary-induced vascular abnormality.

Chronic withdrawal of the highly-palatable diet reversed body weight, and was associated with normalisation of metabolic, vascular, and molecular abnormalities. Although, fenofibrate treatment caused a remarkable reduction in body weight and metabolic improvement, it had very limited beneficial effects on vascular function.

In conclusion, highly-palatable diet-induced obesity is associated with metabolic and vascular related clinical symptoms. Molecular defects in insulin signalling pathways could participate in the induction of such pathological signs. Principally, PI 3-kinase seems to be the key controlling step of insulin transduction, having a major role in inducing local insulin resistance and the subsequent relevant clinical symptoms. Moreover, the augmented levels of circulating and tissue accumulation of triglyceride in diet-induced obese animals could have a central role in mediating the local and systemic insulin resistance. Long term consumption of a high calorie diet irrespective of developing obesity, appears to be the major causative factor in inducing vascular defects leading to cardiovascular disease. Physiological but not pharmacological management of obesity appears to be more effective in correcting vascular abnormalities associated with dietary obesity.

Abbreviations

ADP	Adenosine-Di-Phosphate
AMP	Adenosine-5'-Monophosphate
ATP	Adenosine-Tri Phosphate
BCA	Bicinconinic Acid
BMI	Body Mass Index
C	Chow-fed group
CCh	Carbamylcholine
CCRC	Cumulative Concentration Response Curve
CNS	Central Nervous System
CRP	C-Reactive Protein
DC	Diet-to-Chow group
EC_{50}	Effective Concentration$_{50}$
ELISA	Enzyme-Linked ImmunoSorbent Assay
ET-1	Endothelin-1
eNOS	Endothelial Nitric Oxide Synthase
ERk1/2	Extracellular Regulated Kinase 1/2
F	Fenofibrate
FBF	Forearm Blood Flow
FFA	Free Fatty Acid
G6P DH	Glucose-6-Phosphate Dehydrogenase
GRB-2	Growth Factor Receptor-protein Bound-2
GTP	Gaunosine-Tri-Phosphate
HK	Hexokinase
HOMA	Homeostasis Model Assessment
HPD	Highly-Palatable Diet
HRP	Horse-reddish Peroxidase
IGT	Impaired Glucose Tolerance
IL-6	Interlukin-6
IR-α	Insulin Receptor-α
IR-β	Insulin Receptor-β
IRS-1	Insulin Receptor-Substrate-1
IRS- 2	Insulin Receptor-Substrate-2
KPSS	Potassium Physiological Salt Solution
LCD	Low Calorie Diet
LDL	Low-Density Lipoprotein
MAP	Mitogen Activated-Protein
NA	Nor-Adrenaline
NAFLD	Non Alcoholic Fatty Liver Disease
NBF	Neutral Buffered Formalin
NEFA	Non-Esterified Fatty Acid
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
PAI-1	plasminogen activator inhibitor-1
PBS	Phosphate Buffered saline
PI 3-kinase	Phosphatidylinositol 3-kinase
PKC-β	Protein Kinase C-β
POD	Peroxidase
PPAR-α	Peroxisome Proliferator Activated Receptor-α
PSS	Physiological Salt Solution
RIA	Radio-Immuno Assay
RER	Rough Endoplasmic Reticulum
ROS	Reactive Oxygen Species

SEM	Standard Error of Mean
SDS-PAGE	Soduim-Dedocyl-Sulfate Poly-Acrylamide Gel Electrophoresis
SNP	Soduim Nitroprusside
TBW	Total Body Water
TG	Triglyceride
TBHB	2,4,6-tr-bromo-3-hydroxy-benzoic acid
TNF-α	Tumour-Necrosis Factor-α
TPS	Tris Phosphate Saline
UV	Ultra-Violet
VLCD	Very low Calorie Diet
V	Vehicle group
VSMC	Vascular Smooth Muscle Cells
V / V	Volume / Volume
W / V	weight / Volume
WAT	White Adipose Tissue
WHO	World Health Organization
ZDF	Zucker Diabetic Fatty rats

Table of contents

List of figures

List of tables

Chapter 1

Introduction

1.1 INTRODUCTION

Obesity is a disease of the life style that was largely neglected. In 1997, the world health organization (WHO), issued a statement indicating that "obesity's impact is so diverse and extreme that it should now be regarded as one of greatest neglected public health problems of our time (WHO, 1997). In fact, overweight and obesity is forecast to increase by 50% by 2015 according to a new report from the WHO. Obesity is a worldwide epidemic (James *et al.*, 2001) that is characterized by excess adipose tissue contributing to numerous chronic diseases (Must *et al.*, 1999) and early mortality (Simopoulous and Van Itallie, 1984; Kushner, 1993). This epidemic has received both national and international attention because of its increasing prevalence, its detrimental impact on health, and subsequent enormous economic burden it imposes (Wolf and Colditz, 1998).

Obesity is a chronic (NHLBI, 1998; WHO, 1998) condition that develops as a result of an interaction between a person's social, behavioral, cultural, psychological, metabolic, and genetic factors (Stunkard *et al.*, 1986; WHO, 1998). It causes or exacerbates many health problems both independently and in association with other diseases. In particular, it is associated with the development of coronary heart disease (CHD), non-insulin-dependent diabetes (NIDD), some forms of cancers (Mark *et al.*, 1999), non-alcoholic fatty liver disease (NAFLD), in addition to many other chronic diseases. In 1998, the American Heart Association issued a "call to action" and reclassified obesity as a major modifiable risk factor for CHD (Eckel and Krauss, 1998). Increased intra-abdominal fat, or waist circumference, as a consequence of obesity is related to a constellation of risk factors, the so-called insulin resistance syndrome. Therefore, breakthroughs in our understanding of the molecular mechanisms of insulin resistance and endothelial dysfunction in vasculature may provide potential opportunities for therapeutic intervention and bring renewed hope and vitality to the management of obesity and its complications.

1.2 OBESITY

1.2.1 Definition and measurement

Obesity, defined as excess adiposity for a given body size, results from an imbalance between energy intake and energy expenditure (Kuczmarski *et al.*, 1997). It is assumed that both a predisposition to excess energy intake and a reduced resting metabolic rate contribute to obesity (Ravussin *et al.*, 1988; Roberts and Leibel, 1998). The concept of energy balance is easy to understand. A typical obese patient will have increased body weight by some 20 kg over 10 years. This means a daily energy excess of 30-40 kcal per day at the start of the development of obesity, which gradually has to be increased to maintain the enlarged body mass. The positive energy balance needed for obesity to develop over the years is so small that the patient does not notice it (Bjorntorp, 1997).

Weight gain occurs differently in men and women. In women, the greatest weight gain is in the younger age groups, while, men generally develops obesity at later in life. Furthermore, as women's educational level raises, their obesity decreases, whereas in men, educational level appears not to be related to obesity (Krauss *et al.*, 1998).

The most commonly used method today for classifying an individual as overweight or obese is based on body mass index (BMI), a value that is measured as body weight in kilograms over the square of height in meters (kg/m^2). In adults, BMI between 20 and 25 is considered normal, individuals with a BMI between 25 and 30 are considered overweight and those with a BMI over 30 are obese (Kuczmarski *et al.*, 1997; NHLBI, 1998), regardless of sex. (Table 1.1).

Table1.1 World health organization's Body Mass Index categories based on increasing health risks.

BMI (kg/m^2)	WHO classification	popular description
< 18.5	Underweight	Thin
18.5-24.9	Normal	Healthy normal "acceptable"
25.0-29.9	Grade 1 overweight	Overweight
30.0-39.9	Grade 2 overweight	Obesity
≥ 40.0	Grade 3 overweight	Morbid obesity

The use of BMI to assess weight-related health risk has gained international acceptance because of the associations between BMI and adiposity (Gallagher *et al.*, 2000), BMI and disease risk (James *et al.*, 2001), and BMI and mortality (Calle *et al.*, 1999). BMI values provide valuable information about increasing body fatness. It allows meaningful comparisons of weight status within and between populations and the identification of individuals and groups at risk of morbidity. It also permits identification of priorities of intervention at an individual or community level and for evaluating the effectiveness of such interventions (WHO, 1995; WHO, 1997).

Waist circumference is another clinically feasible measurement that may be used independently or in addition to BMI (Janssen *et al.*, 2002) to assess weight-related health risk. Although, BMI and waist circumference are the recommended (USDHHS, 1998) and most clinically feasible means of identifying patients who are overweight or obese in clinical practice, there are several other methods used in clinical practice to assess body adiposity such as: (1) Skinfold test; a simpler test for measuring body fat, in which a pinch of skin is precisely measured by calipers at standardized points on the body to determine the thickness of the subcutaneous fat layer, and converted to body fat percentage by an equation; (2) Bioelectrical impedance analysis (BIA). is

a commonly used process for estimating body composition. It has come into favor in recent years due to its role in determining an estimate of total body fat. BIA actually determines the electrical impedance of body tissues, providing an estimate of total body water (TBW). TBW in turn, can be used to estimate fat-free body mass and subsequent body fat (WHO, 1995; WHO, 1997).

1.2.2 Prevalence and epidemiology

Obesity should no longer be regarded simply as a cosmic problem affecting certain individuals, but as epidemic that threatens global well being. It is affecting an estimated 300 million people worldwide (WHO, 2002). Its prevalence is increasing in both developed and developing countries throughout the world. In the United States, the prevalence of obesity is greater than it has ever been, with striking increases observed during the past 2 decades (Flegal *et al.*, 1998). Approximately, 64.5 % of adult population in the United States is estimated to be overweight in the period 1999-2000 as compared with 46 % in 1976 to 1980. Most of this increase is attributable to a dramatic rise in the prevalence of obesity from 15% to the recent estimate of nearly 31%.(Flegal, 2002). Obesity is pervasive, affecting people of all ages and at all socioeconomic level. Moreover (Mokdad *et al.*, 1999), in addition to age and sex, race and ethnicity are important factors in the prevalence of obesity (Williamson, 1993; Lovejoy *et al.*, 1996; USDHHS, 2000). The prevalence of obesity increased from 12% in 1991 to 18% in 1998. Increases were seen in both sexes and all socioeconomic classes, with the greatest increase seen in 18-29 year olds (Mokdad *et al.*, 1999). Conservative estimates of the economic costs of obesity in developed countries are between 2 and 7 % of the total health costs, which represents a significant expenditure of national health-care budgets (Seidell, 1996).

Increasing age is associated with an increase in obesity. Body weights of men and women in the United states increase approximately 9.1 kg between the ages of 25 and 55 years (Belanger *et al.*, 1988; Wang *et al.*, 1994). These increases in body weight generally are not explained by increases in fat-free mass, because bone mass peaks around 30 years of age and muscle mass

plateaus and later declines unless the individual engages in the strengthening activities. These changes in body weight and body composition are attributable, in part, to the natural declines in growth hormone, dehydroepandrosterone, and testosterone with aging. In addition, reductions in resting metabolism alter energy balance and contribute to weight gain.

In Europe, on average, 15% of men and 22% of women are considered to be obese, with overweight also being more common among women than men. More than half the adult population between 35 and 65 years of age were either overweight or obese. In England and Wales, the most recent health survey has confirmed an increase in the prevalence of obesity in adults from 6% in men and 8% in women in 1980 to 23% of men and 25% of women in 2002 (Prescott-Clarke and Primatesta, 1998; Rennie and Jebb, 2005). Furthermore, the increasing prevalence of obesity is not confined merely to Europe and America. In Southeast Asia a marked rise is being seen in all populations, and in Japan and China a pronounced increase in the prevalence of overweight and obesity has been observed during the past two decades. The most striking figures come from the Pacific region. In urban Samoa the prevalence of obesity is estimated as greater than 75% of adult women and 60% of adult men (Hodge *et al.*, 1995). High prevalence rates also occur in the Middle East, in the United Arab Emirates is recognized as a major public-health problem that maybe an important factor in the increasing occurrence of other chronic diseases (Musaiger, 1996).

1.2.3 Etiology of obesity

Overweight and obesity result from the interaction of many factors, including genetic, behavioural, environmental, physiological, social and cultural influences that result in energy imbalance and promote excessive fat deposition. The relative contribution of each of these factors has been studied extensively, and although genes play an important role in the regulation of body weight, the WHO consultation on obesity (WHO, 1998) conducted that behavioral and environmental factors (i.e., sedentary lifestyles combined with excess energy intake) are primarily responsible for the dramatic increase in obesity during the past two decades. Moreover, these factors may interact

with genetic susceptibility to produce obesity (Bouchard and Tremblay, 1990). Increasing energy consumption, decreasing energy expenditure, or a combination of both leads to a positive energy balance and consequently increasing body mass of the individuals promoting induction of obesity.

1.2.4 Factors influencing obesity

1.2.4.1 Genetic susceptibility

Although, obesity runs in families, the influence of genotype on the etiology of obesity may be attenuated or exacerbated by nongenetic factors. Apart from rare obesity-associated syndromes, the genetic influences seem to operate through susceptible genes. Such genes increase the risk of developing obese characteristics but are not essential for their expressions or, by themselves, sufficient to explain the development of the disease. Moreover, it is estimated that approximately 40% of the variance in daily energy expenditure (excluding vigorous physical activity) is attributable to genotype (Bouchard and Tremblay, 1990). Thus, there is substantial evidence implicating the role of genetics in body weight regulation. These suggest that differences in genetic susceptibility within population determine those who are most likely to become obese in any given set of environmental circumstances (Bouchard *et al.*, 1990). In other words; implicit to the susceptible-gene hypothesis is the role of environmental factors that unmask latent tendencies to develop obesity.

1.2.4.2 Environmental factors

Energy expenditure. The most variable component of energy expenditure is physical activity, representing 20-50% of total energy expenditure (Prentice *et al.*, 1996). In developed countries there is a relationship between low level of physical activity and obesity (Rissanen *et al.*, 1991). Longitudinal studies of Pima Indians indicate that the risk of 10-kg weight gain during a 4-year follow-up is a seven-fold higher in those in the lowest tertile of relative resting metabolic rate, compared with those in the highest tertile (Ravussin *et al.*, 1988).

Energy intake. It seems that environmental influences act through increasing energy intake and / or decreasing energy expenditure. There is some

evidence that high-fat diets are associated with an increased risk of obesity within populations, but cross-cultural studies have failed to show any consistent relationship between nutritional factors and relative weights (Blundell and Macdiarmid, 1997).

Culture. Evidence for the critical role of environmental factors in the development of obesity comes from the migrant studies and the 'westernisation' of diet and lifestyles in developing countries. Study of Nigerians living in the United States, found that mean BMI for men and women in Nigeria is 21.7 and 22.6, respectively, while those livings in United States have an average BMI of 27.1 for men and 30.8 for women. Moreover, increasing prevalence of obesity is associated with adverse health consequences such as hypertension (adult Nigerian living in Africa is 15%, whereas it is as high as 30% among those living in United States) (Wilks, 1996).

In industrialised countries, a higher prevalence of overweight and obesity is observed in those with lower educational attainments and low income, although the reverse may be seen in developing countries. There is tendency for overweight to increase after marriage and with increasing parity. Hence, a balanced dietary intake and physical activity are crucial in increasingly affluent societies (Kuczmarski *et al.*, 1994).

1.2.5 Complications of obesity

Even small increases in weight across a population can have devastating impact on public health. Close to 300,000 deaths each year in the United States may be attributable to obesity (Allison *et al.*, 1999). Excess weight increases the risk of multiple pathological conditions, including cardiovascular disease, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), hypertension, certain forms of cancer, cerebrovascular disease, gallstones, osteoarthritis, dyslipidemia, sleep apnea, degenerative arthritis (Mark *et al.*, 1999), asthma (USDHHS, 2001), cataracts (Weintraub *et al.*, 2002), benign prostatic hypertrophy (Giovannucci *et al.*, 1994), menstrual irregularities, and pregnancy complications resulting in reduced life expectancy. Moreover,

obesity may lead to depression and social discrimination (USDHHS, 2001), having negative effects on physical functioning, vitality (Coakley *et al.*, 1998), and general quality of life (Fine *et al.*, 1999). The adverse health consequences occur not only in individuals who are in the overweight and obese categories, but disease risk also starts to increase even for those at the upper end of the normal range of BMI (22.0-24.9) (Flegal *et al.*, 1998).

Increasing body fat mass is accompanied by profound changes in physiological function. These changes are, to a certain extent, dependent on the regional distribution of adipose tissue. Generalized obesity results in alterations in total blood volume and cardiac function, whereas the distribution of fat around the thoracic cage and abdomen restricts respiratory excursion and alters respiratory function. The intra-abdominal visceral deposition of adipose tissue, which characterises upper body obesity, is a major contributor to the development of hypertension, elevated plasma insulin concentrations and insulin resistance, diabetes mellitus and hyperlipidaemia.

Obesity is an important determinant of cardiovascular disease. Moreover, there is strong link between obesity and a generalised metabolic disorder of which insulin resistance is an indicator. It is difficult to define the precise contribution of obesity to insulin resistance, but most analyses suggest that it can account for ≥ 50% of the variance in insulin sensitivity in the general population. Insulin resistance is associated with a constellation of metabolic abnormalities, including obesity, diabetes, dyslipoproteinaemia, hypertension, and atherosclerosis. The complex nature of insulin resistance, and it's relation to atherogenesis is not fully understood yet. Therefore further studies are needed to explore whether insulin resistance can promote atherosclerosis independently of other risk factors.

1.2.6 Treatment of obesity

The goals of obesity treatment are to achieve and then to maintain clinically meaningful weight loss, with ultimate goal of reducing the risk for or severity of obesity-related diseases, impairments, and functional limitations. Weight losses of 5% to 10% of initial body weight produce health benefits and are deemed by many health care practitioners to represent a clinical success. Long-term success, however, is dependent on maintenance of a 10% weight loss for at least 1 year (Wing and Hill, 2001). Wing and Hill estimated that approximately 21% of adults who are overweight or obese are successful at 1 year, but longer-term success generally is lower (Wing and Hill, 2001). Effective therapeutic regimens for treating obesity should incorporate multiple approaches to encourage behavioural change or modification and creative strategies to facilitate consistent and long term follow-through. Numerous options are available today, (Rosenbaum *et al.*, 1997; NAASO, 2000) including low-energy diets, physical activity/exercise, behavioural modification, (Wadden and Foster, 2000), pharmacotherapy, (Klein, 1999; Bray and Tartaglia, 2000) and surgery (NIH, 1991). The treatment choice depends on the degree of obesity, the presence of co-morbidities, previous weight loss therapies utilised and the relative success of each, and specific characteristics of an individual's personal life.

1.2.6.1 Behavior and lifestyle modifications:

Behavior modification is an important component of all weight loss programmers (Wadden and Foster, 2000). Behavior strategies frequently are targeted toward identifying stimuli that signal unhealthy behaviours (e.g., binge eating), learning about the role of readiness, in initiating or continuing positive behaviours (Prochaska and Velicer, 1997) and recognising barriers that may compromise healthy pursuits. Most of the various lifestyle approaches have several factors in common, which includes: the use of self-monitoring and goal setting; stimulus control; modification of eating style habits; the use of reinforcement for healthy behaviours; nutrition education; moderate physical activity; and cognitive restructuring, including stress management, relaxing skills, meditation, and relapse-prevention training. In

general, these approaches produce moderate weight loss and have minimal side effects. They are most useful for individuals with mild obesity (BMI of 27 to 30). Individuals who follow such approaches to weight loss maintain on average about two thirds of their initial weight loss 12 months after treatment termination (Krauss *et al.*, 1998). Although no single theoretical framework for behavioural intervention has been shown to be superior, success in modifying patterns of eating and physical activity generally is dependent on consistency, support, and long-term modification of lifestyle, rather that on one specific diet or exercise program (Racette *et al.*, 2003).

1.2.6.2 Diet:

Dietary approaches form the basis of most weight loss interventions and rely on a reduction in total energy intake. Although many diets focus on dietary fat reductions, the main determinant of weight loss is the total energy content of the diet (Kennedy *et al.*, 2001) (relative to total energy expenditure), rather than the composition of macronutrients. Therefore, whether diet therapy is based on a high complex-carbohydrate, low-fat meal plan, as advocated by the American Heart Association (Krauss *et al.*, 2000) and many nutrition professionals, (NAASO, 2000; St Jeor *et al.*, 2001) or relies primarily on carbohydrate restriction, its success is dependent on a relative energy deficit. Weight-reducing diets may be very-low-calorie diets (VLCDs, 800 kcal/d) or low-calorie-diets (LCDs, 800/1500 kcal/d) and may consist of liquid formulas, pre-packaged meals, nutritional bars, regular foods, or a combination. Very-low-calorie diets are very effective for weight reduction, (Saris, 2001) and the nutritionally adequate formulas used today have fewer associated health problems (e.g., gout, cholelithiasis, hair loss) as compared with the VLCD formulas used in the 1970s.

Despite rapid and clinically meaningful weight loss with VLCDs, however, maintenance of the reduced weight is variable and generally poor. Instead, LCDs are used more commonly because they are safer, have fewer side effects, enable better adherence, and therefore may result in comparable weight loss as VLCD regimens (Rossner and Flaten, 1997). Diets containing more than 1,200 kcal/day produce slower weight losses, but they are

advantageous because they can be incorporated more easily into individual lifestyles and generally can be followed for longer periods of time without adverse health effects (Wing, 2001).

1.2.6.3 Exercise and physical activity:

Exercise generally does not produce considerable weight loss when used independently, but is a very important adjunct to a weight-reducing diet (Jakicic *et al.*, 1999) because it increases energy expenditure, enhances loss of adipose tissue, (Racette *et al.*, 1995) and improves dietary adherence. (Racette *et al.*, 1995). Although aerobic exercise has been used most frequently for weight loss and control because of the caloric expenditure required, strength training has numerous benefits and may help to preserve fat-free mass during diet-induced weight loss (Geliebter *et al.*, 1997). In addition to formal exercise, daily physical activity plays critical role in energy balance, (Saltzman and Roberts, 1995) weight control, disease prevention, and achievement and maintenance of overall health. Prospective studies indicate that physical activity protects against obesity, (DiPietro *et al.*, 1998) specifically influencing abdominal obesity, (Tremblay *et al.*, 1990) and reduces the incidence of several diseases associated with obesity (such as cardiovascular disease, (Leon *et al.*, 1997; Blair *et al.*, 2001) type 2 diabetes (Eriksson and Lindgarde, 1991; Helmrich *et al.*, 1991), depression, (Hughes, 1984; Taylor *et al.*, 1985) and premature death (Leon *et al.*, 1997; Lee and Paffenbarger, 2000). Furthermore, exercise adherence and habitual physical activity *are the* greatest determinants of weight maintenance following weight loss (Andersen *et al.*, 1999; Jakicic *et al.*, 1999). The effects of physical activity on fitness and health are dramatic (Blair *et al.*, 2001) and there is evidence that physically active individuals who are obese have a lower risk for morbidity and mortality than sedentary individuals of normal weight. (Bray, 2000).

1.2.6.4 Pharmacotherapy:

Pharmacological agents may be used in conjunction with diet, exercise, and behavioural strategies when non-pharmacological approaches alone fail to produce or sustain meaningful weight loss. Several appetite suppressant

drugs are approved for weight loss, (Bray, 2000; Yanovski and Yanovski, 2002) but the treatment duration for most is limited to twelve weeks or less. The chronic nature of obesity necessities longer-term therapy, because drug cessation usually leads to weight regain. Two drugs approved for the long-term treatment of obesity include:

Orlistat (Xenical): is indicated with meals (120 mg) and acts to reduce the absorption of fatty acids by inhibition of triglyceride hydrolysis through its action as a gastric and pancreatic inhibitor (Hill *et al.*, 1999; Finer *et al.*, 2000). Orlistat proved more effective than diet alone for weight loss, with improvements in total cholesterol, low density lipoprotein and the low density lipoprotein to high density lipoprotein ratio and glycemic control. Orlistat represents an overall safe treatment for obesity, given that the drug is minimally absorbed. Side effects of Orlistat include malabsorption of fat-soluble vitamins and steatorrhea.

Sibutramine (meridia): acts on CNS and exerts its effects by acting as a norephinephrine, serotonin, and dopamine reuptake inhibitor (Luque and Rey, 1999). Sibutramine treatment is indicated for weight loss, and applied in combination with reduced-calorie diet. Daily doses of 10 mg and 15 mg sibutramine lead to sustained weight loss over a period of about six months. Sibutramine is contra-indicated in patients with poorly controlled hypertension and patients with a history of cardiovascular heart disease (weber, 1999).

It is worth noting that there are many other compounds and hormones in clinical trials such as: Axokine, β3-adrenergic agonists, cannabinoid receptor antagonists, and leptin.

1.2.6.5 Surgery:

Surgery is reserved for cases of extreme obesity (BMI of ≥ 40 kg/m^2) or for more moderate obesity (BMI of ≥ 35 kg/m^2) when obesity-related co-morbidities are present. The most commonly surgical procedure is the gastric bypass, in which the upper portion of the stomach is stapled to create a small (10-30 ml) reservoir (Deitel and Shikora, 2002). The net result is a substantial weight loss within 6 months. Weight losses of 45 kg (100 lb) (Pories *et al.*,

1995), or 60 to 70% of excess body weight (Sugerman *et al.*, 1987), have been observed 1 year after gastric bypass, and large losses have been maintained for up to 15 years (Pories *et al.*, 1995). Vertical banded gastroplasty is another, less commonly used surgical procedure in which a band constricts the upper portion of the stomach, effectively reducing its capacity. In contrast to the surgical procedures used in the past decades, the current techniques have low mortality rates of approximately 1.3% (Melinek *et al.*, 2002) to 1.5% (Pories *et al.*, 1995). However, morbidity associated with wound infections, incisional hernia, and anastomotic leak with peritonitis may be higher, particularly when additional surgery is necessary. Additional risks of surgical treatment for obesity include steatorrhea, vitamin and mineral deficiencies, and osteoporosis (Sugerman, 2000).

1.3 INSULIN AND INSULIN RESISTANCE

With obesity and diabetes epidemic proportions in the developed world (WHO, 2000), the role of insulin resistance and its sequelae is gaining prominence. Understanding the roles of insulin across a wide range of physiological process and the influences on its synthesis and secretion, alongside its actions from the molecular to the whole body level, has significant implications for much chronic disease seen in westernised populations today. Therefore, the study of insulin and insulin resistance remain in the forefront of medical research, relevant at all levels from bench to bedside to public health policy.

1.3.1 The discovery of Insulin

In 1889 two Germans, Minkowski and Mering noted, from their experimental work with animals, that total pancreatectomy led to the development of severe diabetes (Bliss, 1993). They hypothesized that a substance secreted by the pancreas was responsible for the metabolic control. In 1921, Banting and McLeod, administered chilled saline extracts of pancreas intravenously to dogs rendered diabetic by pancreatectomy and observed lowering of blood glucose. Insulin was finally isolated, purified and made available in a form capable of therapeutic administration, which lead to the award of Nobel Prize, in 1923 to Banting and McLeod.

1.3.2 Definition

Insulin is a peptide hormone secreted by the β-cells of the pancreatic islets of Langerhans and maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism, promoting cell division and growth via mitogenic effects.

1.3.3 Structure of Insulin

In 1928 polypeptide nature of insulin was defined and its amino acid sequence was identified in 1952. It is in fact a dipeptide, containing A and B chains respectively, linked by disulphide bridges, and containing 51 amino acids, with a molecular weight of 5802 (Home, 1997). The A chain comprises 21 amino acids and the B chain 30 amino acids (Dodson and Steiner, 1998).

1.3.4 Synthesis and release of Insulin

Insulin is synthesized in the β-cells of the pancreatic islets of Langerhans as its precursor, proinsulin. Proinsulin is synthesized in the ribosomes of the rough endoplasmic reticulum (RER) from the mRNA as pre-proinsulin. Pre-proinsulin is formed by sequential synthesis of signal peptide, the B chain, the connecting (C-) peptide and then the A chain comprising a single chain of 100 amino acids. Removal of the signal peptide forms proinsulin, which acquires its characteristic three dimensional structure in the endoplasmic reticulum. Secretory vesicles transfer proinsulin from the RER to the Golgi apparatus, whose aqueous zinc-containing and calcium rich environment favours formation of soluble zinc-containing proinsulin hexamers (Dodson and Steiner, 1998). As immature storage vesicles form from Golgi, enzymes acting outside the Golgi convert proinsulin to insulin and C-peptide (Malaisse, 1997). Insulin forms zinc-containing hexamers which are insoluble, precipitating as chemically crystals at pH 5.5. When mature granules secreted into circulation by exocytosis, insulin and an equimolar ratio of C-peptide are released. Proinsulin and zinc typically comprise no more than 6% of the islet cell secretion (Dodson and Steiner, 1998).

1.3.5 Mechanism of action of insulin secretion

Increased levels of glucose induce the "first phase" of the glucose-mediated insulin secretion by release of insulin from secretory granules in the β-cell. Glucose entry into the β-cell is sensed by glucokinase, which phosphorylates glucose to glucose-6-phosphate (G6P), generating ATP (De Lonlay and Saudubray, 2000). Closure of K+/-ATP-dependent channels results in membrane depolarization and activation of voltage dependent calcium channels leading to an increase in intracellular calcium concentrations; this triggers pulsatile insulin secretion (Soria *et al.*, 2004). Other mediators of insulin release include activation of phospholipases and protein kinase A, (e.g., by acetylcholine) and by stimulation of adenylyl cyclase activity and activation of β-cell protein kinase A (Bratanova-Tochkova *et al.*, 2002).

1.3.6 Sites of action of insulin

1.3.6.1 Vasculature

In addition to conventional responses on skeletal muscle and adipose tissue, insulin also exerts important biological effects on the vasculature (Baron and Brechtel, 1993; Baron, 1994).

Insulin-stimulated vasodilation. Insulin-stimulated vasodilation has been attributed to several mechanisms, including interaction with the sympathetic nervous system at the vascular level, the activation of ion channels (Hasdai *et al.*, 1998), the release of adenosine (McKay and Hester, 1996) and increase in the generation of NO by the vascular endothelium (Scherrer *et al.*, 1994; Steinberg *et al.*, 1994). Studies in isolated vessels and cultured endothelial cells have improved our understanding of the molecular mechanisms by which insulin stimulates relaxation of isolated vascular strip preparation (Laight *et al.*, 1998), and blunts the response to a number of vaosconstructing reagents (Wambach and Liu, 1992). Therefore variant insulin signalling proteins may contribute to the genetic predisposition to develop endothelial dysfunction and cardiovascular disease. Insulin-stimulated PI 3-kinase activity results in activation of PKB (protein kinase B)/AKt, which has been demonstrated to mediate many metabolic and vascular effects of insulin

(Whiteman *et al.*, 2002). In cultured endothelial cells, stimulation of AKt (PKB) by insulin has been proposed to phosphorylate eNOS at specific residue, Ser^{1177} (human sequence), increasing its activity several folds (Michell *et al.*, 1999; Kim *et al.*, 2001). Majority of studies have demonstrate that insulin stimulate NO production in endothelial cells. In a number of insulin-resistant states, the ability of supraphysiological concentrations of insulin to stimulate blood flow is attenuated (Yki-Jarvinen and Utriainen, 1998), indicating that direct effects of insulin on NO production is of importance in the vascular endothelium (Yki-Jarvinen, 2003).

Vasoconstrictive actions of insulin. It has also been suggested that insulin has vasoconstrictor action, thought to be principally mediated by Endothelin-1 (ET-1) (Webb, 1997). Insulin stimulate ET-1 production in endothelial cells both *in vivo and in vitro,* and this is thought to influence both normal blood flow and changes in blood pressure in patients with hyperinsulinaemia and insulin resistance (Piatti *et al.*, 2000). During eNOS inhibition and hyperglycemia, insulin stimulates vasoconstriction (Schroeder *et al.*, 1999), mediated by ET-1 (Eringa *et al.*, 2002). NO-depending vasodilation is attenuated in insulin-resistant subjects, whereas acute stimulation of ET-1 release is unaffected (Ferri *et al.*, 1995). Insulin also activates MAPK (mitogen-activated protein kinase) which is thought to be a key step in vasoconstriction and ET-1 activation in skeletal muscle arterioles, an effect to antagonize PI 3-kinase-depending vasodilation (Eringa *et al.*, 2004).

Other vascular effects of insulin. Studies in cultured endothelial cells, have shown that, insulin up-regulates eNOS expression (Aljada *et al.*, 2000; Ding *et al.*, 2000) and inhibits the expression of the pro-atherogenic transcription factor NF_kB (nuclear factor $_kB$).

1.3.6.2 Muscle

Glucose uptake into muscle is essentially insulin dependent via GLUT 4, and muscle accounts for about 60-70% of whole-body insulin mediated uptake (Smith, 2002). In fed state, insulin promotes glycogen synthesis via activation of glycogen synthase, enabling energy release *via* glycolysis (e.g. during

intense muscular activity). Muscle cells do not rely on glucose (or glycogen) for energy during basal state, when insulin levels are low. Insulin suppresses protein catabolism while insulin deficiency promotes release of amino acids for gluconeogenesis (Giorgino *et al.*, 2005). In insulin resistance, muscle glycogen synthesis is impaired; this appears mediated by reduced intracellular glucose translocation (Hunter and Garvey, 1998).

1.3.6.3 Liver

While glucose uptake into the liver is not insulin dependent, it accounts for about 30 % of whole body insulin-mediated glucose disposal (Smith, 2002). In liver glycogen synthesis is stimulated and protein synthesis and lipoprotein metabolism are modulated (Denton and Tavare, 1997). Gluconeogenesis and ketone body production are inhibited (Hunter and Garvey, 1998). Alterations of lipoprotein metabolism represent a major manifestation of insulin resistance. Increased free fatty acid delivery, and reduced VLDL catabolism by insulin resistant adipocytes, results in increased hepatic triglyceride content and VLDL secretion (Krauss, 2004). Moreover, synthesis of CRP in hepatocytes is induced in response to adipocyte-derived pro-inflammatory cytokines such as IL-6 and TNF-α (Devaraj *et al.*, 2004).

1.3.7 Physiological role of Insulin

Insulin is the pivotal hormone regulating cellular energy supply and macronutrient balance, thus directing anabolic processes of the fed state (Burks and White, 2001). Insulin is essential for the intracellular transport of glucose into insulin-dependent organs such as muscle and adipose tissue. Signalling abundance of exogenous energy, adipose tissue breakdown is suppressed and its synthesis promoted. In muscle cells, glucose entry promotes glycogen synthesis and storage, and for carbohydrates, rather than fatty acids, (or amino acids) to be utilized as the immediately available energy source for muscle contraction. Insulin therefore, promotes glycogen and lipid synthesis in muscle cells, while suppressing lipolysis and gluconeogenesis from muscle amino acids. In the presence of an adequate supply of amino acids, insulin is anabolic in muscle (Karam, 1997). In vascular tissue, insulin has several specific physiological roles which includes induction of

vasodilation, *via* insulin signalling pathway "PI 3-kinase" in which activated Akt enzyme phosphorylate and activate eNOS enzyme leading to increased production of NO which act as a vasodilator (Zeng and Quon, 1996; Fleming and Busse, 2003).

1.3.8 Insulin signalling

Insulin mediates its actions through binding to insulin receptor (Fig. 1.1). The insulin receptor was first described in 1971. It consists of a heterotetramer consisting of 2 α and 2 β glycoprotein subunits linked by disulphide bonds and is located on the cell membrane (Kido *et al.*, 2001). Insulin binds to the extracellular α subunit, resulting in conformational change enabling binding of ATP to intracellular component of the β subunit (Wolever, 1990). ATP binding in turn, triggers phosphorylation of β subunit conferring tyrosine kinase activity. This enables tyrosine phosphorylation of intracellular substrate proteins known as insulin receptor substrates (Rosenbaum *et al.*, 1997; Kido *et al.*, 2001). There are four known specifically-named IRS proteins: IRS 1 and 2 have widely overlapping tissue distribution (Withers and White, 2000). Based on results from specific knockout models, the most important proteins in the regulation of carbohydrate metabolism appear to be IRS-1 and -2 (White, 2002). In humans, rare mutations of the IRS-1 protein are associated with insulin resistance (Whitehead *et al.*, 1998) and disruption of the IRS-1 gene in mice results in insulin resistance mainly of muscle and fat (Yamauchi *et al.*, 1996). Interestingly, knockout mice not only show insulin resistance of muscle, fat and liver, but also develop and manifest diabetes as a result of β-cell failure (Previs *et al.*, 2000). Therefore, it is tempting to speculate that dysfunction of IRS-2 and its downstream targets might represent a common feature of both peripheral insulin resistance and β-cell failure. Furthermore, circulating free fatty acids and the adipokine TNF-α increase serine phosphorylation of IRS proteins, thereby causing impaired insulin signal transduction (White, 2002). In addition, prolonged stimulation with insulin as commonly found in diabetic patients with hyperinsulinaemia may result in regulated degradation of IRS proteins (Rui *et al.*, 2001).

Down-stream of IRS proteins, the phosphatidyl-inositol-3-kinase (PI 3-kinase) is a central mediator of the effect of insulin. PI 3-kinase isoforms have been subdivided into three classes Ia, Ib, and II. Class Ia PI 3-kinases are thought to be the major effector of insulin signaling and activation of protein kinase B (PKB) also called (AKt) (Alessi and Cohen, 1998). Tyrosine phosphorylated IRS-1 or IRS-2 then binds to src-homology 2 (SH2) domains of intracellular proteins, including the p85 regulatory subunit of phosphatidyl-inositol (PI) 3-kinase. The interactions of IRS and p85 subunit of PI 3-kinase results in the activation of p110 catalytic subunit of PI 3-kinase. Activation of PI 3-kinase increases serine phosphorylation of Akt which in turn, will mediate the effects of insulin on glucose transport, glycogen synthesis, protein synthesis, lipogenesis, and suppression of hepatic gluconeogenesis. AKt regulates both, glucose uptake via facilitated glucose transporters (GLUTs) and intracellular glucose metabolism in insulin sensitive tissue such as skeletal muscle (Fig. 1.1) (Alessi and Cohen, 1998).

As is the case for other growth factors, insulin stimulates the mitogen-activated protein (MAP) kinase extracellular signal regulated kinase (ERK) (Fig. 1.1). This pathway involves the tyrosine phosphorylation of IRS proteins and/or Shc, which in turn interact with the adaptor protein Grb2, recruiting the Son-of-sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. The activation of Ras also requires stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2 (Fig. 1.1). Once activated, Ras operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Ras, MEK, and ERK. Activated ERK can translocate into the nucleus, where it catalyses the phosphorylation of transcription factors such as P62, initiating a transcriptional programme that leads to cellular proliferation or differentiation (Boulton *et al.*, 1991). Blockade of the pathway with dominant negative mutants or pharmacological inhibitors prevents the stimulation of cell growth by insulin, but no effect on the metabolic actions of the hormone (Lazar *et al.*, 1995).

In vascular tissue, in addition to the above insulin siganlling pathways and metabolic effects, the activated AKt " by phosphorylated PI 3-kinase" will directly phosphorylates eNOS on serine 1177 and activates the enzyme, leading to increased NO production and thus providing vascular protection (normal vasodilation) (Fig. 1.1) (Zeng and Quon, 1996; Fleming and Busse, 2003).

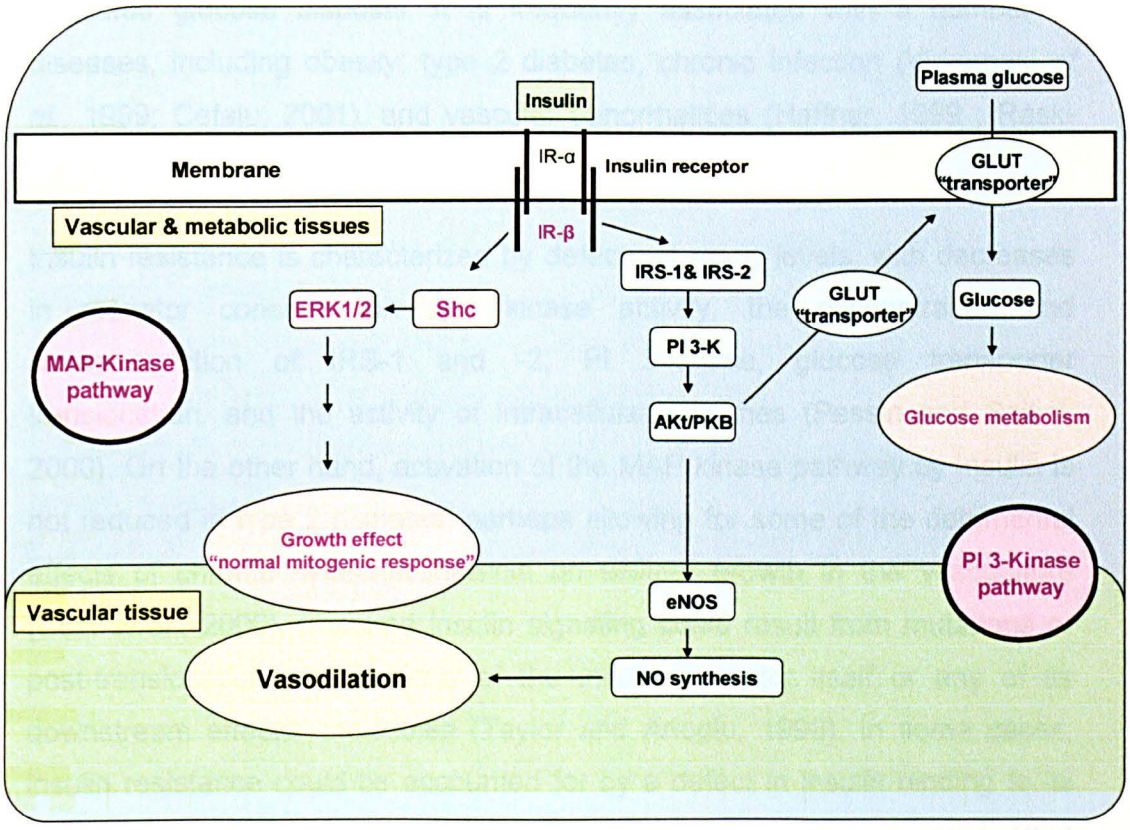


Figure 1.1 Insulin signalling pathways in vascular and metabolic tissues

1.3.9 Insulin resistance

Insulin resistance is a common pathologic state in which targets cells fail to respond to ordinary levels of circulating insulin, thus producing an attenuated biological response. Classically this refers to impaired sensitivity to insulin mediated glucose disposal. It is frequently associated with a number of diseases, including obesity; type 2 diabetes, chronic infection (Virkamaki *et al.*, 1999; Cefalu, 2001), and vascular abnormalities (Haffner, 1999 ; Rask-Madsen and King, 2005).

Insulin resistance is characterized by defects at many levels, with decreases in receptor concentration and kinase activity, the concentration and phosphorylation of IRS-1 and -2, PI 3-kinase, glucose transporter translocation, and the activity of intracellular enzymes (Pessin and Saltiel, 2000). On the other hand, activation of the MAP kinase pathway by insulin is not reduced in type 2 diabetes, perhaps allowing for some of the detrimental effects of chronic hyperinsulinaemia on cellular growth in the vasculature (Cusi *et al.*, 2000). Impaired insulin signaling could result from mutations or post-translocation modifications of the insulin receptor itself or any of its downstream effector molecules (Taylor and Arioglu, 1998). In some cases, insulin resistance could be accounted for by a defect in insulin binding to its receptor (Roach *et al.*, 1994); however, insulin resistance is most often attributed to post binding defect in insulin action. A marked reduction in the receptor kinase activity was observed in several patients (type A) with extreme resistance to insulin, but with normal binding (Grunberger *et al.*, 1984; Grigorescu *et al.*, 1986). Similarly, severe defects in receptor kinase activity are associated with naturally occurring mutations of the insulin receptor gene (Taylor *et al.*, 1992), indicating that genetic and acquired factors can profoundly influence insulin sensitivity. Nonetheless, genetic defects in the insulin receptor are relatively rare, but represent the most severe forms of insulin resistance and are exemplified by leprechaunism, the Rabson Mendenhall, and the type A syndrome of insulin resistance (Taylor and Arioglu, 1998).

1.3.9.1 Insulin resistance and metabolic function:

Insulin resistance affects many tissue functions and a variety of metabolic processes in the body. Hyperinsulinaemia causes exaggerated responses in tissues that remain sensitive to insulin. For example, hyperinsulinaemia stimulates the sympathetic nervous system, which may contribute to hypertension (Reaven *et al.*, 1996; Ginsberg, 2000), or increasing ovarian androgen production, which is integral to hormonal imbalances observed in the polycystic ovary syndrome (Franks, 1995; Dunaif and Thomas, 2001). Similar to the muscle and liver cells, there are various cellular functions that also exhibit resistance to insulin. In particular, insulin resistance in fat cells leads to increased lipolysis with release of fatty acids and a variety of sequelae, including dyslipidaemia and vascular abnormalities caused by excessive amounts of circulating free fatty acids (Ginsberg, 2000). High free fatty acid concentrations also contribute to resistance to insulin's action by enhancing glucose output from the liver and reducing glucose disposal in skeletal muscle (Boden, 1999).

1.4 INSULIN RESISTANCE AND OBESITY

Increased adipose tissue, especially that in an upper body or "android" deposition, was first associated with diabetes and vascular disease by French endocrinologist jean Vague in 1956 (Vague, 1996). Insulin resistance increases with increasing body mass index, waist circumference, and in particular waist-hip ratio (Aronne and Segal, 2002), reflecting increased adiposity especially increased levels of visceral adipose tissue. Visceral adipose tissue refers to intra-abdominal fat around the intestines and correlates closely with liver fat accumulation.

1.4.1 Fatty acid-induced insulin resistance

Metabolic characteristics of visceral adipose tissue differs from that of the subcutaneous fat. Visceral adipose tissue is metabolically more active; increased free fatty acid turnover that promotes insulin resistance at cellular level (Giorgino *et al.*, 2005). The insulin resistance seen in obesity is believed to involve primarily muscle and liver, with increased adipocyte- derived free

fatty acids promoting triglyceride accumulation in these tissues. (Perseghin *et al.*, 2003). Circulating FFAs derived from adipocytes are elevated in many insulin-resistant states and have been suggested to contribute to the insulin resistance of diabetes and obesity by inhibiting glucose uptake, glycogen synthesis, glucose oxidation, and by increasing hepatic glucose output (Bergman and Ader, 2000). Elevated FFAs are also associated with a reduction in insulin-stimulated IRS-1 phosphorylation and IRS-1 associated PI 3-kinase activity (Shulman, 2000). There are two hypotheses that links increased circulating FFAs with insulin resistance:

1) Randle et al. demonstrated that fatty acids compete with glucose for substrate oxidation in isolated rat heart muscle and rat diaphragm muscle (Fig. 1.2). They speculated that increased fat oxidation causes the insulin resistance associated with obesity (Randle *et al.*, 1963; Randle *et al.*, 1965). The mechanism they proposed to explain the insulin resistance was that an increase in fatty acids caused an increase in the intramitochonderial acetyl CoA/CoA and $NADH^+/NAD^+$ ratios, with subsequent inactivation of pyruvate dehydrogenase (Fig. 1.2). This in turn would cause intracellular citrate concentrations to increase, leading to inhibition of phosphofructokinase, a key rate-controlling enzyme in glycolysis. Subsequent accumulation of glucose-6-phosphate would inhibit hexokinase II activity, resulting in an increase in intracellular glucose concentrations and decreased glucose uptake.

2) Alternative mechanism for fatty acid-induced insulin resistance has been proposed based on studies on human skeletal muscle (Fig. 1.3). An increase in delivery of fatty acids to muscle or a decrease in intracellular metabolism of fatty acids, leads to an increase in intracellular fatty acid metabolites such as diacylglycerol, fatty acyl CoA, and ceramides. These metabolites activate a serine/threonine kinase cascade (possibly initiated by protein kinase C) leading to phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1 and IRS-2), which in turn reduces the ability of the insulin receptor substrates to activate PI 3-kinase. As a consequence, glucose transporter activity and other events downstream of insulin receptor signaling are diminished (Gavrilova, 2000; Kim *et al.*, 2000).

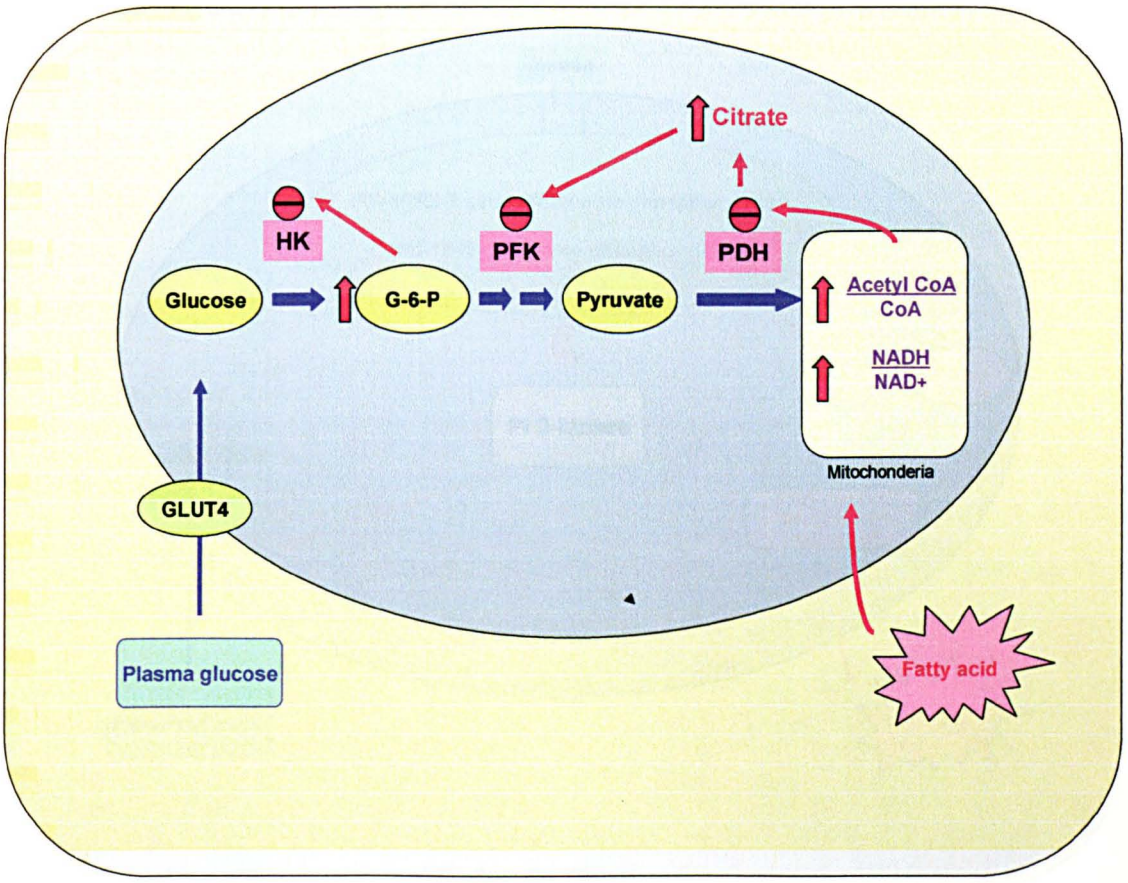


Figure 1.2 Mechanism of fatty acid-induced insulin resistance in skeletal muscle as proposed by Randle et al.

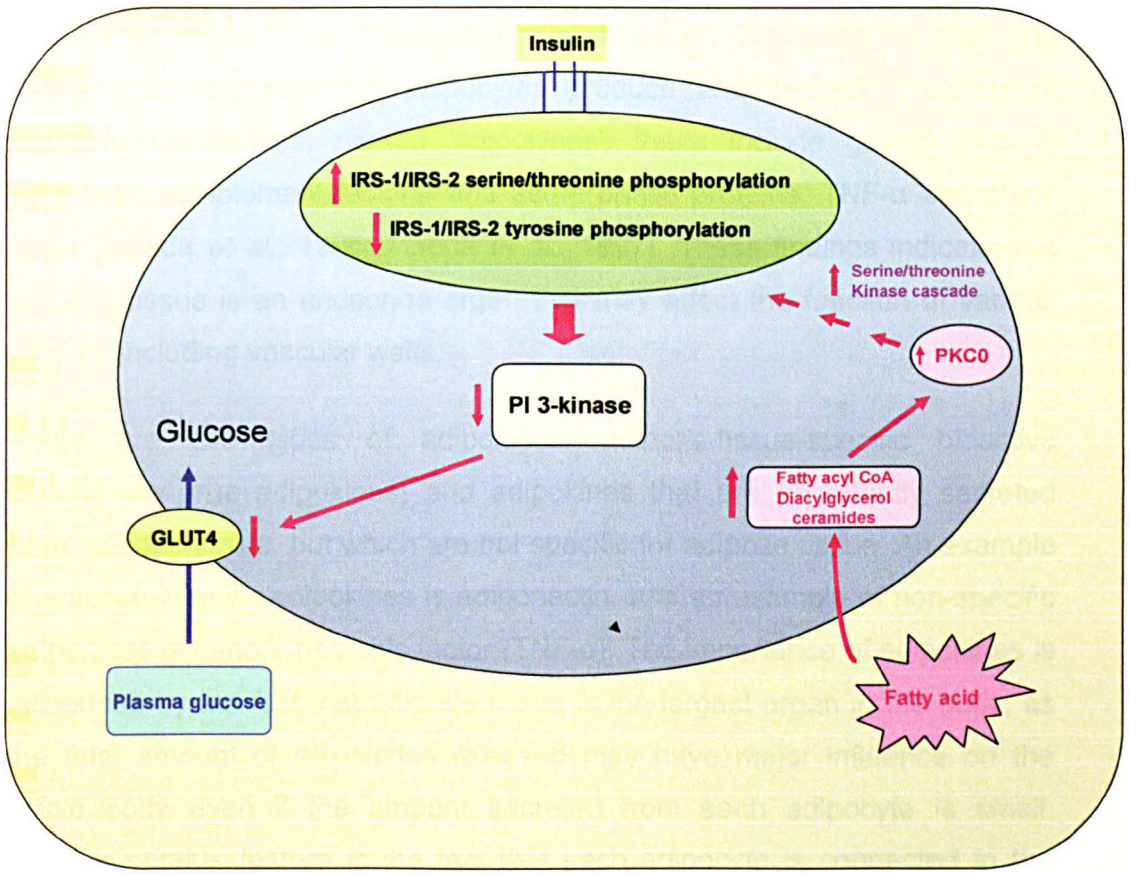


Figure 1.3 Proposed alternative Mechanism for fatty acid-induced insulin resistance in skeletal muscle.

1.4.2 Adipokines and insulin resistance

There is a close relationship between insulin resistance and visceral adiposity. It has become apparent that insulin sensitivity is modulated by adiposity, and this has led to significant interests in adipokines, which may play a pivotal role in the regulation of insulin signalling and its action. Research on adipocyte biology has revealed that adipocytes produce and secrete a variety of bioactive substances, named 'adipokines'; these include growth factors, cytokines, complement factors, and acute phase proteins, TNF-α and many more (Maeda *et al.*, 1996; Maeda *et al.*, 1997). These findings indicate that adipose tissue is an endocrine organ that may affect the function of various organs, including vascular walls.

There are two types of adipokines: adipose-tissue-specific bioactive substances (true adipokines) and adipokines that are abundantly secreted from adipose tissue, but which are not specific for adipose tissue. An example of adipose-specific adipokines is adiponectin, and an example of non-specific adipokines is tumour necrosis factor (TNF-α). The importance of adipokines is influenced by the fact that adipose tissue is the largest organ in the body, as the total amount of adipokines released may have major influence on the whole body even if the amount secreted from each adipocyte is small. Another notable feature is the fact that each adipocyte is connected to the vascular network, therefore, released adipokines can easily reach the systemic circulation.

Dysregulation of adipokine production and secretion is involved in the development of metabolic (including insulin resistance) and cardiovascular diseases. Metabolic syndrome has been receiving an increased interest recently, as a highly atherogenic state that is characterized by the clustering of multiple risk factors such as impaired glucose metabolism, hyperlipidaemia and hypertension in a given individual. In metabolic syndrome, intra-abdominal visceral fat accumulation is shown to play a key role in the

development of a variety of metabolic and circulatory disorders through the dysregulation of adipokines.

1.4.2.1 TNF-α:

Is expressed as a 26 kDa cell surface transmembrane protein, subsequently cleaved to a 17 kDa active biological form, produced by a broad range of tissues, but mainly by adipocytes (Kern *et al.*, 1995; Wajant *et al.*, 2003). It has been shown that adipose TNF-α mRNA and plasma TNF-α protein are increased in most animal models and human subjects with obesity and insulin resistance. Neutralising the blood TNF-α in obese rats with a soluble TNF-α receptor, immunoglobulin G fusion protein, markedly improves insulin resistance. This finding indicates that higher production of TNF-α accumulated in adipose tissue may be causative for obesity-associated insulin resistance. Furthermore, vascular endothelium is a principal target of actions of TNF-α (Locksley *et al.*, 2001; Wajant *et al.*, 2003). Bioavailability of NO is reduced in cultural endothelial cells incubated with TNF-α (Myers *et al.*, 1994). In addition, endothelium-dependent vasodilatation is impaired in animals and human subjects after infusion with TNF-α (Wang *et al.*, 1994; Bhagat and Valance, 1997). Possibly by reducing eNOS mRNA stability or increasing super oxide production (Yoshizumi *et al.*, 1993; De Keulenaer *et al.*, 1998).

1.4.2.2 IL-6:

Is a 26 kDa protein produced by several tissues, including adipose and endothelium (Mohamed-Ali *et al.*, 1997). Adipose tissue has been postulated to contribute a significant production (approx. 30 %) of the total circulating IL-6 (Mohamed-Ali *et al.*, 1997; Yudkin *et al.*, 1999). Elevated IL-6 concentrations is associated with insulin resistance and obesity (Yudkin *et al.*, 1999). Furthermore, IL-6 results in impaired NO-mediated relaxation of systemic vessels in pregnant rats (Orshal and Khalil, 2004), and stimulates endothelial cell proliferation and angiogenesis (Holzinger *et al.*, 1993; Hernandez-Rodriguez *et al.*, 2003). Moreover, IL-6 has been reported to stimulate the synthesis and secretion of CRP, that contributes to atherosclerosis (Bhakdi *et al.*, 1999).

1.4.2.3 Leptin:

Leptin, the product of the *ob* gene, is a plasma protein secreted by adipocytes and is involved in the control of body weight, mainly through its hypothalamic effects. Obese subjects have been shown to have up to a 10-fold greater plasma leptin concentration than lean subjects (Considine *et al.*, 1996), but the anorexigenic effects of leptin become less pronounced indicating development of leptin resistance. However, the exact mechanism of this proposed 'leptin resistance' is not fully clear yet (Bjorbaek *et al.*, 1999; Banks and Farrell, 2003).

The presence of leptin receptors on endothelial cells, (Bouloumie *et al.*, 1998) has led to the hypothesis that leptin interacts with the vascular wall and may contribute to atherosclerotic change and angiogenesis. Atherosclerosis is initiated by endothelial cell damage. As the atherosclerotic plaque progresses it develops its own microvascular network (neovascularisation), the disruption of which is thought to play a role in plaque destability. Leptin enhances formation of capillary-like tubes and neovascularization *in vitro and in vivo* (Bouloumie *et al.*, 1998; Sierra-Honigmann *et al.*, 1998). Moreover, studies in normal adolescents with variable BMIs found increased leptin concentration to be associated with impaired vascular function independent of the metabolic and inflammatory disturbances associated with obesity (Singhal *et al.*, 2002). Furthermore, *in vitro* stimulation of cultured endothelial cells with leptin leads to an increase in the production of (Reactive oxygen Species) ROS, *via* hydrogen peroxide (Bouloumie *et al.*, 1999) and superoxide production from mitochondria (Bouloumie *et al.*, 1999). Migration of endothelial cells is a key event in angiogenesis contributing to pathological states such as diabetic vasculopathy. Leptin has been shown to induce cell migration in human umbilical vein endothelial cells (HUVECs), mediated by the PI 3-K/AKt (pkB)/eNOS and MAPK pathways (Goetze *et al.*, 2002). In contrast, number of studies have indicated that leptin stimulates vasodilation (Lembo *et al.*, 2000; Matsuda *et al.*, 2003). Despite this insulin-mimetic action of leptin that may mediate beneficial cardiovascular effects, the majority of studies

performed to date indicate that hyperleptinaemia is atherogenic. Therefore, to further elucidate the exact role of leptin in obesity-related endothelial dysfunction and its mechanisms, more *in vivo* and *ex vivo* studies are needed.

1.4.2.4 C-reactive protein (CRP):

An acute phase reactant marker produced by the liver and adipose tissue in response to systemic inflammation. The circulating level of CRP rises with BMI (Visser *et al.*, 1999; Pannacciulli *et al.*, 2001; Bullo *et al.*, 2003; Anty *et al.*, 2006), and elevated levels of this inflammatory marker have been associated with both obesity and diabetes, while its levels fall with a weight loss (Tchernof *et al.*, 2002). A very recent study, (unpublished results, IS Wood and P Trayhurn) using conventional RT-PCR have found that there are very small amount of CRP levels in human white adipose tissue (WAT), or adipocytes. The very low levels of CRP expression would suggest that adipocytes are unlikely to be a significant contributor of circulating CRP levels. Secretion of IL-6 from adipose tissue is increased in obesity. IL-6 is the major cytokine regulating the hepatic production of CRP (Heinrich *et al.*, 1990; yudkin *et al.*, 1999). Thus, WAT may be a major player in the raised circulating levels of CRP in obesity, but through the indirect route of adipocyte-derived IL-6. CRP levels are frequently elevated in patients with acute ischaemia and myocardial infarction (Biasucci *et al.*, 1999; Buffon *et al.*, 1999) indicating its active role in the atherosclerosis process (Yeh *et al.*, 2001; Verma *et al.*, 2002). Thus, CRP should be regarded as an indirect, albeit important measure of endothelial function.

1.5 INSULIN RESISTANCE AND VSACULAR SYSTEM

1.5.1 Pathways of insulin action in vasculature

When insulin activates its receptor, 2 major pathways are switched on (Fig. 1.1). The first pathway involves activation of phosphatidylinositol 3-kinase, which is an important mechanism for glucose transport in skeletal muscle. This pathway has also recently been shown to be an important factor for endothelial nitric oxide production that leads to insulin-induced vasodilation. The second insulin-activated pathway is the MAP-K pathway, which promotes insulin-mediated growth of the vascular smooth muscle cells (VSMC).

Therefore, activated ERK can translocate into the nucleus, thus initiating a transcriptional programme that lead to cellular proliferation or differentiation (Boulton *et al.*, 1991; Lazar *et al.*, 1995). Moreover, MAP-K also can stimulates migration induced by insulin (Wang *et al.*, 2003).

1.5.2 Endothelium and endothelial function.

The endothelium is the layer of thin, flat cells that lines interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells line the entire circulatory system, from the heart to the smallest capillary. The importance of endothelium in vascular function has been known for decades. Endothelial cells not only provide the physical lining of the blood vessels but also play a vital role in vascular homeostasis, vascular tone regulation, vascular smooth muscle cells proliferation, trans-endothelial leukocyte migration, thrombosis, and thrombolysis. Endothelial cells are involved in many aspects of vascular biology including:

- vasoconstriction & vasodilation, and hence the control of blood pressure
- blood clotting
- atherosclerosis
- formation of new blood vessels (angiogenesis)
- inflammation and swelling (oedema)

Endothelial cells also control the passage of materials and the transit of white blood cells into and out of the bloodstream. In some organs, there are highly differentiated endothelial cells to perform specialised 'filtering' functions. Examples of such unique endothelial structures include renal glomerulus and blood-brain barrier. Endothelial tissue is a specialised type of epithelium tissue (one of the four types of biological tissue in animals (Alberts *et al.*, 2002). In response to various mechanical and chemical stimuli, endothelium synthesise and release a large number of vasoactive substances; that include NO and prostaglandins (Quyyumi, 1998). Clinical problems may develop when these functions or processes are in imbalance.

1.5.3 Endothelial dysfunction

It can be defined as the partial or complete loss of balance between vasoconstrictor and vasodilators, growth promoting and inhibiting factors, pro-atherogenic and anti-atherogenic factors, and pro-coagulant and anti-coagulant factors (Quyyumi, 1998). In other words, endothelial dysfunction is a physiological dysfunction of normal biochemical processes carried out by the endothelium, the cells that line the inner surface of all blood vessels including arteries and veins. Compromise of normal function of endothelial cells is characteristic of endothelial dysfunction. Endothelial dysfunction can result from disease processes, as occurs in septic shock, hypertension, hypercholesterolaemia, diabetes as well as from environmental factors, such as smoking tobacco products. Endothelial dysfunction is now regarded as an early pivotal event in atherogenesis (Ross, 1999) and has been shown to precede the development of clinically detectable atherosclerotic plaques in the coronary arteries (Mano *et al.*, 1996). It has also been considered an important event in the development of microvascular complications in diabetes (Tooke, 1995).

A key feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to appropriate stimuli. In animal models, this can be tested using myograph study. This technique is thought to stimulate the endothelium to release nitric oxide (NO), which diffuse into the surrounding vascular smooth muscle causing vasodilatation.

Dysfunctional endothelial cells are unable to produce NO to the same extent (or there maybe an increased and rapid destruction of NO) as healthy endothelial cells and therefore vasodilatation is reduced. Endothelial function can be improved significantly by exercise and improved diet. Other factors have been identified as improving endothelial function includes cessation of smoking, loss of weight and treatment of hypertension and hypercholesterolaemia amongst various other measures.

1.5.4 Nitric Oxide synthesis

Nitric oxide is the major factor in arteries mediating endothelial dependent relaxation. NO is produced in endothelial cells through the conversion of L-arginine to L-citrulline (Fig. 1.4), in the presence of molecular oxygen and NADPH, *via* the activity of endothelial enzyme Nitric Oxide Synthase (eNOS), and its cofactors tetrahydrobiopterin, flavin adenine dinucleotide and flavin mononucleotide (Fig. 1.4). Insulin enhances tetrahydrobiopterin production by stimulating its biosynthetic enzyme GTP cyclohydrolase, and thus stimulates eNOS activity by calcium-independent phosphorylation of eNOS at serine and threonine residues *via* PI 3-kinase/AKt pathway, resulting in enhanced nitric oxide production. Nitric oxide then diffuses to the underlying vascular smooth muscle cells to stimulate the production of cyclic guanosine monophosphate (cGMP) through the activation of enzyme guanylate cyclase. The increase in cGMP will lead to a reduction in intracellular calcium ions concentration, inducing vasodilation (Fig. 1.4).

There are various pharmacological and non-pharmacological stimuli that can be used to assess the production of nitric oxide in endothelial cells *in vivo* as well as its effect on the underlying vascular smooth muscle cells (endothelium-dependent and independent vasodilation, respectively). The intra-arterial infusion of acetylcholine or methacholine has widely been used to stimulate endothelial cell production of nitric oxide, whereas sodium nitroprusside has been used to directly stimulate vascular smooth muscle cells. Another approach to the assessment of endothelial function is to measure plasma levels of markers of low-grade inflammation such as C-reactive protein (CRP), IL-6, and TNF-α. Apart from vasodilatory effects, NO reduces vascular smooth muscle cell migration and growth (Sarkar *et al.*, 1996), platelet aggregation and thrombosis (Antl *et al.*, 2006), monocyte and macrophage adhesion, and inflammation (Peng *et al.*, 1998).

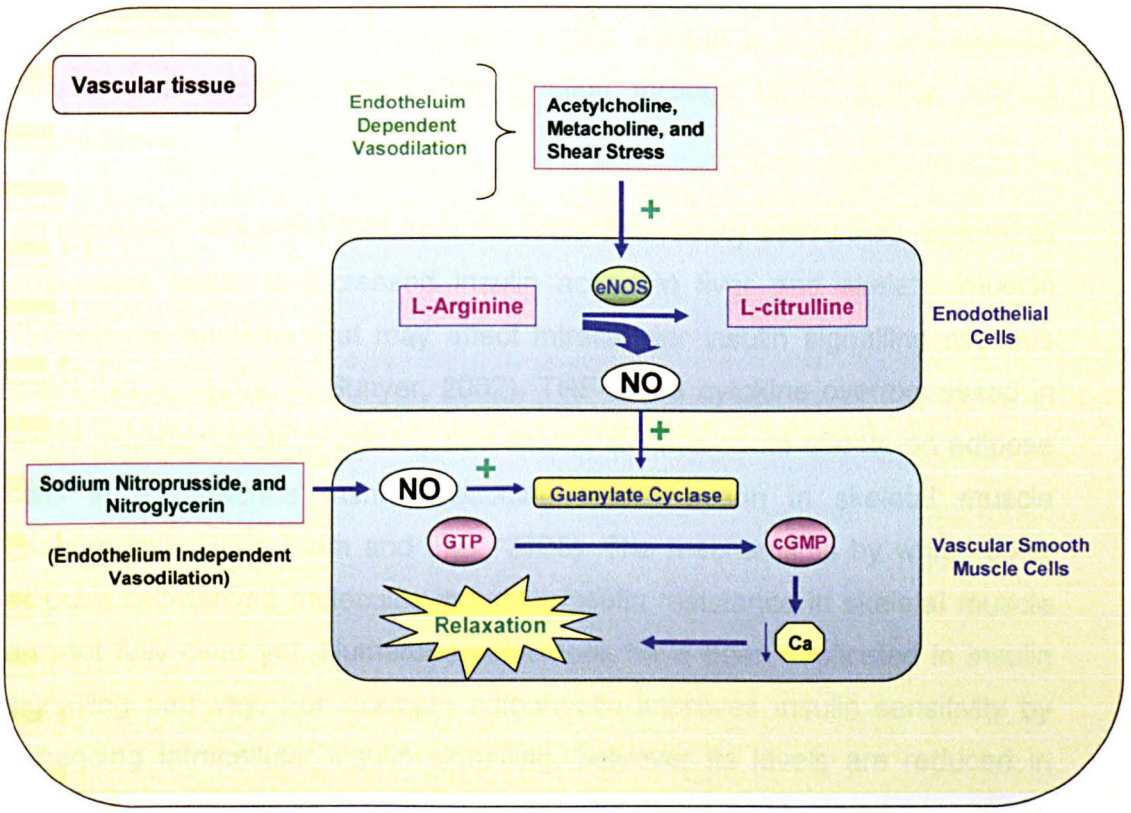


Figure 1.4 Synthesis and function of Nitric oxide as vasodilator in vascular system

1.6 OBESITY, INSULIN RESISTANCE, ENDOTHELIAL DYSFUNCTION, AND ATHEROSCLEROSIS: THE LINK

Obesity, particularly "central or abdominal" adiposity, leads to an imbalanced production of several metabolic products, hormones, and cytokines (adipokines), which favor decreased insulin sensitivity in liver and skeletal muscle and impaired endothelial function through direct and/or indirect mechanisms.

An increased efflux of FFAs from the more lipolytically active intra-abdominal adipocytes leads to increased insulin action in liver and skeletal muscle through mechanisms that may affect intracellular insulin signalling cascade (Griffin *et al.*, 1999; Pi-Sunyer, 2002). TNF-α is a cytokine overexpressed in some animal models of obesity that seems to have some effects on adipose cells in a paracrine fashion reducing insulin action in skeletal muscle (Hotamisligil, 1999; Kahn and Flier, 2000). The mechanisms by which other adipose cell-derived molecules increase insulin resistance in skeletal muscle are not fully clear yet. Numerous adipokines have been implicated in insulin signalling pathway. For example adiponectin improves insulin sensitivity by enhancing intracellular insulin signalling, however its levels are reduced in states of obesity/insulin resistance (Yamauchi *et al.*, 2001; Yamauchi, 2003). Resistin, another fat-derived hormone, has also been found to be linked to obesity and insulin resistance in animal models; yet, its role in human obesity seems to be insignificant (Nagaev and Smith, 2001; Steppan *et al.*, 2001).

In addition to inducing insulin resistance, certain fat-derived products might have a direct effect on vascular function. FFAs (Steinberg and Baron, 2002), TG (Naderali *et al.*, 2001) and adipokines IL-1, IL-6, and TNF-α been closely linked to endothelial dysfunction and subclinical inflammation (Winkler *et al.*, 1999; Yudkin *et al.*, 2002). IL-6 is a potent stimulus for the production of CRP in the liver which, in turn, may have some direct deleterious effects in the vascular wall (Yudkin *et al.*, 1999). CRP is considered an excellent marker of low-grade inflammation in the vascular wall, a well recognized mechanism in the development of atherosclerosis (Libby *et al.*, 2002).

The mechanisms by which insulin resistance leads to endothelial dysfunction are certainly multiple and complex. All major abnormalities that are part of the insulin resistance syndrome, such as hyperglycaemia, hypertension, dyslipidaemia, and altered coagulation/fibrinolysis, are directly and independently linked to endothelial dysfunction. Despite that, it is still not clear whether insulin resistance *per se* causes endothelial dysfunction and ultimately atherosclerosis. Nonetheless, hyperinsulinaemia, a surrogate marker for the presence of insulin resistance, was found to be an independent risk factor for cardiovascular disease in nondiabetic individuals (Despres *et al.*, 1996).

New insights into a more direct link between insulin resistance and the vasculature come from the observation that insulin has direct vascular effects. Insulin is known to have a direct vasodilatory effect mediated through stimulation of nitric oxide production in endothelial cells (Kuboki *et al.*, 2000). In insulin-resistant state, the ability of insulin to stimulate nitric oxide production in the endothelium is diminished. Interestingly, the stimulation of nitric oxide production in endothelial cells and the stimulation of glucose uptake in muscle and fat tissue by insulin occur through the phosphatidylinositol 3-kinase (PI3-K) and Akt pathway (Cusi *et al.*, 2000; Montagnani and Quon, 2000). In contrast, other effects of insulin action on the vasculature, including the stimulation of migration and growth of smooth muscle cells and the production of plasminogen activator inhibitor (PAI-1), are mediated through the mitogen-activated protein kinase (MAP-K) pathway (Hsueh and Law, 1999).

Many other abnormalities may certainly contribute to the link between insulin resistance and endothelial dysfunction, such as the production of reactive oxygen species, i.e., superoxide (Cai and Harrison, 2000), inflammation (Yudkin *et al.*, 1999), production of cytokines such as TNF-α (Winkler *et al.*, 1999), activation of the renin-angiotensin system (Folli *et al.*, 1999), and elevation of ET-1 (Piatti *et al.*, 2000). Therefore, a large number of mediators with complex mechanisms closely link obesity, insulin resistance, and endothelial dysfunction in a multidirectional and dynamic framework.

Jiang et al. found that Zucker rats, a well-established model of insulin resistance, decreased insulin-mediated glucose uptake in muscle and decreased insulin-mediated nitric oxide production in endothelial cells (Jiang *et al.*, 1999) are mediated through the PI3-K pathway, while insulin-mediated stimulation of VSMC migration and growth and PAI-1 production, *via* MAP-K pathway, is preserved. Extrapolating these findings to a theoretical framework in humans, could suggest that individuals who exhibit the insulin resistance syndrome may have an abnormality in nitric oxide production by endothelial cells (Arcaro *et al.*, 2002). However, it is not known if the mechanism of abnormal selective signalling pathways described in animal model of genetic obesity would also operates in dietary obese animals. Furthermore, whether or not there is an increased secretion of adipocytokines and inflammatory markers in dietary-obese animals merits further investigation.

Support for a closer association of obesity, insulin resistance, and endothelial dysfunction comes from studies that have assessed the effects of weight loss on an improvement in insulin sensitivity and endothelial function. As described above increased adipose tissue, particularly in the abdominal region, is associated with the production of several hormones, mediators, and cytokines that impact insulin sensitivity in liver and skeletal muscle and vascular function. Thus, weight loss and/or an improvement in insulin sensitivity whether physiologically or pharmacologically may lead to a more favorable profile of these fat-derived substances, which would translate into an improvement of metabolic and endothelial function and a reduction of the low-grade inflammatory state. In fact, several studies now support this concept (Dandona *et al.*, 1998; Ziccardi *et al.*, 2002).

1.7 THE PEROXISOME PROLIFERATORS ACTIVATED RECEPTOR-α AGONIST (FENOFIBRATE)

Peroxisome proliferators are group of chemicals that have several applications such as: therapeutically used drugs (e.g., hypolipidaemic agents), plasticizers, and organic solvents used in the chemical industry, herbicides, and naturally occurring hormones. Peroxisome proliferators, as the name implies cause an increase in the number and size of peroxisomes in the liver

(associated with increased liver size), kidney and heart tissue of susceptible species, such as rats and mice. Long-term administration of peroxisome proliferators can cause liver cancer in these animals (Reddy and Rao, 1992). Whether frequent exposure to peroxisome proliferators represents a hazard to human subjects is unknown; however, increased cancer risk has been shown to be associated with long-term therapeutic administration of the hypolipidemic drugs gemfibrozil, fenofibrate, and clofibrate. The peroxisome proliferator activated receptor-α (PPAR-α), a member of the nuclear receptor superfamily, has been found to be activated by different ligands including naturally occurring eicosanoids and certain long-chain fatty acids, as well as synthetic compounds such as lipid-lowering fibric acids (i.e. fenofibrate, gemfibrozil and the research compound WY14643). Moreover, recent studies have revealed that humans have considerably lower levels of PPAR-α in liver than rodents, and this difference may, in part, explain the species differences in the carcinogenic response to peroxisome proliferators (Reddy and Rao, 1992; Gonzalez *et al.*, 1998).

1.7.1 PPAR-α and peroxisome proliferation in the liver

In hepatocytes, peroxisomes are membrane-bound organelles that contain enzymes responsible for β-oxidation of fatty acids, the biosynthesis of cholesterol, and other biochemical pathways (Mannaerts and Van Veldhoven, 1993). Peroxisome proliferation coincides with increase in many peroxisomal and mitochondrial enzymes involved in fatty acid β-oxidation (Reddy and Chu, 1996). PPAR-α mediates the activation of genes encoding these enzymes through dimerization with the retinoid X receptor α and binding to cis-acting regulatory elements upstream of the promoter regions in target genes (Green and Wahli, 1994; Schoonjans *et al.*, 1997). In rats and mice, PPAR-α is expressed at high levels in the liver and kidney, the primary sites of peroxisome proliferation. (Lee *et al.*, 1995).

In addition to PPAR-α, two other nuclear receptors share significant sequence similarity to PPAR-α. PPAR-γ is highly expressed in white and brown adipose tissues and macrophages; it is not found at significant levels in the liver (Lemberger *et al.*, 1996). PPAR-γ is required for the differentiation of

adipocytes and the tissue-specific regulation of genes in white and brown fat cells (Spiegelman and Flier, 1996). It is the target for the thiozolidinedione drugs used to control adult-onset diabetes (Lehmann *et al.*, 1995). In contrast to PPAR-α and PPAR-γ, PPAR-β is more ubiquitously expressed and is most abundant in the central nervous system (Lemberger *et al.*, 1996). The target genes and physiological functions of PPAR-β are currently unknown.

1.7.2 Physiological role of PPAR-α

On the basis of target gene expression patterns, PPAR-α appears to have critical roles in regulation of fatty acid metabolism, including fatty acid β-oxidation, apolipoproteins, and fatty acid transport proteins (Lee *et al.*, 1995; Auwerx *et al.*, 1996; Aoyama *et al.*, 1998). Moreover, PPAR-α regulates serum cholesterol, in particular, high-density lipoprotein cholesterol, and triglyceride levels, as indicated by the results obtained in mice lacking PPAR-α. Constitutive levels of serum cholesterol are markedly higher in PPAR-α-null mice than in wild-type controls, and fibrate-induced reductions of serum triglycerides do not occur in these mice as well. PPAR-α affects constitutive expression of mitochondrial enzymes involved in fatty acid catabolism as well as induction of mitochondrial and peroxisomal fatty acid β-oxidation pathways by peroxisome proliferators and perhaps other fatty acid metabolites (Aoyama *et al.*, 1998). Thus, genes encoding peroxisomal and mitochondrial fatty acid-metabolising enzymes, apolipoproteins, and fatty acid transport proteins are all regulated by PPAR-α, indicating that this receptor has important physiological roles in control of fatty acid metabolism.

1.7.3 Functions of peroxisome proliferator-activated receptor-α in the endothelium

PPAR-α is expressed in monocytes/macrophages, vascular smooth muscle cells (VSMCs), and endothelial cells, with evidence for regulation by PPARs of relevant vascular target genes (Marx *et al.*, 1999; Marx *et al.*, 2000). There are several studies argue for anti-inflammatory and anti-atherosclerotic effects through PPAR-α. It has been demonstrated that fenofibrate (PPAR-α activator) caused activation for eNOS expression in endothelial cells leading to increased NO production and finally vasodilation (Goya *et al.*, 2004;

Murakami *et al.*, 2006). Moreover, in VSMCs, PPAR-α activators (WY14643, fenofibrate) inhibites interleukin1-induced production of interleukin-6 and prostaglandin as well as expression of cyclo-oxygenase-2 (Staels *et al.*, 1998). Induction of adhesion molecule expression in the endothelium is considered one of the earliest steps in atherogenesis. The endothelium has also been suggested to defend against oxidative stress and to maintain appropriate redox status (Kunsch and Medford, 1999; Inoue *et al.*, 2001). In this sense, data implicating PPARs in redox responses are also relevant. Inoue *et al.*(Inoue *et al.*, 2001) found that both PPAR-α and PPAR-γ activators induced expression of the superoxide scavenger enzyme Cu^{2+}, Zn^{2+} - superoxide dismutase (Inoue *et al.*, 2001).

Interestingly, despite the extensive PPAR-α mediated anti-inflammatory and anti-atherosclerotic effects, recent studies crossing PPAR-α-deficient mice with apolipoprotein-E-deficient mice found less, rather than more, atherosclerosis (Tordjman *et al.*, 2001). The reasons for these differences between *in-vitro* and *in-vivo* findings are unclear. Certainly, the available evidence with PPAR agonists in clinical trials appears to support beneficial effects, with no obvious evidence for an increase in atherosclerosis among such patients.

1.8 ANIMAL MODELS OF OBESITY

1.8.1 Obesity induced by spontaneous mutations:

Several strains of rodents have been identified and propagated because of spontaneous mutations that resulted in an obese phenotype. The animals are not only useful when studying biology of the underlying a specific mutation, but also studying the mechanism of obesity itself.

1.8.1.1 ob/ob ($Lepr^{ob}$/ $Lepr^{ob}$ mice):

The leptin gene is one of the most extensively studied in obesity research because a spontaneous mutation of this gene that prohibits secretion of mature leptin causing hyperphagia, hypothermia, hypercorticosteronaemia, hyperglycaemia, hypothyroidism, growth hormone deficiency,

hyperinsulinaemia, reduced energy expenditure, decreased linear growth, infertility, and early-onset morbid obesity (Friedman, 1998).

This mutation is a single base substitution that results in premature termination of leptin synthesis. This mouse serves as an ideal model to study both leptin function and morbid early onset obesity. The mutation is rare in humans (Tschop and Heiman, 2001).

1.8.1.2 db/db ($Lepr^{db}$/ $Lepr^{db}$ mice):

The $Lepr^{db}$/ $Lepr^{db}$ mice have a phenotype similar to $Lepr^{ob}$/ $Lepr^{ob}$ mice, but they are resistant to leptin because of a mutated leptin receptor. $Lepr^{db}$/ $Lepr^{db}$ mice suffer from morbid obesity with hyperleptinaemia, insulin resistance, and diabetes (Coleman, 1978; Halaas *et al.*, 1995). This mouse provides an excellent model to study leptin resistance, early onset morbid obesity, and is also a useful model to study type II diabetes (Friedman *et al.*, 1997; Tschop and Heiman, 2001).

1.8.1.3 The fa/fa mutation in Zucker, Koletsky- and Zucker Diabetic Fatty (ZDF) rats:

Obese Zucker and Koletsky rats have mutations in the extracellular domain of the leptin receptor and exhibit morbid obesity, the *fa/fa* mutation observed in Zucker rats reduces the expression of the leptin receptor on the cell surface with marked intracellular retention, decreased leptin binding and diminished signal transduction. ZDF rats were isolated from a colony of obese Zucker rats and become diabetic after about 8 weeks when presented with a high fat diet (Chua *et al.*, 1996; Friedman *et al.*, 1997). These rats provide an excellent model to study leptin resisatnce and early onset morbid obesity (Tschop and Heiman, 2001).

1.8.2 Environmentally induced obesity:

In general, genetic models of rodents' obesity show severe insulin resistance and β-cell dysfunction, features which are not usually typical of human type 2 diabetes. This has led to the search for a model more representative of the human form of the disease; i.e. one which has impaired glucose tolerance

(IGT) and mild insulin resistance as a consequence increased intake of a high-energy, highly palatable diet-induced obesity.

Many investigators use wild-type rodents on a high fat diet as a model to study obesity. The rats can be weaned onto obesity-inducing diet (a highly-palatable diet; HPD). Hundred days is the recommended age to start an obesity study, because at this age rats cease to gain lean mass but readily accrue fat mass. Throughout the present study this type of obesity model has been used, which resembles common human obesity better than monogenic rodent models. The increased energy intake is not associated with abnormal leptin or leptin signalling (i.e. absence of a genetic defect in the production of leptin or its receptor). There is extensive literature to show that rats fed high-fat or highly palatable diet (HPD) become progressively more obese than chow-fed littermates, developing hyperleptinaemia and insulin resistance similar to obese human subjects (Chang *et al.*, 1990; Kraegen *et al.*, 1991; Pickavance *et al.*, 1999 ; Harrold *et al.*, 2000). Moreover, the development of obesity on a polygenic background makes this an attractive model.

1.9 AIMS OF THE PROJECT

Our understanding of insulin signal transduction pathways and insulin resistance has evolved extremely rapidly over the past few years. Still, further studies are required to unravel the mechanisms controlling these intricate regulatory processes that presumably mediate, at least in part, the insulin resistance associated with obesity and hyperinsulinaemia.

Metabolic factors associated with obesity, such as free fatty acids can modulate insulin signalling pathways, so this study speculate that there are molecular processes in insulin signalling cascade that are susceptible to inhibition or activation by metabolic factors associated with obesity and thus the spectrum of responses results from the interactions between these factors yielding insulin resistance. Moreover, insulin resistance is associated with accelerated atherosclerosis and a major question is whether or not insulin resistance occurs in cardiovascular tissues and whether this is the driving force for accelerated cardiovascular disease. Furthermore, hepatic and

muscular resistance to the actions of insulin considered the major causative factors in inducing systemic insulin resistance. However, the molecular events of insulin signalling cascades and its correlation to metabolic and vascular abnormalities merit further investigation.

Visceral fat-derived metabolic products, hormones, and cytokines play a major role in effecting insulin action in skeletal muscle and in creating a state of low-grade inflammation and endothelial dysfunction. Some specific and shared insulin signalling abnormalities in muscle, fat, and endothelial cells may also contribute to this rather complex association. Further studies are needed to define the missing steps in the insulin-signalling network, elucidate the mechanisms of cross-communication in the system, and determine the molecular defects that give rise to insulin resistance. Such studies will provide new insights into diabetes and insulin resistance, perhaps allowing a more focused and individualised approach to therapy or prevention of these disorders. Furthermore, Nonpharmacological and pharmacological interventions targeting obesity and/or insulin resistance demonstrate an amelioration of endothelial dysfunction and low-grade inflammation. These strategies, particularly in individuals at risk of type 2 diabetes, may improve clinical cardiovascular outcomes. Investigating the molecular mechanisms related to protein expression of insulin signalling pathways, in addition to determining the vascular and metabolic changes associated with both therapeutical approaches may provide valuable information on the mechanisms and strategies of both types of treatment.

That obesity is associated with vascular dysfunction is well known, but, importantly, such dysfunction can develop after only short-term consumption of an energy-rich diet, long before the onset of frank obesity (Naderali and Williams, 2001b). However, the roles played by different dietary components remains unclear. Thus, the first chapters of the present study investigated the effects of consumption of different high-energy diets of differing macronutrient compositions on the genesis of obesity, induction of vascular abnormalities and metabolic changes.

Also, this study will focus on investigating the protein expressions and deficiencies in the cascade of insulin signalling pathways of insulin resistance vascular and metabolic tissues (aorta, liver, and skeletal muscle) under circumstances related to diet-induced obesity and consumption of high energy diets. Furthermore, the present study will investigate possible association of the above detrimental factors with the development of related clinical disorders such as atherosclerosis and cardiovascular abnormalities, and non-alcoholic fatty liver disease. Moreover, the beneficial effects of the therapeutical approaches of obesity such as non-pharmacological (lifestyle modification, diet withdrawal) and pharmacological (fenofibrate administration) will also be investigated.

It is possible that changes in the levels of protein expression of insulin signaling pathway in vascular and metabolic tissues may induce insulin resistance and alter vasorelaxant property of insulin. Moreover, the level of protein expression of these kinases could fluctuate and respond differently to any pathological, physiological, or pharmacological conditions, such as dietary obesity and its treatment. Therefore, investigating the protein expression of these kinases under certain obesity-related experimental conditions could provide vital information about obesity-induced insulin resistance in vascular and metabolic tissues. Consequently, this study aims to investigate the protein expression of insulin signalling components in aorta, liver and skeletal muscle different experimental groups.

Chapter 2

Methods

2. 1 ANIMAL MODEL

Diet-induced obesity resembles common human obesity better than monogenic rodent models; the increased energy intake is not associated with abnormal leptin or leptin signalling. There is extensive literature to show that rats fed high-fat or highly-palatable diets (HPD) become progressively more obese than chow-fed littermates, and develop insulin resistance and hyperleptinaemia (Wilding *et al.*, 1992a; Pickavance *et al.*, 1999; Harrold *et al.*, 2000) similar to obese humans.

2.1.1 Induction of obesity and energy distribution of the various diets

Out bred male Wistar rats were obtained from Charles River UK Ltd. (Margate, UK), and were selected with a starting weight ranging between 170 and 200 g.

For all experimental protocols unless otherwise stated, animals were fed a highly-palatable diet (HPD) for 7 to 15 weeks to induce obesity (under several experimental conditions), which was defined as a significant increase in fat-pad masses than those of the chow fed controls. The highly- palatable diet provided 1010 KJ/100 g as total energy content, consisted of 33% powdered chow, 33% condensed milk (Nestle UK Ltd., York, UK) and 7% sucrose (Tate & Lyle, London, UK) by weight with the reminder being the tap water. This provided 65% energy as carbohydrate, 19% as protein and 16% as fat (Wilding *et al.*, 1992a). Age-matched controls were fed standard laboratory pelleted chow (chow-fed group)(CRM (P), Biosure, Cambridge, UK) which provide 1310 KJ/100 g as total energy content, consisted of 60% energy as carbohydrate, 30% as protein, and 10% as fat. The diet was made by hand and stored at 4°C. On the other hand, several other types of diets were also included in this study. The High-energy Diet ("Galaxy", milk smooth and creamy chocolate (Mars)) which provided 2256 KJ/100 g as total energy content (42.2% as carbohydrate, 52.7% as fat, and 5.1% as protein) (chocolate-supplemented group). Chocolate-Biscuit supplemented diet ("McVitie's Hobnobs", biscuit covered one side with chocolate) (Biscuit-fed group) which provided 2035 KJ/100g as total energy content (55% as

carbohydrate, 15% as protein and 30% as fat). And fat-enriched diet (Research Diets Inc, New Brunswick, NJ, USA) (high-fat fed group) providing 1980 KJ/100 g as total energy content (35% of energy as carbohydrate, 20% as protein, and 45% as fat) (Table 2.1).

Table 2.1 Summary for energy and micronutrient distribution for the different diets Used in this study

Type of diet	Total energy (KJ)		Energy distribution (% & KJ) per 100g		
	Per 1 gm	Per 100 gm	Carbo.	Fat	Protein
Standard lab. chow	13.1	1310	60 %	10 %	30 %
			786 KJ	131 KJ	393 KJ
Highly-palatable diet (HPD), obesity-inducing diet	10.1	1010	65 %	16 %	19 %
			656.5 KJ	161.6 KJ	191.9
Highly-energy diet (HED), "Galaxy"	22.56	2256	42.2 %	52.7 %	5.1 %
			952.032 KJ	1188.9 KJ	115.1 KJ
Chocolate-Biscuit Diet (CBD), "McVitie's"	20.35	2035	56 %	30 %	15 %
			1119.25 KJ	610.5 KJ	305.25KJ
Fat-Enriched Diet (FED)	19.8	1980	35 %	45 %	20 %
			693 KJ	891 KJ	396 KJ

2.2 EXPERIMENTAL PROTOCOLS

2.2.1 Experimental protocol No. 1

Adult male Wistar rats were randomly assigned to three groups (*n*=6/group) which, throughout a 10-week period, were each fed diets of differing compositions. This duration was chosen to model human chronic consumption of high-energy food inducing excessive weight gain. Control animals received standard pelletted laboratory chow (chow-fed group). One of the experimental groups received chow supplemented with chocolate biscuit (McVitie's 'HobNobs') (biscuit-fed group), and the second received a fat-enriched diet (high-fat-fed group). Animals were housed singly under controlled environmental conditions (19-22°C; 30-40% humidity) on a 12-h light/dark cycle (lights on at 07:00 h) and had free access to food and water throughout the study. Food intake was measured daily and the body weights weekly. On the day of experiment, the rats were killed by CO_2 inhalation after 2 hours of fasting (Fig. 2.1)

2.2.2 Experimental protocol No. 2

Adult male Wistar rats (n=21) were randomized and assigned to two groups which were fed on either a standard pelletted laboratory chow throughout (n=7; control group), or given chow plus "Galaxy" chocolate (n=14; chocolate-supplemented group). After 14 weeks, half of the chocolate-supplemented group was given fenofibrate (50 mg/kg/day) and the remainder was given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, UK) by oral gavage daily for 7 days, before being sacrificed. Daily energy intake was determined in all three groups for the last 7 days of the study. On the day of experiment, the rats were killed by CO_2 inhalation after 2 hours of fasting (Fig.2.2)

2.2.3 Experimental protocol No. 3

Adult male Wistar rats (n=30) were randomised and assigned to a control group (n=10) and a test group (n=20). Controls were fed a standard laboratory pelleted diet (chow-fed group), while test group had free access to a highly-

palatable diet. Chow-fed controls remained on their prospective diet for 15 weeks, while after 8 weeks, highly-palatable-diet fed animals were subdivided into two groups. The highly-palatable diet was removed from one group and the standard chow diet was reintroduced (diet-to-chow) for further 7 weeks, while the remaining subgroup was maintained on the highly-palatable diet (diet-fed) for another 7 weeks. After 15 weeks from the start of the study, rats were killed by CO_2 inhalation after 2 hours of fasting (Fig. 2.3).

2.2.4 Experimental protocol No. 4

Adult male Wistar rats (n=30) were randomised and assigned to a control group (n=10) and a test group (n=20) that was fed a highly palatable diet for 15 weeks. Controls were fed a standard laboratory pelleted diet (chow-fed). The test group had free access to a highly-palatable diet. After 8 weeks, half of the test group were given fenofibrate (50 mg/kg/day) and the remainder were given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, UK) by oral gavage, daily for 7 weeks. On the day of experiment, the rats were killed by CO_2 inhalation after 2 hours of fasting (Fig. 2.4).

2.2.5 Experimental protocol No. 5

Adult male Wistar rats (n=40) were randomized and assigned in the first period of the experiment (8 weeks) to a control group (n=10) and a test group (n=30). Controls were fed standard laboratory pelleted chow (chow-fed group), while the test group had free access to a highly-palatable diet. In the second period (7 weeks), chow-fed controls remained on their prospective diet, while the highly-palatable diet fed animals were subdivided into three subgroups (each subgroup 10 animals). In the first subgroup, highly-palatable diet was removed and the standard chow diet was re-introduced (diet-to-chow), while the second subgroup was given fenofibrate (fenofibrate-treated group, 50 mg/kg/day) and the third group (diet-fed group) was given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, UK), by oral gavage daily for 7 weeks. On the day of experiment, the rats were killed by CO_2 inhalation after 2 hours of fasting (Fig. 2.5).

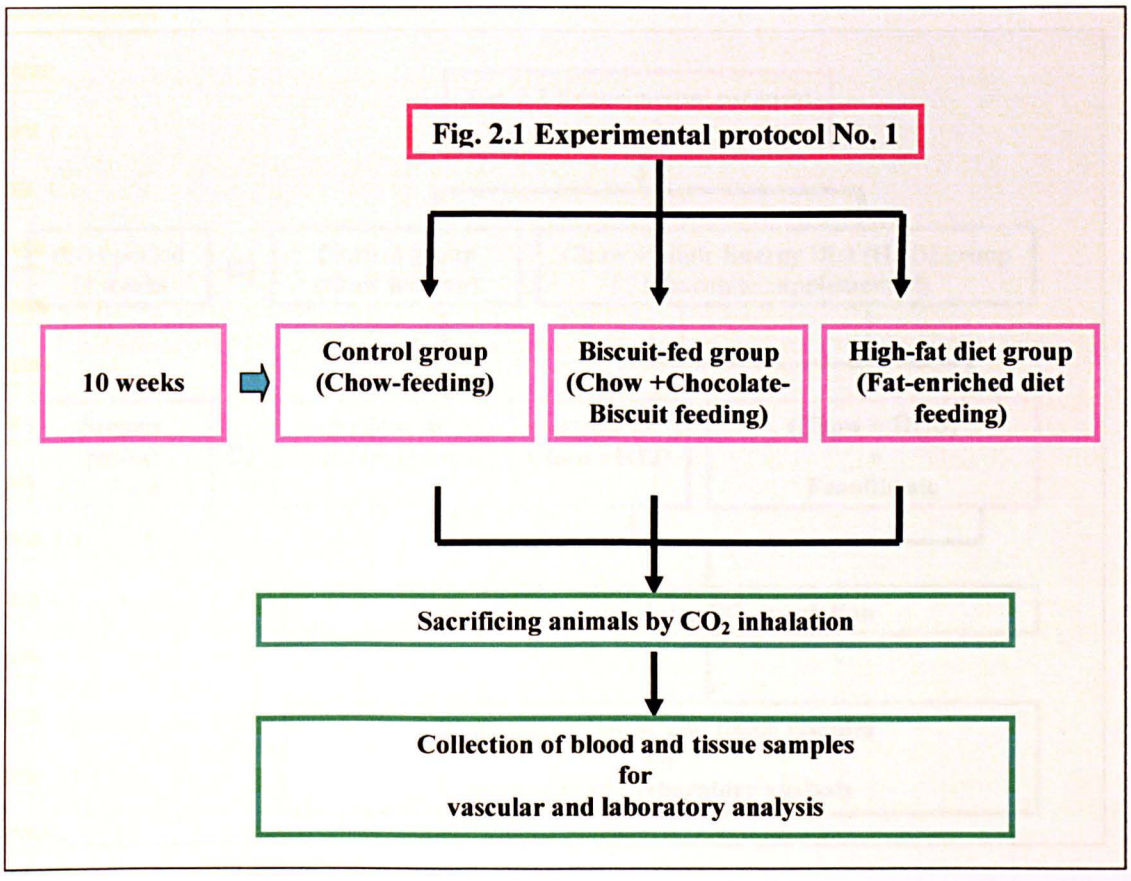


Fig. 2.1. Experimental protocol No. 1

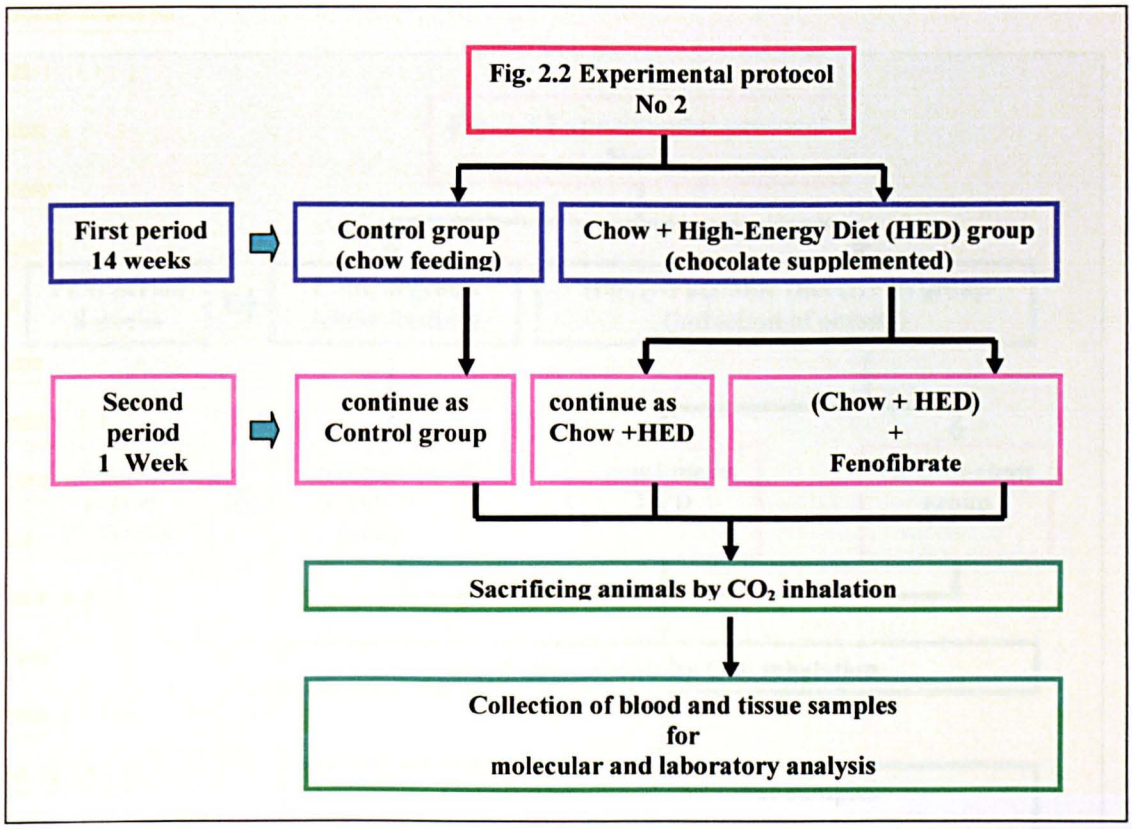


Fig. 2.2. Experimental protocol No. 2

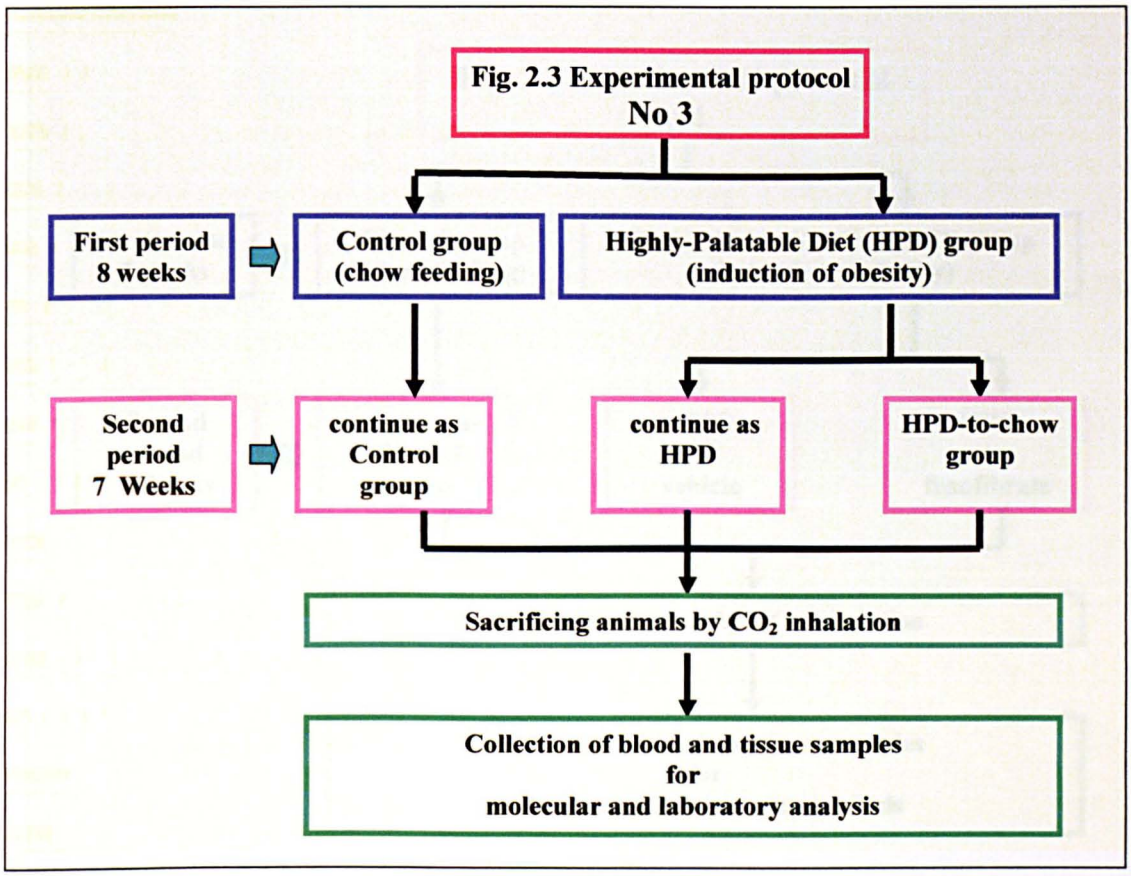


Fig. 2.3. Experimental protocol No. 3

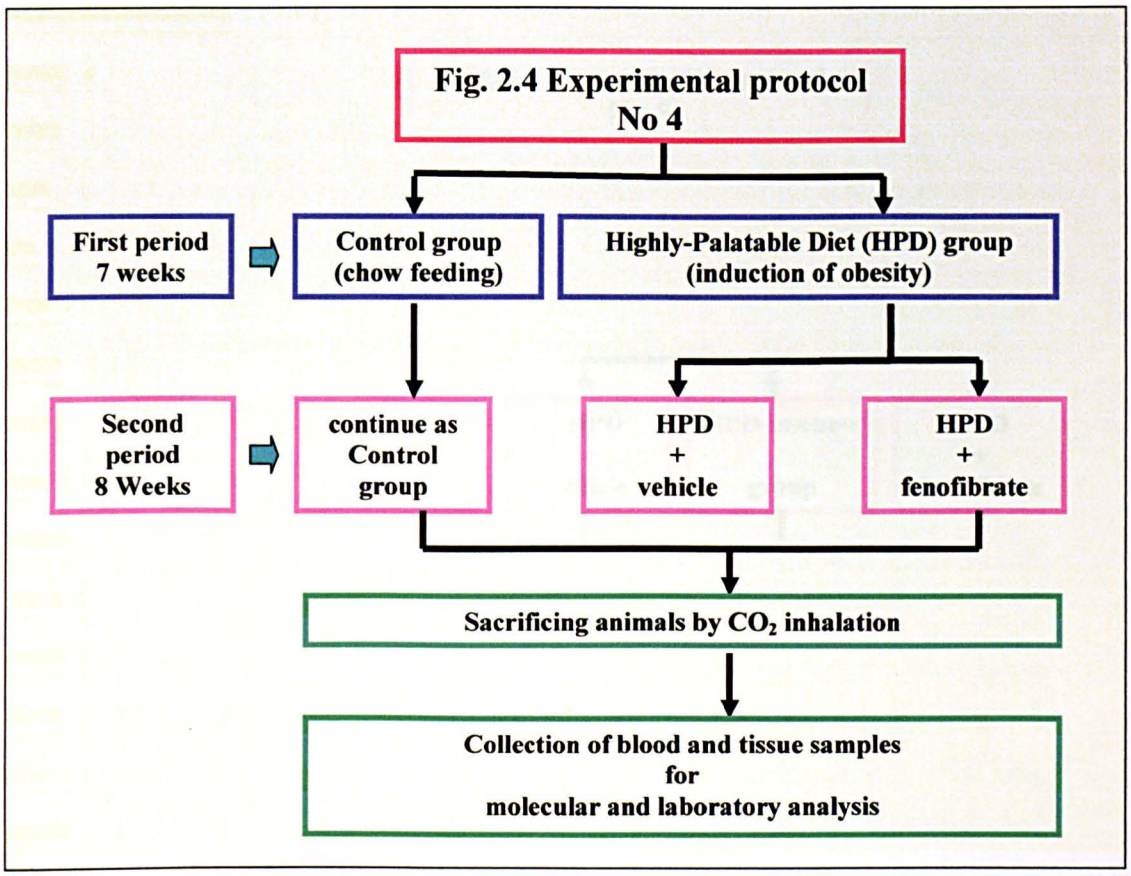


Fig. 2.4. Experimental protocol No. 4

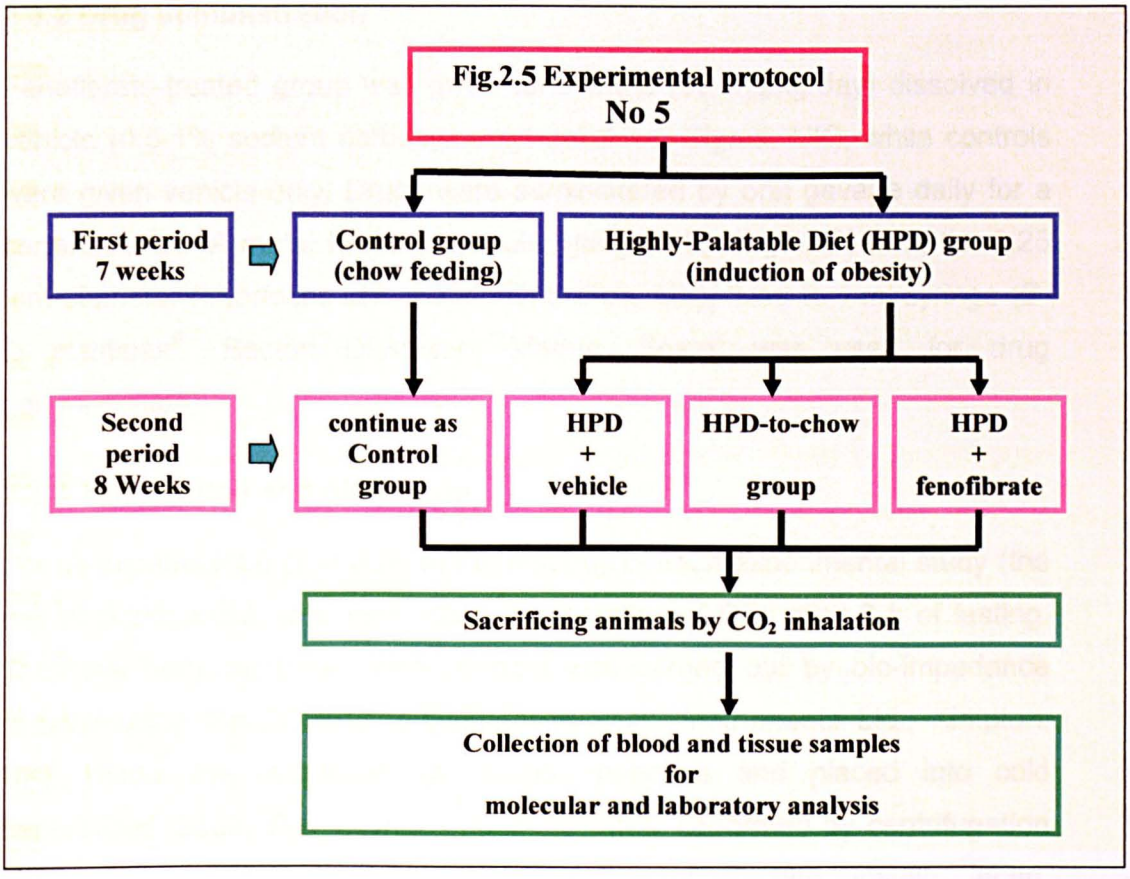


Fig. 2.5. Experimental protocol No. 5

2.3 HOUSING, MAINTENANCE, AND ANIMAL TERMINATION

2.3.1 Housing and maintenance

All animals had free access to tap water throughout the experiment and were housed individually or in groups of 2 in solid-bottomed cages during the period of the experiment, under controlled environmental conditions (19-22 °C; 30-40% humidity) and a 12 h light-dark cycle (lights on at 07:00h).

2.3.2 Drug administration

Fenofibrate-treated group was given fenofibrate (50 mg/kg/day) dissolved in vehicle (0.5-1% sodium carboxymethyl cellulose, Sigma, UK), while controls were given vehicle only. Drugs were administered by oral gavage daily for a certain period. A metal feeding cannula (gauge: 18; length: 50 mm; tip: 2.25 mm diameter (Interfocus Ltd., Haverhill, Suffolk, UK)] fixed to 1-ml syringe (B-D plastipak®, Becton Dickinson, Madrid, Spain) was used for drug administration.

2.3.3 Termination and dissection

For all experimental protocols, In the last day of each experimental study (the day of termination), rats were killed by inhalation of CO_2 after 2 h of fasting. The total body fat mass measurement was carried out by bio-impedance method using the TOBEC® equipment (biotech. Instruments Ltd., Kimpton, UK). Blood was withdrawn by cardiac puncture and placed into cold heparinized tubes. The plasma was immediately separated by centrifugation before being frozen for later measurements of glucose, insulin, leptin, nonesterified free fatty acids (NEFA), triglycerides (TG), C-reactive protein (CRP), adiponectin, and tumour necrosis factor-alpha (TNF-alpha). The aorta, as well as gonadal, epididymal and perirenal fat pads, gastrocnemius and soleus muscles and liver were dissected, weighed and snap frozen in liquid nitrogen for later molecular and experimental studies. The heart and lungs were also dissected and their weights were determined. Six third-order mesenteric arteries were also carefully dissected from each animal for assessment of vascular contractility.

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 Protein extraction

Fifty milligram of tissue (aorta; liver or gastrocnemius muscle) was homogenized at 4 °C in 500 µl buffer containing 120 mM NaCl, 10% glycerol, 2 mM Na_3VO_4, 1% Nonidet-p40, 1 mM PMSF, 10 mM $Na_4p_2O_7$, 100 mM NaF, 20 mM Tris (pH 7.6) and a complete mini® protease inhibitor cocktail with polytron homogenizer. The homogenates were then incubated on a rocking platform at 4°C for 30 min. After 3 x 10 second bursts of sonication, tubes were subsequently centrifuged for 45 min at 13000 x g at 4°C. Supernatants were collected, and protein concentrations were determined by Bicinconinic acid (BCA) method (as per kit instructions-Sigma, UK).

2.4.2 Western immunoblotting

2.4.2.1 SDS-PAGE

A discontinuous acrylamide gel system was used. A stacking gel (5%) was set above a 10% (depending on protein of interest) resolving gel. Samples were standardized to 4-6 mg protein/ml with a lysis buffer. Twenty microlitre of sample protein was boiled in 20 µl 2x electrophoresis sample buffer, for 10 min and then subjected to SDS-PAGE (Tris-glycine buffer, 100 V). Resolved proteins were electro blotted onto nitrocellulose membranes in a buffer containing 25 mM Tris, 190 mM glycine, 1% SDS (W/V) and 20% (V/V) methanol (100 V for 1 hour). Staining immobilized proteins on nitrocellulose with Ponceau S assessed a successful transfer. Nitrocellulose blots were submerged in 0.2% Ponseau S for 15 min with agitation. Blots were then washed with 1 x TBS containing 1% Tween-20 until proteins could be visualized, after which blots were further washed until protein bands had disappeared.

2.4.2.2 Immunoblotting

Non-specific binding proteins were discriminated by incubating the blot with a blocking buffer (5% milk powder, 1x PBS) at room temperature for one hour, followed by immunoblotting with appropriate primary antibody (1:500 dilution) made up in blocking buffer, left overnight at 4 °C. The following morning, blots were washed in 1 x PBS with 1% Tween and incubated with secondary antibody (1:1000), a Horse-reddish peroxidase (HRP) linked anti-rabbit (or

anti-goat, depending on the source of the species used for primary antibody) for 1 hour at room temperature. Proteins were detected using enhanced chemiluminescence method. Positive controls were included for standardization of samples between blots, while molecular weights markers were used for sizing the bands. Densitometry was used to quantify the bands obtained.

2.5 CHEMICALS AND ANTIBODIES

SDS-PAGE and immunoblotting equipments were obtained from Bio-Rad (Richmond, California, USA). Tris, phenylmethyl-sulfonylfluoride (PMSF), aprotinin, dithiotheitol, Tween-20, glycerol, SDS "Lauryl sulfate", B-mercabtoethanol, Bromophenol blue, Temed, ammonium persulfate, and Ponceau S stain, Noradrenaline (NA), carbamylcholine (CCh), sodium nitroprusside (SNP), indomethacin, carboxymethyl cellulose, fenofibrate, and BCA (Bicinconinic acid) method kit for prötein determination were all obtained from Sigma Chemicals (Sigma Ltd., UK). The commercial ELISA kit for measuring plasma TNF-α was obtained from PeproTechEC Ltd. Plasma insulin was measured using ELISA kit from Mercodia. Complete mini® protease inhibitor cocktail, the commercial kits for measuring plasma glucose and triglycerides were all obtained from Roche Diagnostics Ltd. (East Sussex, UK). NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaCO_3$, KH_2PO_4, and EDTA, were all obtained from BDH laboratory supplies (Poole, UK). The commercial kit for measuring plasma C-reactive protein was obtained form Life Diagnostics, Inc. (West Chester (PA), USA). Acrylamide and molecular weight marker were obtained from Bio-Rad laboratories, Inc. (Hercules (CA), USA). Running buffer (Glycine/SDS) and transferring buffer (Tris/glycine) were obtained from National Diagnostics (Atlanta, Georgia, USA). Secondary antibody, an HRP-linked anti-rabbit was purchased from Serotic (Oxford, UK). Polyclonal antibodies against beta subunit of insulin receptor (C-19, sc-711), IRS-1 (C-20, sc-559), IRS-2 (A-19, sc-1556), eNOS (NOS3, C-20, sc-654), Akt1 (C-20, sc-1618), Shc (C-20, sc-288: specific for Shc p46, p52 and p66) and ERK1 (C-16, sc-93: reactive with ERK1 p44 and, to lesser extent, ERK2 p42) and PI 3-kinase were purchased from Santa Cruz Biotechnology (Santa Cruz,

California, USA). Polyclonal antibody against PKC-β was purchased from UPSTATE (UPSTATE house, Dundee, UK).

NA, CCh and SNP were dissolved in double distilled water, while indomethacin was dissolved in ethanol. NA and SNP were kept away from the light throughout the experiment. All water-soluble solutions were freshly made on the day of experiment.

2.6 HOMEOSTASIS MODEL ASSESSMENT (HOMA)

HOMA, an index of insulin resistance, employs the measures of fasting plasma concentrations of glucose and insulin. Thus, terminal concentrations of glucose and insulin were used to calculate an index of insulin resistance where insulin concentration is expressed in units of μU/ml and glucose in mM.

[insulin x [glucose] / 22.5

2.7 ASSESSMENT OF VASCUALR FUNCTION

2.7.1 Myograph technique set up

Six third-order mesenteric arteries (150-220 μm diameter, 2 mm length) were carefully dissected from each animal. Each artery was freed of fat and connective tissue and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK), based on the principle of Mulvany's myograph which measures isometric tension generated in response to various stimuli. Arterial segments (two at a time) were incubated in a 5-ml organ bath containing physiological salt solution (PSS; composition [in mM]: NaCl 119; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.17; $NaHCO_3$ 25; KH_2PO_4 1.18; EDTA 0.026; and glucose 5.5) gassed with 95% O_2 and 5% CO_2 at 37 °C. After 30 min equilibration, the length–tension characteristics for each vessel were determined using the law of Laplace (P = T/r, where P is the transmural pressure, T is the tension, and r is the vessel radius). Each vessel was then set to its normalized diameter, i.e. the diameter it would achieve at rest *in vivo* under a transmural pressure of 100 mmHg. The computer also calculated the target tension that each vessel should develop in response to a

maximal stimulus. Arteries were then allowed a further 30 min to equilibrate before being depolarised twice with high-potassium physiological salt solution (KPSS) in which the NaCl in normal PSS was replaced by an equimolar concentration of KCl (125 mM). Cumulative concentration–response curves to either KCl (10–125 mM) or noradrenaline (NA, 0.5–6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded.

However, examples of the original myograph tracing involving the administration of vasoconstrictors and vasodilators to illustrate the experiment and data acquisition have been shown in the figures (2.6 to 2.10).

2.7.2 Assessment of defect in vascular relaxation

Endothelium-dependent and -independent defects were investigated by exposing NA-precontracted arteries to carbamylcholine (CCh) or sodium nitroprusside (SNP), respectively. To eliminate possible interference by vasoactive prostanoids, 10 mM indomethacin was added to the organ bath, prior to contraction with NA (8 μM). When contraction reached a plateau (after 2 min), concentration–response curves were performed to either CCh, histamine or SNP (for all, 10 nM–100 μM).

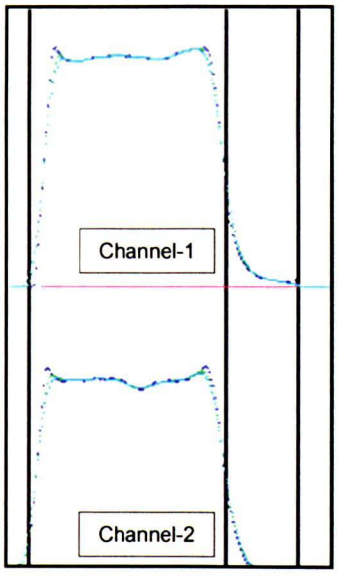


Fig. 2.6. An example for the original myograph tracing for KCl (125mM).

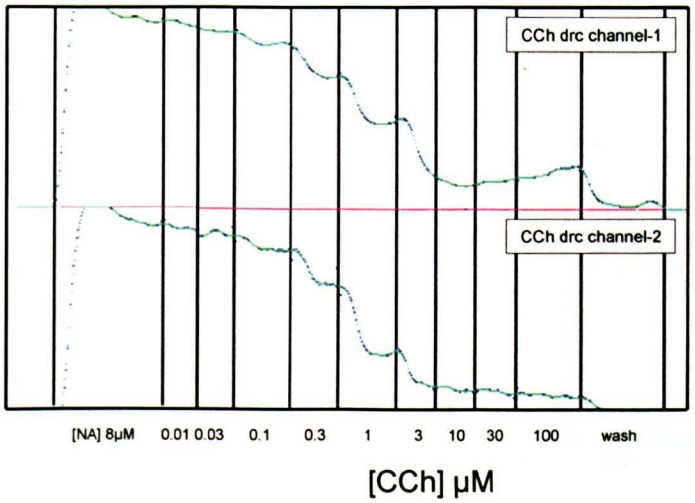


Fig. 2.7. An example of the original myograph tracing for CCh (10nM -100μM) dose-response curve.

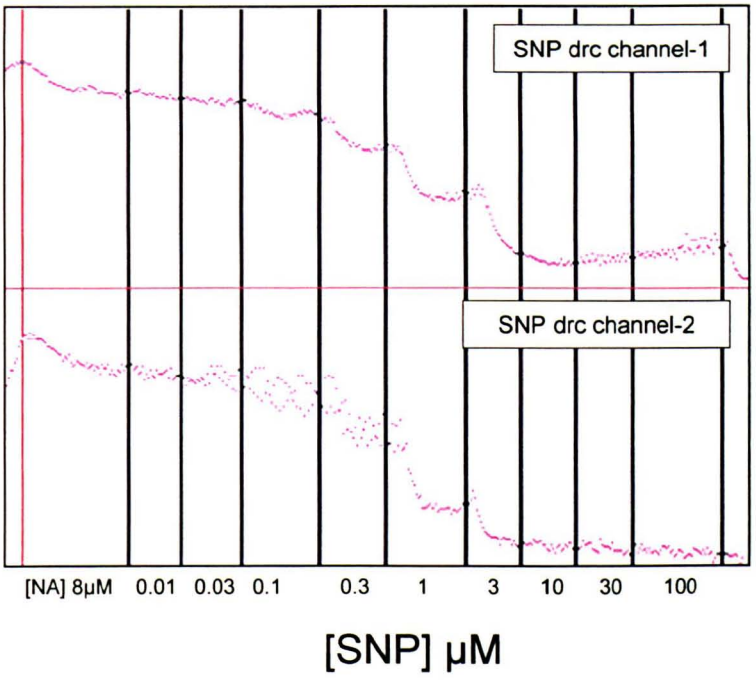


Fig. 2.8. An example of the original myograph tracing for SNP (10nM-100μM) dose-response curve.

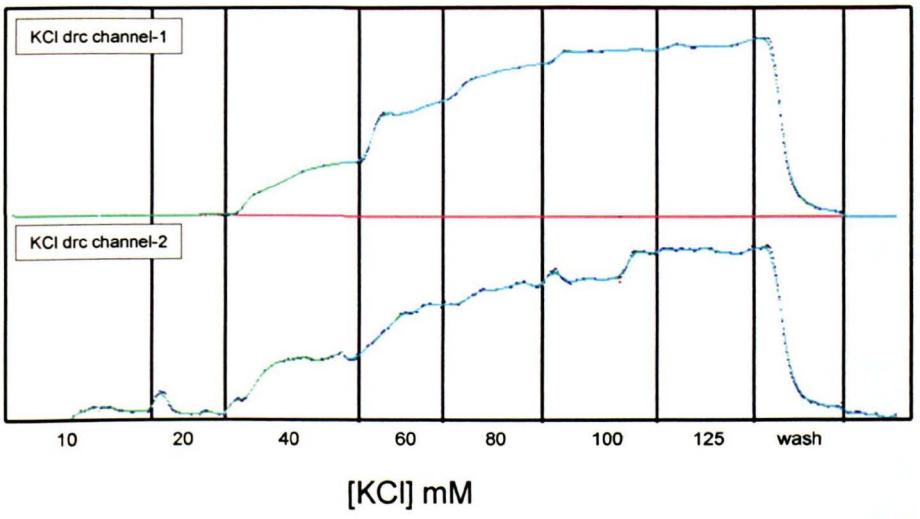


Fig. 2.9. An example of the original myograph tracing for KCl (10-125mM) dose-response curve.

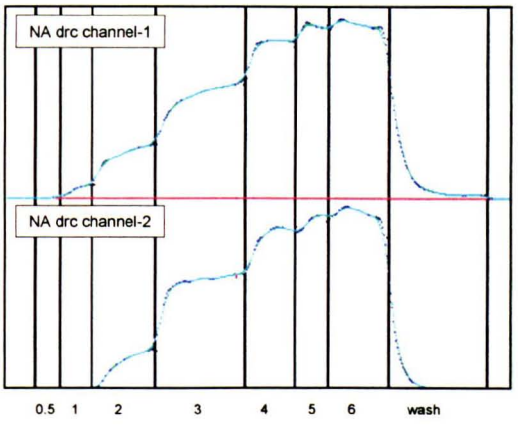


Fig.2.10. An example of the original myograph tracing for NA (0.5-6 µM) dose-response curve.

2.8 DATA INTERPRETATION AND STATISTICAL ANALYSIS

Changes in body weights of each group were collected weekly and are expressed as absolute total body weights. For Western blotting, the data from chow-fed (control) animals were expressed as 100% response, and the results from other test groups (in each experiment) were normalized and subsequently expressed as the percentage of their respective controls.

In myograph study, vasoconstriction in response to NA and KCl were expressed as absolute force generated. Relaxation in responses to CCh, SNP, or histamine were calculated as the percentage reduction from the maximal tension generated at the supramaximal concentration of NA (8 μM). The average response for all the vessels from a given animal was determined before group analysis.

In the vascular experiment (myograph), in order to determine the statistically significant difference between the experimental groups, ANOVA test was used, specifically, MANOVA tests were applied to perform multiple dataset analysis. Multivariate analysis of variance (MANOVA) is simply ANOVA with several dependent variables. However, the main objective of using MANOVA is to determine if the response variables are altered by the observer's manipulation of the independent variables. If the overall multivariate test is significant, we could conclude that the respective effect is significant.

In all experiments, data are expressed as mean ± standard error of mean (SEM). Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student t-test' or repeated-measures (Bratanova-Tochkova *et al.,* 2002), Bonferroni "t-test" or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p<0.05$ levels. All statistical analysis were performed using Microsoft Office Excel 2003.

2.9 LIVER HISTOLOGY

The tissue samples of liver were prepared for histological examinations using ordinary tissue processing and stained by Haematoxylin and Eosin (H and E) stains protocol. Ten liver specimens (10 animals) of each experimental group were interpreted by a single pathologist who was blinded to all data of liver samples and the experimental groups under investigation. The liver samples were analysed microscopically for the presence or absence of the following histological features: steatosis, steatohepatitis, fibrosis and hepatocellular carcinoma. Histological features were interpreted according to the schema outlined by Brunt et al. (Brunt *et al.*, 1999). Briefly, steatosis was graded on a 3-point scale: grade 1=steatosis involving < 33 % of hepatocytes, grade 2= 33-66%, grade 3 > 66%. Inflammation (steatohepatitis) was graded on a 4 point scale: grade 0=no or negligible inflammation, grade 1=mild, grade 2=moderate, grade 3=severe. Fibrosis was staged on a 5 point scale: stage 0=no fibrosis, stage 1=zone 3 perisinusoidal/ perivenular fibrosis, stage 2=zone 3 and periportal fibrosis, stage 3=septal/bridging fibrosis, stage 4=cirrhosis.

2.9.1 Procedure of liver histology

2.9.1.1 Tissue processing:

1- Liver tissues were fixed overnight in 10% neutral buffered formalin (NBF).

2- Tissue samples were dehydrated with ascending grade of alcohol (70%, 80%, 90%, and 100%). Each for an hour.

3- Tissue were then cleared with xylene; two changes each in an hour.

4- Tissues were impregnated with molten paraffin wax two changes each in an hour.

5- Tissues were then picked from molten paraffin and embedded in embedding paraffin to obtain tissue embedded paraffin blocks.

6- Tissues were then cut using microtome into 4 µm thickness and now the samples ready for H and E staining.

2.9.1.2 Heamatoxylin and Eosin staining protocol:

1- Slides were deparaffinised by immersing in xylene, two changes 3 minutes each.
2- Slides were rehydrated in descending grades of alcohol (100% two changes 5 minutes each, 90%, 70%, 5 minutes each).
3- Sections were left in running water for 5 minutes.
4- Sections were stained in haematoxylin by dipping for few minutes.
5- Sections were placed in running water until water looks clean and sections became blue.
6- Sections were places in 1% acid alcohol (99 ml 70% alcohol + 1 ml concentrated HCl) for two seconds.
7- Sections were washed in running tap water.
8- Sections were stained with eosin for 2 minutes.
9- Sections were washed in running tap water.
10- Sections were dehydrated in ascending grades of alcohol, cleared in xylene two changes.
11- Sections were mounted with DPX.

2.10 PLASMA AND TISSUE MEASUREMENTS AND ASSAYS

2.10.1 Plasma Insulin

2.10.1.1 Principle

Mercodia rat insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample react with peroxidase-conjugate anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction is stopped by adding acid to give colorimetric endpoint that is read spectrophotometrically.

2.10.1.2 Assay procedure

Plasma insulin concentrations were measured using a commercial ELISA kit. Standards (0.0; 0.15; 0.4; 1; 3; and 5.5 µg/l) and samples were added in duplicate to 96 well microtitre plate coated with anti-insulin antibodies and mixed with 50 µl conjugate solution, and incubated in a shaker for 2 hours at room temperature. The reaction volume was aspirated, and 350 µl washing solution was added to each well. The washing solution was then aspirated completely. The aspiration and washing procedure was repeated 5 times, after which to all wells, 200 µl of peroxidase substrate was added, and the plate was then incubated for further 15 minutes. Finally, 50 µl of stop solution was added to the wells. The plate was shaken for approximately 5 seconds to ensure mixing of substrate and stop solution. The absorbance was measured at 450 nm using 96 well plate reader. The concentrations of the samples were measured as µg/l from the obtained standard curve.

2.10.2 Plasma Leptin

2.10.2.1 Principle

Plasma leptin concentrations were measured with a commercial redioimmunoassay (RIA) kit (mouse leptin RIA; Linco, Dorset, UK). Mouse anti-leptin serum exhibits 100% cross-reactivity with rat leptin.

2.10.2.2 Assay procedure

Standards and samples (100 µl) were added to GL4 tubes (Beaumont, Leicester, UK) in duplicate and mixed with 100 µl of mouse leptin antibody. Tubes were vortexed, covered and left to incubate overnight at 4 °C. 100 µl of ^{125}I-mouse leptin was added; tubes were vortexed and incubated overnight at 4°C. Precipitating reagent (1 ml) was then added, and tubes were vortexed and incubated for 20 min at room temperature. After which tubes were centrifuged at 3000 x g at 4°C for 40 min. the tubes were decanted in one movement, careful not to lose the pellet. Bound radioactivity was counted on an automated gamma counter (Cobra auto-gamma; Berks, UK). The intra-assay coefficient of variation was below 10% and the detection limit was 0.2 ng/ml.

2.10.3 Plasma TNF-α

2.10.3.1 Principle

Plasma TNF-α concentrations were measured using a commercial ELISA kit (PeproTechEC Ltd, London, UK). Rat TNF-α development kit contains the key components required for the quantitative measurement of rat TNF-α in a sandwich ELISA format within the range of 32 - 3000 pg/ml.

2.10.3.2 Assay procedure

The standard was diluted from 3 ng/ml to zero in diluent. 100 µl of standard or sample was added to each well in duplicate, and incubated at room temperature for at least 2 hours. The solution in the plate was then aspirated and the plate washed 4 times. 100 µl detection antibody, diluted in diluent to a concentration of 0.5 µg/ml, was added to each well. The plate was then incubated for 2 hours at room temperature. The solution was aspirated again and the wells washed further 4 times. 100 µl of avidin peroxidase (1: 2000 diluted) was added to each well. The plate was then incubated at room temperature for further 30 minutes.

The plate then aspirated and washed further 4 times. Substrate solution (100 µl) was added to each well and the plate incubated at room temperature for colour development. The colour development was monitored with an ELISA plate reader at 405 nm. The concentrations of the samples were measured as pg/ml from the obtained standard curve.

2.10.4 Plasma C-Reactive Protein (CRP)

2.10.4.1 Principle

The rat CRP ELISA is based on a solid phase enzyme-linked immunosorbent assay (Filippatos *et al.,* 2005). The assay uses affinity purified anti-rat CRP antibodies for solid phase (microtiter wells) immobilization and HRP conjugated anti-rat CRP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. These results in CRP molecules being sandwiched between the

immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB reagent is added and incubated for 20 minutes at room temperature. This results in the development of blue colour. Colour development is stopped by the addition of stop solution, changing the colour to yellow, and optical density is measured spectrophotometrically at 405 nm. The concentration of CRP is proportional to the optical density of test sample.

2.10.4.2 Assay procedure

Standards (31.25, 15.6, 7.8, 3.9, 1.95, 0.98, and 0 ng/ml) and samples were added in duplicate to the wells of microtiter plate which coated with anti-rat CRP antibody, and incubated on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 °C) for 45 minutes. The incubation mixture then removed by flicking plate into an appropriate Bio-waste container. The microtiter wells were rinsed and flicked 6 times with distilled water. The wells were then striked sharply onto absorbent paper towels to remove all residual water droplets. Enzyme conjugate reagent (100 µl) was added to each well and incubated on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 °C) for further 30 minutes. The wells were washed as detailed above (by flicking, rinsing, and striking the wells sharply onto absorbent paper or towels to remove all residual water droplets). TMB reagent (100 µl) was then added to each well and gentle mixed on an orbital micro-plate shaker at 100-150 rpm at room temperature for another 20 minutes. The reaction was stopped by adding 100 µl of stop solution to each well and gently mixing (it's important to make sure that all the blue colour changes to yellow). The optical density was read at 405 nm with a microtiter plate reader within 15 minutes. The concentrations of the samples were measured as ng/ml from the obtained standard curve.

2.10.5 Triglyceride content of muscle and liver tissues

Muscle tissue (160-200 mg) or liver tissue (80-100 mg) were homogenised in 500 µl distilled water with polytron homogeniser. The homogenate were centrifuged for 5 minutes at 13,000 x g, and the supernatants were transferred

to eppendurff tubes. The triglycerides assay was performed on the supernatant to detect the concentration of TG in muscle or liver tissue.

2.10.6 Plasma triglycerides:

2.10.6.1 Principle

The series of reactions involved in the assay system are as follows:

Triglycerides + H_2O ------ Lipase ------ Glycerol + Free Fatty Acids

The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP).

Glycerol + ATP ------ GK ------ Glycerol-3-phosphate ADP

Glycerol-3-phoaphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxide (H_2O_2).

Glycerol-3-phosphate + O_2 ------ GPO ------ DAP + H_2O_2

in a trinder type colour reaction catalyzed by peroxidase (POD), the H_2O_2 reacts with 4-aminoantipyrine (4 AAP) and 3,5 dichloro-2 hydroxybenzene sulfonate (3,5 DHBS) to produce a red colored dye. The absorbance of this dye is proportional to the concentration of triglycerides present in the sample.

H_2O_2 + 4AAP + 3,5DHBS ------ POD ------ Quinoneimine dye + $2H_2O$

2.10.6.2 Assay procedure

Serial dilutions of standards were prepared as following: 125; 62.5; 31.25; 15.512; 7.756; and 0.0 mg/dl. In 96 well micro titre plate, 3 µl of each sample or standard in duplicate plus 300 µl of reagent solution was added to the wells. The plate was then incubated for 5 minutes at room temperature. Finally, the absorbance was read at 520 nm using micro titre plate reader and the concentrations of unknown samples were obtained from the standard curve.

2.10.7 Plasma Glucose

2.10.7.1 Principle

The test kit for glucose, non-colorimetric (Roche Diagnostics; Milton Keynes, UK) is based on the following principle: D-glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (Bratanova-Tochkova *et al.,* 2002) and adenosine-5'phosphate (ATP), with the simultaneous formation of adenosine-5'-diphosphate (ADP):

D-glucose + ATP ------ HK ------ G-6-P + ADP

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH):

G-6-P + $NADP^+$ ------ G6P-DH ------ D-gluconate-6-phosphate + NADPH + H^+

The amount of NADPH formed in this reaction is stiochiometric to the amount of D-glucose. The increase in NDAPH is measured by means of its light absorbance at 340 nm in the UV range.

2.10.7.2 Assay procedure

First, plasma samples had to be de-proteinised. This involved mixing 1 ml of 3% perochloric acid with 100 µl of samples in a 1.5-ml Eppendorf tube and centrifuging for 5 minutes at 5000 rpm. Then, 1 ml of a solution containing NADP and ATP was added to an empty 3-ml plastic cuvette (the "blank") as well as to all the cuvettes containing 100-µl volumes of the de-proteinised plasma solution. The volume was made up to 3 ml by adding 2 ml of distilled water to all cuvettes. The final concentrations of NADP and ATP were 0.8 mg/ml and 1.9 mg/ml, respectively. The solutions were mixed and the first set of absorbencies (A1) read at 340 nm at room temperature after about 3 min, referencing against the blank. Next, 20 µl of a suspension containing Hexokinase (Bratanova-Tochkova *et al.*, 2002) and glucose-6-phosphate-dehydrogenase (G6P-DH) (final concentrations 1.94 U/ ml and 0.97 U/ml, respectively) we added to each cuvette and the solutions mixed. After allowing

the reaction to proceed for 15 min (the endpoint of reaction). The second set of absorbencies were read (A2) at 340 nm. These absorbencies were then applied to the general equation for calculating concentrations (C):

$$C = V X \Delta A / v x d x \varepsilon$$

Where

V = final volume (ml)

v = sample volume (ml)

d = light path (cm)

ε = absorption coefficient of the dyestuff at the wavelength,; in this case, ε=extinction coefficient of NADPH at 340 nm = 6.3

ΔA = the absorbance difference (A2 – A1)

This reduces to the formula

52.49 x ΔA

To yield the concentration of glucose in plasma in mM units.

2.10.8 Free Fatty Acid (FFA)

2.10.8.1 Principle

FFAs are, in the presence of the enzyme acyl-CoA synthetase (acyl CS), converted by ATP and coenzyme A (CoA) into acyl-coenzyme A (acyl-CoA), resulting in adenosine-5'-monophosphate (AMP) and pyrophosphate. Acyl CoA reacts with oxygen (O_2) in the presence of acyl-CoA oxidase to form 2,3-enoyl-coenzyme A (enoyl-CoA).

The resulting hydrogen peroxide (H_2O_2) converts 2,4,6-tribromo-3-hydroxy-benzoic acid (TBHB) and 4-aminoantipyrine to a red dye in the presence of peroxidase (POD). The dye is measured in the visible range at 546 nm.

FFAs + CoA + ATP ------ Acyl CS ------ acyl-CoA + AMP + pyrophosphate

Acyl-CoA + O2 ------ ACOD ------ enoyl-CoA + H_2O_2

H_2O_2 + 4-AAp + TBHB ------ POD ------ red dye + 2 H_2O + HBr

2.10.8.2 Assay procedure

One milliliter of a solution containing ATP, CoA, acyl CS, POD and 4-AAp and TBHB was added to an empty 2-ml plastic cuvette (the blank) as well as to cuvettes containing 50 µl volumes of sample. The volume of the blank was made up with 50 µl of distilled water. The solutions were mixed and allowed to sit for 10 minutes at room temperature. Fifty µl of a solution containing N-ethyl-maleinimide was then added to all cuvettes for the removal of an existing surplus of CoA before the oxidation of the activated FFAs by ACOD. The solutions were then mixed and the first set of absorbencies (A1) read at 546 nm measured against water. The reaction was started by adding 50 µl of a solution containing ACOD to all cuvettes. The solutions were mixed again and the end of the reaction reached at room temperature after 15 min. the second set of absorbencies (A2) were then read, again at 546 nm. The absorbance differences (A2 – A1) were calculated for both blank and unknown (plasma) and applied to the formula:

$$\Delta A = \Delta A_s - \Delta A_b,$$

Where ΔA_s and ΔA_b are the absorbance differences of each unknown sample and the blank respectively. These volumes were then applied to the formula

$$1.192 \times \Delta A$$

Derived from the general formula for calculating the concentration outlined above (in glucose assay) and where:

$$\varepsilon = \text{absorption coefficient of the red dye at 546 nm} = 19.3$$

to yield the concentration of FFAs in plasma in mM units.

Chapter 3

Results: Differential effects of high-energy diets on vascular reactivity precede significant obesity: Combined high-carbohydrate and high-fat induces greater vascular dysfunction than high-fat alone

3.1 Introduction

Obesity is an important risk factor for atheroma, and is thought to be a major factor in the development of hypertension (Lakka *et al.*, 2002). Arterial dysfunction and structural abnormalities, ranging from alterations in release of vasoactive substances from endothelial cells to changes in arterial wall elasticity and agonist-induced vasodilatory responses, have been seen in obese human subjects and in animal models that reflect some aspects of human obesity (Cowan *et al.*, 1991; Steinberg *et al.*, 1996; Arcaro *et al.*, 1999; Dobrian *et al.*, 2000; Perticone *et al.*, 2001; Naderali, 2001b). Thus, the link between obesity and vascular dysfunction is well-established. Critically, however, many of the components of the metabolic syndrome, including cardiovascular disorders, are manifest in preobesity (Ota *et al.*, 2002). Indeed, acute vascular dysfunction is evident in humans after only a couple of high-energy meals (Ong *et al.*, 1999), and animal models reveal that irreversible vascular impairment can arise as early as two days after consumption of a high-energy diet (Naderali, 2001b; Naderali, 2003).

The causes of vascular dysfunction in obesity and related disorders remain uncertain, but plasma non-esterified fatty acids (NEFAs), triglycerides (TGs), leptin and insulin, all of which are increased in obesity, are prime candidates (Despres *et al.*, 1996; Steinberg, 1997; Tka *et al.*, 1997; Glueck *et al.*, 1999; Soderberg *et al.*, 1999; Wallace *et al.*, 2001; Naderali, 2001b). Of these, only NEFAs and TGs are raised by short-term high-energy feeding, suggesting that they may play the more critical role (Naderali, 2001b; Naderali, 2003). Consumption of diets high in either carbohydrate or saturated fat results in atherogenic dyslipidaemia (German and Dillard, 2004), further suggesting a link between dietary components and vascular abnormalities. It is less clear whether or not these components have different effects on vascular function and, if so, what these are.

It is reasonable to suggest that the current obesity epidemic, at least in the Western world, is due to consumption of energy-dense foods which are relatively high in both carbohydrate and fat, rather than one or the other.

Thus, it was of value to test the vascular effects of such a diet. As a secondary aim, it was also of value, whilst exploring the obesity-inducing effects of various diets in our laboratory, to identify those which, after a prolonged period of consumption, failed to induce significant obesity.

The hypothesis, therefore, is that different dietary components would differentially effect vascular function, that a combination of such components would exacerbate dysfunction, and that these effects would be measurable in a prevailing phase of overweight. This would be shown by measuring vascular function in rats fed diets high in various atherogenic components for a prolonged period.

3.2 METHODS

3.2.1 Experimental protocol

Adult male Wistar rats (192 ± 4 g) were randomly assigned to three groups (n=6/group) which, throughout a 10-week period, were each fed diets of differing compositions. This duration was chosen to model human chronic consumption of high-energy food while inducing weight gain, representing a state of obesity. Control animals received standard pelletted laboratory chow, providing 63% of energy as carbohydrate, 28% as protein and 10% as fat (chow group). One of the experimental groups received chow supplemented with chocolate biscuit, providing 56% of energy as carbohydrate, 15% as protein and 30% as fat (biscuit-fed group), and the second received a fat-enriched diet, providing 35% of energy as carbohydrate, 20% as protein and 45% as fat (high-fat-fed group). A more detailed breakdown of the macronutrient composition of the diets is given in Table 1. Animals were housed singly under controlled environmental conditions. Food intake was measured daily and body weight weekly.

At the end of the experiment, after two hours fasting, the rats were killed by CO_2 inhalation. A single epididymal fat pad and gastrocnemius muscle was dissected free from each animal and weighed. As an index of body composition, these values were expressed as a ratio. Blood was collected into cold heparinized tubes and plasma separated by centrifugation and stored

frozen for later measurement of analytes such as: glucose, insulin, leptin, non-esterified fatty acids (NEFA) and triglyceride (TG).

3.2.2 Assessment of vascular function

Six third-order mesenteric arteries were carefully dissected from each animal and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK). Pairs of arteries were incubated in a 5-ml organ bath containing physiological salt solution (PSS) gassed with 95% O_2 and 5% CO_2 at 37 °C.

After a 30-min equilibration, the length-tension characteristics for each vessel were determined, as described previously in chapter two, section 2.7.1. Arteries were allowed a further 30 min to equilibrate before being depolarized twice with high-potassium PSS (KPSS), in which NaCl in normal PSS was replaced by an equimolar concentration of KCl (final $[K^+]$ = 125 mM). Any vessel failing to reach its predetermined target tension in response to KPSS was discarded. Arterial contractility was evaluated from concentration-response curves to KCl (10-125 mM) and noradrenaline (NA; 0.5-6 μM).

Arterial relaxation was measured in NA-pre-constricted arteries following exposure to two endothelium-dependent vasodilators, carbamylcholine (CCh) and histamine, and an endothelium-independent vasodilator, sodium nitroprusside (SNP). Arterial segments were contracted with a supramaximal concentration of NA (8 μM). When contraction reached a plateau (after 2 min), responses to CCh, histamine and SNP over the range of 10 nM-100 μM were carried out in random order (for more details about the assessment of vascular function, see chapter two, sections 2.7.1 & 2.7.2).

3.2.3 Statistical analyses

Vasoconstriction in response to NA and KCl was expressed as absolute force generated, whilst relaxation in response to CCh, histamine and SNP concentrations was calculated as percentage reduction from the maximal tension induced by 8 μM NA.

Data are expressed as mean ± S.E.M. An average response for all the vessels from a given animal was determined before group analysis. EC_{50} values were determined to evaluate changes in agonist-induced responses. Significance of statistical differences between vascular responses were tested using repeated measures analysis of variance (Bratanova- *et al.*, 2002), followed by *post-hoc* Bonferroni *t*-tests. Differences between diet-fed groups and chow-fed controls for all other parameters were tested by student's (unpaired) "*t*-test" or Mann-Whitney test, as appropriate. Within-group differences were analysed by paired "*t*-test". Vasorelaxation data were first quantified by calculation of area under the curve (AUC) using the trapezoidal rule. Differences were considered statistically significant at $p<0.05$.

3.3 RESULTS

3.3.1 Body weight, food intake and metabolic data

Animals fed the biscuit- or high-fat diet gained more weight than their chow-fed counterparts, although this did not reach significance (+13% and 16%, respectively; both $p>0.05$; Fig. 3.1). This was in accordance with higher daily energy intakes for both groups (+27% and 8%), but only that of biscuit-fed animals rose significantly ($p<0.01$). Consistent with these findings, both biscuit- and high-fat-fed groups had increased epididymal fat pad masses (+72% and 48% *vs* controls, Fig. 3.4), although only significantly so in the former ($p<0.05$). In contrast, gastrocnemius muscle mass was comparable in all three groups (Fig. 3.5), resulting in a significant change in fat/lean ratio (body composition) only on the biscuit-supplemented diet ($p<0.05$ *vs* controls; Fig. 3.6).

Although neither diet affected circulating concentrations of glucose, insulin or leptin (all $p>0.05$ *vs* chow-fed controls, Figs. 3.7 – 3.9), triglycerides rose by 41% in the biscuit-fed group ($p<0.05$). The high-fat diet increased circulating NEFAs by twice the proportion of the biscuit-supplemented diet (+38% and 17% *vs* controls; $p<0.01$ and $p<0.05$, respectively; Fig. 3.11).

3.3.2 Arterial contractile responses

Neither of the diets altered vessel diameter (chow-fed: 190 ± 10 μm; biscuit-fed: 200 ± 10 μm; high-fat-fed: 200 ± 15 μm; both $p>0.05$ *vs* chow-fed). KCl- and NA-induced vasoconstriction in arteries from all three groups produced a characteristic sigmoid relationship. Both agonists increased vasoconstriction in the biscuit-fed group ($p<0.01$ and $p<0.001$, respectively), but had no effect in the high-fat-fed group (both $p>0.05$). Maximal KCl- and NA-induced contractions induced were the following, respectively: chow-fed, 5.29 ± 0.52 and 6.58 ± 0.89 mN/mm; biscuit-fed, 6.79 ± 0.58 and 11.14 ± 0.11 mN/mm; high-fat-fed, 5.25 ± 0.21 and 6.55 ± 1.1 mN/mm (Figs. 3.12 & 3.13).

3.3.3 Endothelium-dependent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM-100 μM), achieving a maximum of 89 ± 5% at a concentration of 100 μM, with EC_{50} values of 0.11 ± 0.01 μM. Arteries from biscuit-fed and high-fat-fed rats that were similarly exposed to CCh displayed a significant rightward shift of concentration-response curves compared with those of chow-fed controls (EC_{50} biscuit-fed: 0.28 ± 0.01; high-fat-fed: 0.52 ± 0.04 μM; both $p<0.001$; Fig. 3.14). Maximal vasorelaxation to 100 μM CCh was reduced by 13% in both biscuit- and high-fat-fed rats (both $p<0.01$ *vs* chow-fed), but was more attenuated in arteries from high-fat- than biscuit-fed rats at lower CCh concentrations (3.16-316 nM; $p<0.01$; Fig. 3.14). Histamine-induced vasorelaxation was similar in all three groups, the concentration-response curves being virtually superimposable. Maximum vasorelaxations induced (at 100 μM) were 81± 3%, 86 ± 5% and 75 ± 7% for chow-, biscuit- and high-fat-fed rats, respectively (Fig. 3.15).

3.3.4 Endothelium-independent vasorelaxation

The endothelium-independent vasodilator, SNP, also induced concentration-dependent vasorelaxation in arteries from all three groups, the curves being significantly shifted to the right in the biscuit- and high-fat-fed groups (EC_{50} biscuit-fed: 1.58 ± 0.02; high-fat-fed: 0.24 ± 0.01; both $p<0.01$ *vs* chow-fed: 0.13 ± 0.02). However, maximum SNP-induced responses were similar in all

three groups. Compared with the chow-fed control and high-fat-fed groups, SNP-induced relaxation in biscuit-fed rats was attenuated by more than 2-fold (AUC for chow-fed: 10.89 ± 0.31; biscuit-fed: 3.59 ± 0.23; high-fat-fed: 8.91 ± 0.07; $p<0.001$, biscuit-fed *vs* both control and high-fat-fed). Although the attenuation of SNP responses was less pronounced in high-fat-fed animals, it nevertheless reached statistical significance (AUC for high-fat *vs* control: $p<0.05$; Fig. 3.16).

Table 3.1 Breakdown of digestible macronutrients (% of energy) in different diets. Values were calculated from manufacturers' nutritional analyses. Summed macronutrient values may not exactly match total values due to small amounts of calorific ingredients which do not fit under these categories and/or to slightly discrepant values given in manufacturers' analysis sheets. Abbreviations: CHO = carbohydrate.

Groups	Total CHO	Complex CHO	Simple CHO	Total fat	Saturated fat	MUFA	PUFA	Total protein
Chow	60.0	55.7	4.3	10.0	1.7	3.0	5.3	30.0
Biscuit	55.0	37.0	18.0	30.0	12.9	10.6	5.4	15.0
High-fat	35.0	16.9	16.9	45.0	39.1	0.0	5.5	20.0

Table 3.2 Daily macronutrient intake (kJ) in male Wistar rats fed different diets for 10 weeks. Statistical significance *vs* chow controls: * $p<0.05$; ** $p<0.01$; *** $p<0.0001$ (unpaired *t*-test; n=6/group). Abbreviations: CHO = carbohydrate; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

Diet	Overall total	Total CHO	Complex CHO	Simple CHO	Total fat	Saturated fat	MUFA	PUFA	Total protein
Chow	361.1+15.3	227.6+9.6	208.5+8.8	19.1+0.8	37.2+1.6	6.1+0.2	11.0+0.5	20.0+0.8	101.6+4.3
Biscuit	459.0+13.6**	255.6+6.8	167.8+3.2**	88.1+4.2***	140.6+6.5***	60.8+3.1***	50.0+2.4***	24.8+0.7**	67.0+1.2***
High-fat	390.2+13.3	136.6+4.7***	66.3+2.3***	66.3+2.3***	175.6+6.0***	152.5+5.2***	0.0	21.5+0.7	78.0+2.7**

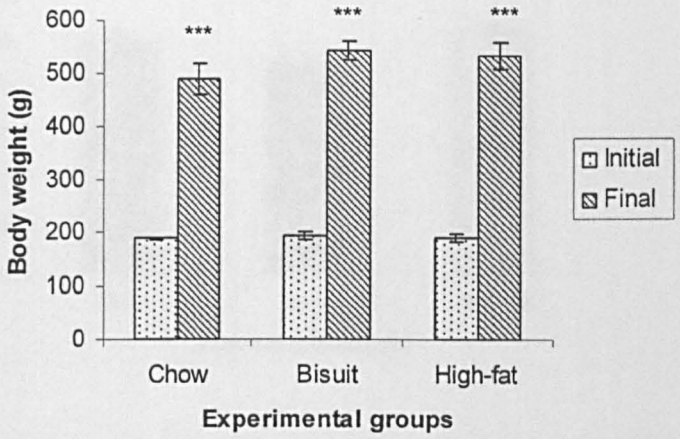


Fig. 3.1. Comparison between initial and final body weight of three experimental groups: chow, biscuit, and high-fat feeding. Statistical significance vs initial body weight: *** $p<0.0001$ (paired t-test; n=6/group).

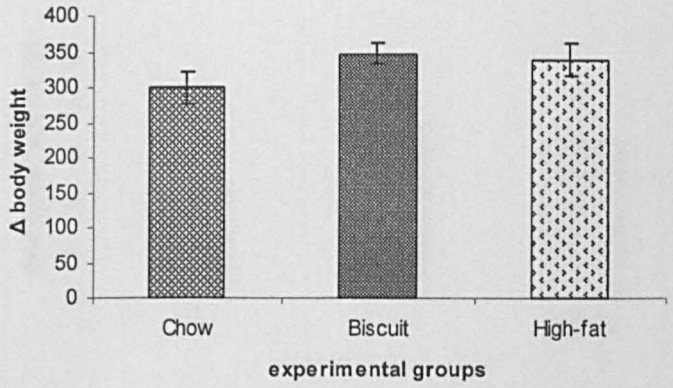


Fig. 3.2. Δ body weight of the three experimental groups: chow, bisciut, and high-fat.

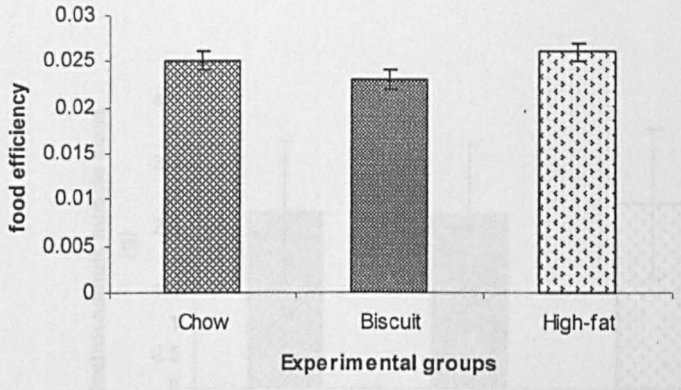


Fig. 3.3. Food efficiency for the three experimental groups: chow, biscuit, and high-fat feeding. There were no significant differences in comparison to chow feed control.

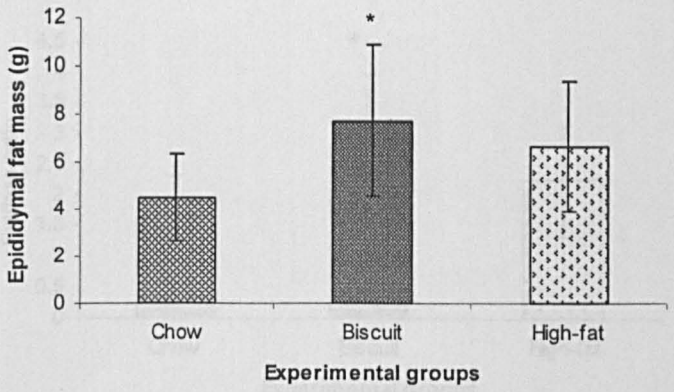


Fig. 3.4. Epididymal fat mass was measured in all three experimental groups: chow, biscuit, and high-fat feeding. The fat mass in biscuit group was significantly more heavier than that of chow-fed control.

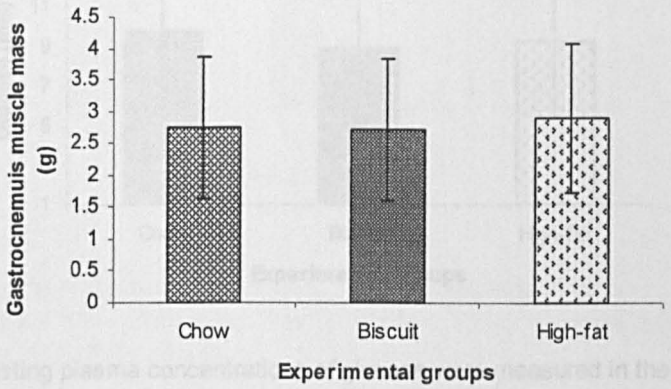


Fig. 3.5. Gastrocnemuis muscle mass was measured in all three experimental groups: chow, biscuit, and high-fat. No significant differences were seen between the three groups.

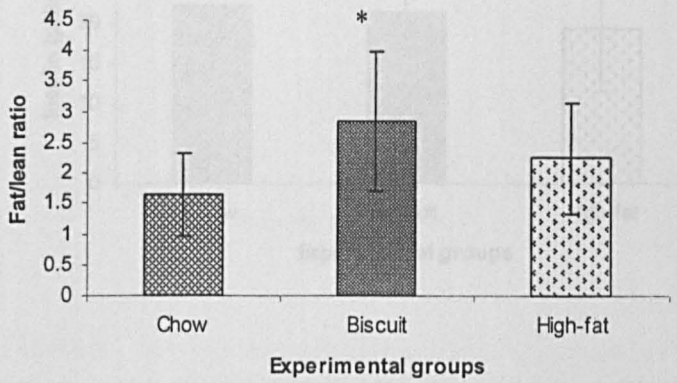


Fig. 3.6. Fat/lean ratio for the three experimental groups: chow, biscuit, and high-fat feeding. There was significant increase in biscuit group in comparison with chow fed control.

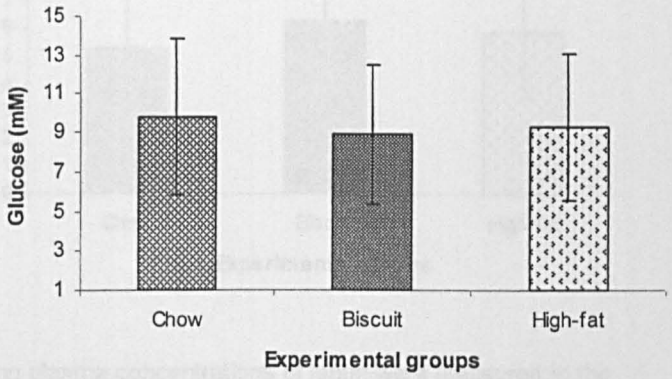


Fig. 3.7. Fasting plasma concentrations of glucose were measured in the experimental groups: chow, biscuit, and high-fat feeding. No significant differences were seen between the three groups.

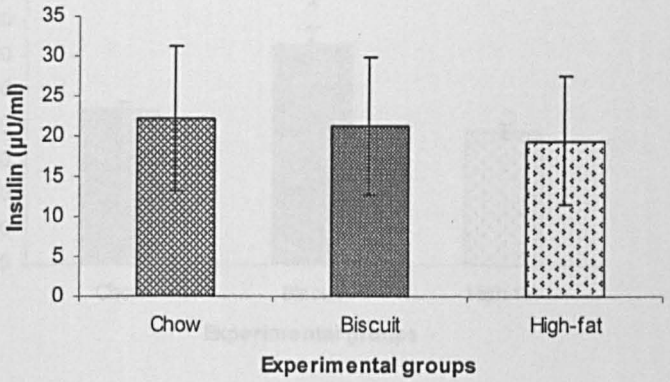


Fig. 3.8. Fasting plasma concentrations of insulin were measured in the experimental groups: chow, biscuit, and high-fat feeding. No significant differences were seen between the three groups.

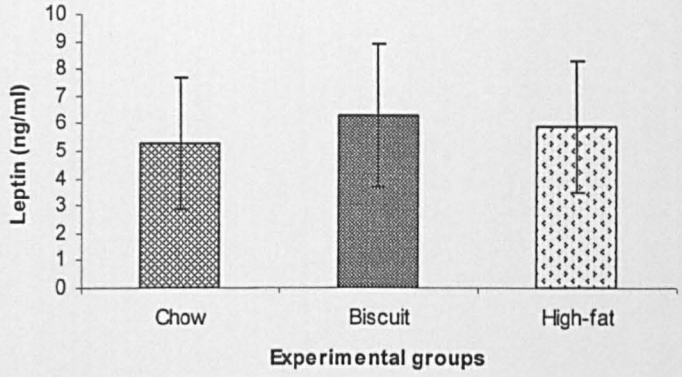


Fig. 3.9. Fasting plasma concentrations of leptin were measured in the experimental groups: chow, biscuit, and high-fat feeding. No significant differences were seen between the three groups.

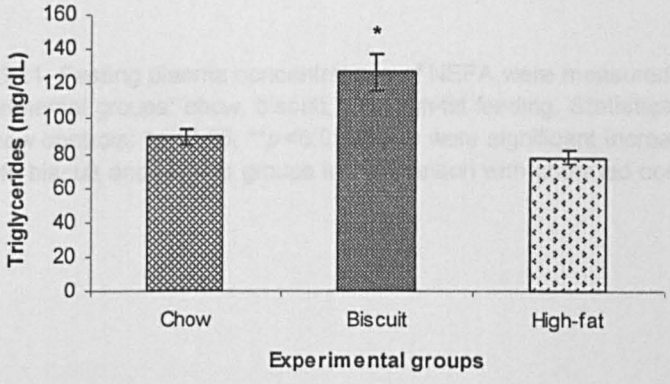


Fig. 3.10. Fasting plasma concentrations of triglycerides were measured in the experimental groups: chow, biscuit, and high-fat feeding. Statistical significance vs chow controls: *$p<0.05$. Biscuit- but not high-fat-fed group had significantly higher TG than their chow-fed counterpart.

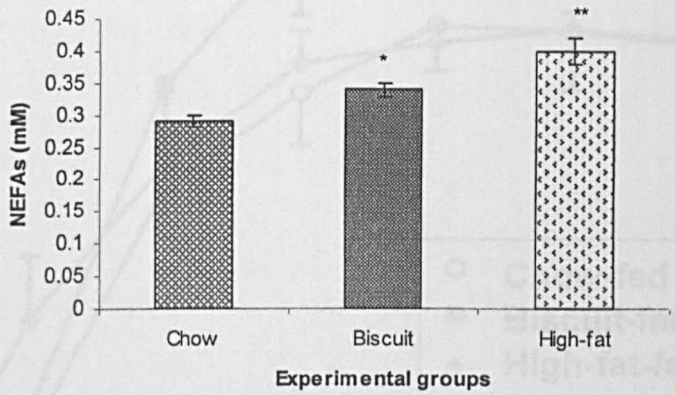


Fig. 3.11. Fasting plasma concentrations of NEFA were measured in the three experimental groups: chow, biscuit, and high-fat feeding. Statistical significance vs chow controls: *$p<0.05$; **$p<0.01$. There were significant increase in the levels of both biscuit and high-fat groups in comparison with chow fed control.

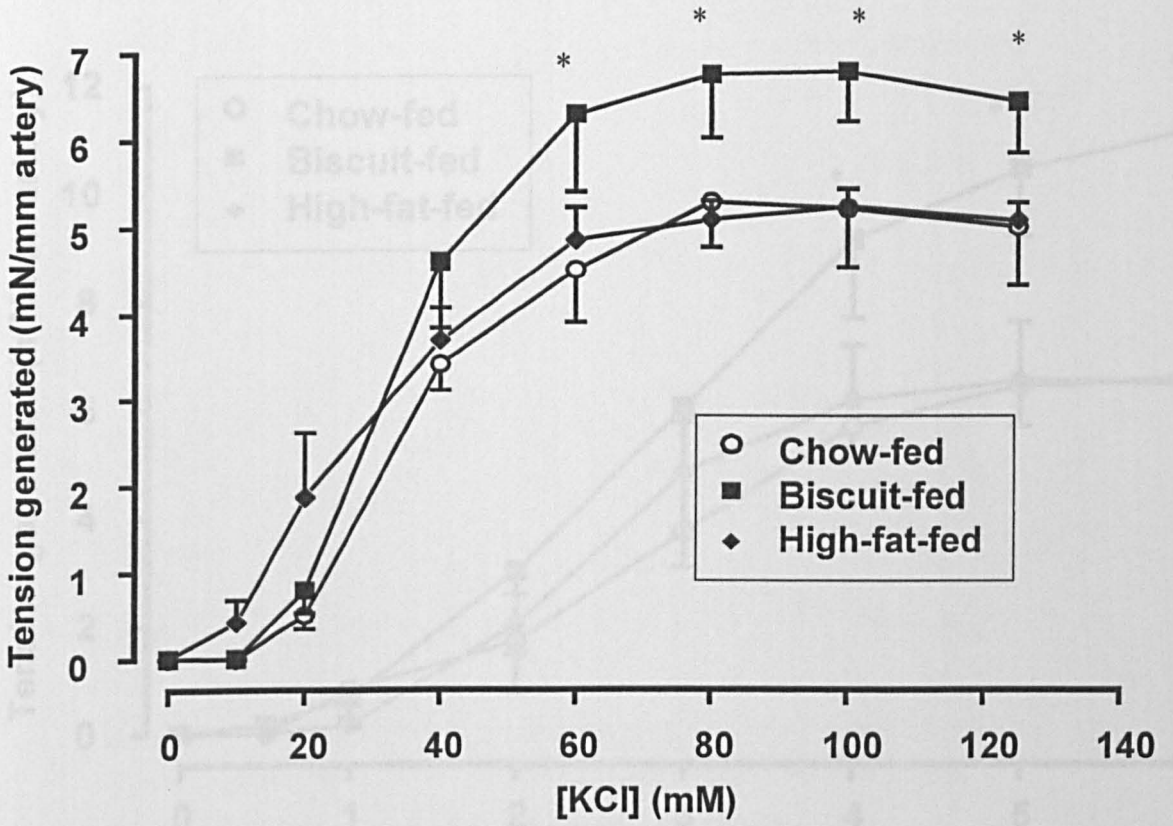


Fig. 3.12 Concentration-response curves for KCl (10-125 mM) in arteries from male Wistar rats fed chow, biscuit-supplemented or high-fat diet for 10 weeks. Data are expressed as mean ± S.E.M. Compared with chow-fed controls, KCl-induced vasoconstriction was significantly augmented in arteries from biscuit-fed animals (*p<0.01; ANOVA (Bonferroni test); n=6/group).

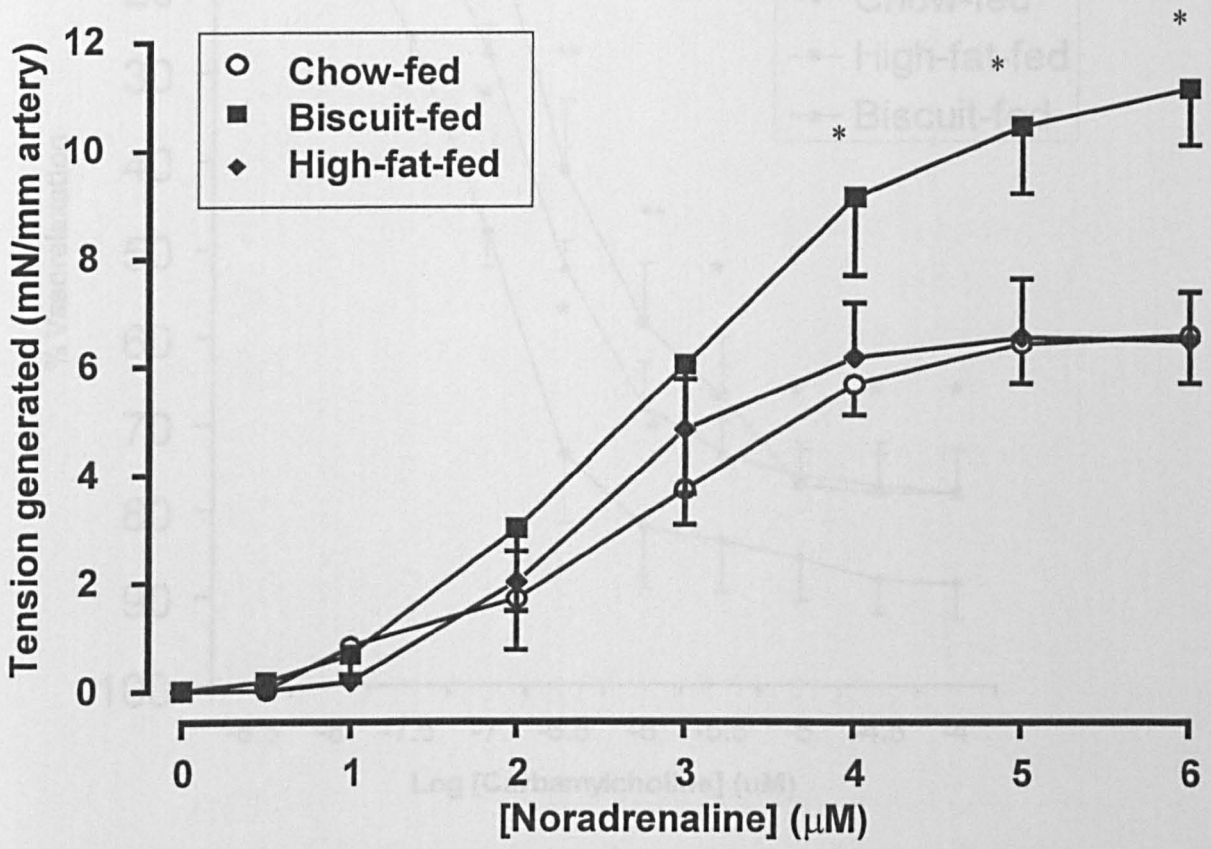


Fig. 3.13 Concentration-response curves for noradrenaline (NA; 0.5-6 μM) in arteries from male Wistar rats fed chow, biscuit-supplemented or high-fat diet for 10 weeks. Data are expressed as mean ± S.E.M. Compared with chow-fed controls, NA-induced vasoconstriction was significantly augmented in arteries from biscuit-fed animals (*p<0.01; ANOVA (Bonferroni test); n=6/group).

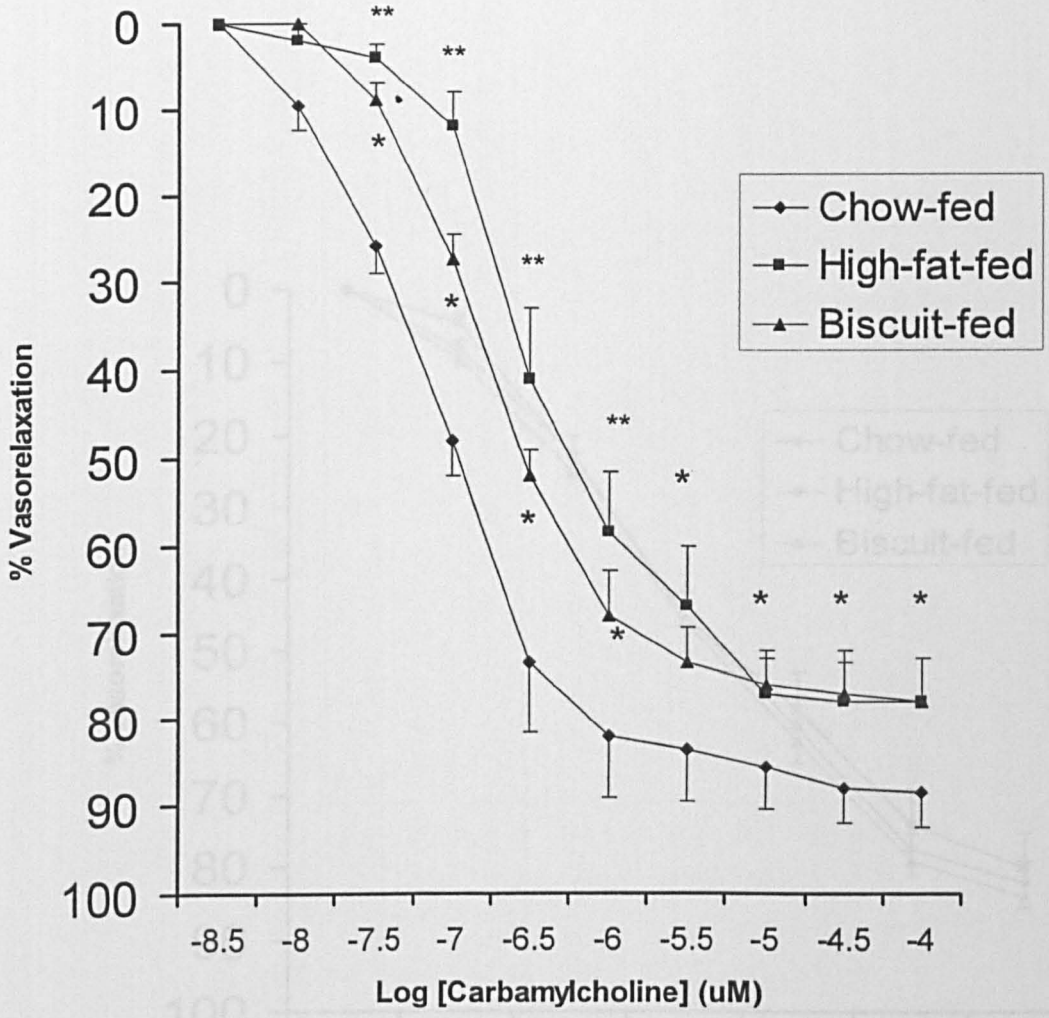


Fig. 3.14 Concentration-response curves for carbamylcholine in noradrenaline (NA)-precontracted arteries from male Wistar rats fed chow, biscuit-supplemented or high-fat diet for 10 weeks, as described in Methods. Data represent mean ± S.E.M. Compared with chow-fed controls, vasorelaxation in response to CCh was significantly attenuated in biscuit-fed and high-fat-fed animals (*$p<0.01$ and **$p<0.001$, respectively; ANOVA (Bonfferoni test); n=6/group).

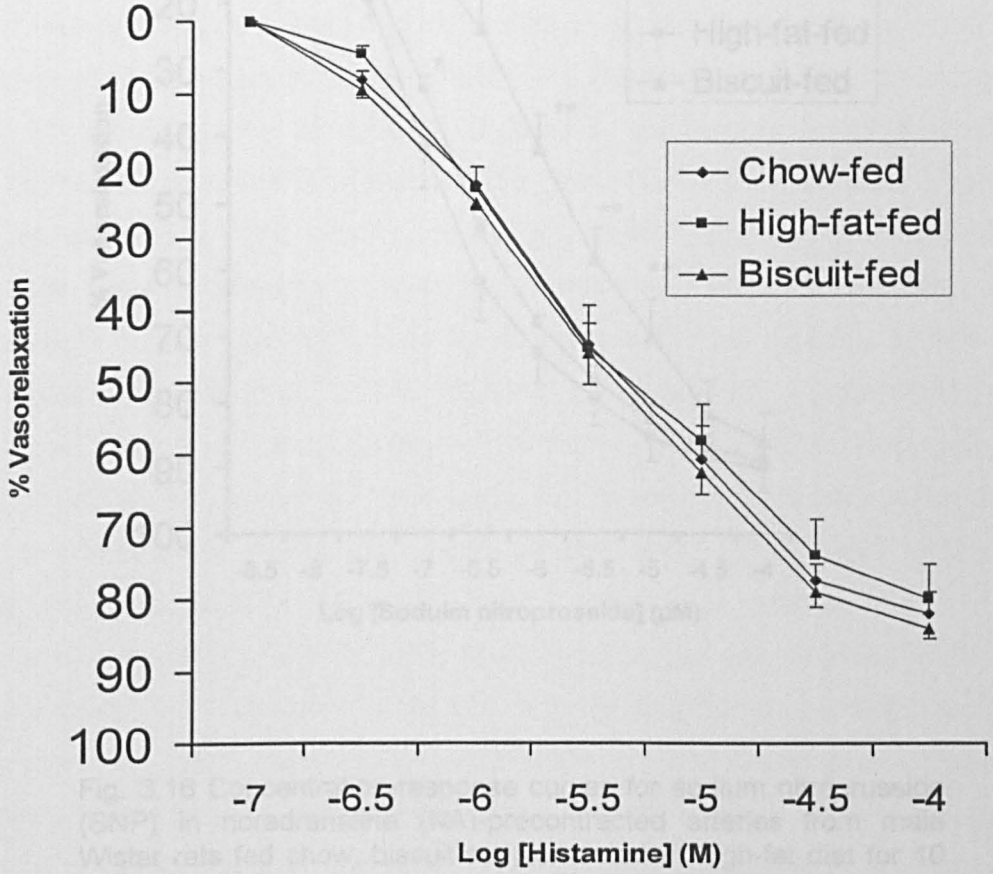


Fig. 3.15 Concentration-response curves for histamine in noradrenaline (NA)-precontracted arteries from male Wistar rats fed chow, biscuit-supplemented or high-fat diet for 10 weeks, as described in Methods. Data represent mean ± S.E.M. No significant changes were seen between all three groups (n=6/group).

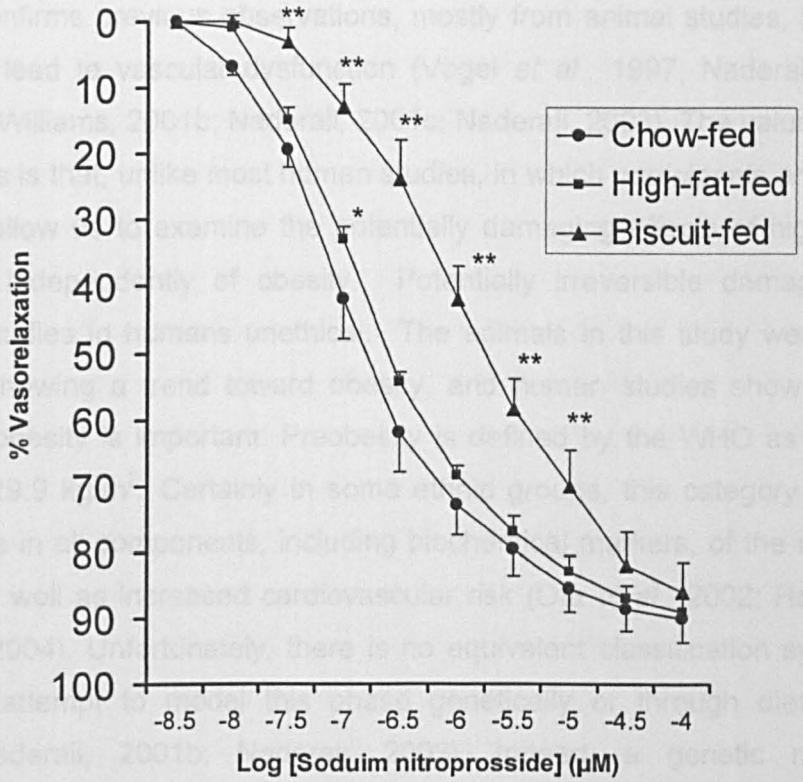


Fig. 3.16 Concentration-response curves for sodium nitroprusside (SNP) in noradrenaline (NA)-precontracted arteries from male Wistar rats fed chow, biscuit-supplemented or high-fat diet for 10 weeks, as described in Methods. Data represent mean ± S.E.M. SNP-induced relaxation was greatly attenuated in biscuit-fed and, to a lesser extent, in high-fat-fed rats (*P<0.05 and **p<0.01, respectively; ANOVA (Bonferroni test); n=6/group).

3.4 DISCUSSION

This study confirms previous observations, mostly from animal studies, that high-energy diets lead to vascular dysfunction (Vogel *et al.*, 1997; Naderali, 2001b; Naderali and Williams, 2001b; Naderali, 2001c; Naderali, 2003). The value of these animal studies is that, unlike most human studies, in which participants are already obese, they allow us to examine the potentially damaging effects of high-energy consumption independently of obesity. Potentially irreversible damage would make such studies in humans unethical. The animals in this study were clearly overweight, showing a trend toward obesity, and human studies show that this period of preobesity is important. Preobesity is defined by the WHO as having a BMI of 25.0-29.9 kg/m^2. Certainly in some ethnic groups, this category shows a significant rise in all components, including biochemical markers, of the metabolic syndrome, as well as increased cardiovascular risk (Ota *et al.*, 2002; Hop le and Xuan Ngoc, 2004). Unfortunately, there is no equivalent classification system for rodents, but attempt to model this phase genetically or through diet can be achieved (Naderali, 2001b; Naderali, 2003). Indeed, a genetic model of atherosclerosis, the JCR:LA-cp rat, prior to development of frank obesity, shows profound alterations in fat and muscle LDL activity (Mantha *et al.*, 2002). It is arguably more important, however, to demonstrate relevant changes in a model more closely resembling the multifactorial nature of most forms of human obesity, which arise on a polygenic background in response to consumption of energy-dense food. In previous studies, vascular damage has been induced prior to obesity onset with short-term feeding of high-energy diets (Naderali, 2001b; Naderali, 2003). It is logical that this would therefore also be true after chronic consumption of a high-energy diet, but this has not been demonstrated until now, as most feeding paradigms of this nature would be expected to induce frank obesity. This study shows, how intriguing it is that, depending on the particular dietary stimulus (in this case, composition), vascular damage can prevail in the absence of significant phenotypic changes reflecting obesity. Thus, one could suggest that some humans are consuming atherogenic diets for prolonged periods,

but are failing to be alerted to their increased cardiovascular risk because they remain relatively slim.

The high-fat and biscuit-supplemented diets used in this study induced similar degrees of weight gain and associated metabolic abnormalities despite having different macronutrient compositions. This suggests that these differences in composition account for the differences observed in vascular reactivity. The biscuit-supplemented diet exerted more pronounced effects on vascular reactivity overall. It heightened contractility, as shown by augmentation of both KCl- and NA-induced vasoconstriction, whereas the high-fat diet had no effect on either of these responses. It also attenuated vasorelaxation in response to the cGMP-mediated actions of both CCh and SNP, as did the high-fat diet. This means that this effect of both diets is mediated through both endothelium and vascular smooth muscle. Consumption of other high-energy diets has shown this dual mechanism (Naderali, 2001b; Naderali and Williams, 2001b; Naderali, 2003), although some such diets exert their effects only through one mechanism (see chapter four), further highlighting the specificity of effects depending on dietary components. The failure of either diet to alter cAMP-mediated vasorelaxation (i.e., histamine-induced responses) also appears to be a common feature of high-energy diets (Naderali, 2001b; Naderali, 2003).

Intriguingly, the degree of abnormality seen in both endothelium- and smooth muscle-mediated vasodilation also differed according to diet. CCh-induced vasorelaxation was more severely attenuated by the high-fat diet, whilst the SNP response more so by the biscuit-supplemented diet. These findings suggest that different dietary components affect vasorelaxation by different mechanisms, that a diet high in fat has greater deleterious effects on the arterial endothelial lining, whereas a diet relatively rich in carbohydrate and fat largely compromises the vascular smooth muscle. There is substantial supporting evidence that dietary components play a role in vascular function. Humans and animals fed diets high in fat, particularly saturated fat (Vogel *et al.*, 1997; German and Dillard, 2004; Seo *et al.*, 2005; Verhamme *et al.*, 2002), or low in protein (Koumentaki *et al.*, 2002) show endothelial abnormalities. On the other hand, the protective effects of high-

carbohydrate (low-saturated fat) diets and diets high in unsaturated fats, are contentious (Ong *et al.*, 1999; German and Dillard, 2004; Pelkman *et al.*, 2004; Keogh *et al.*, 2005; Volek and Feinman, 2005). The biscuit-supplemented diet, although lower in both total and saturated fat than the high-fat diet, contained a significantly greater amount of monounsaturated fats (MUFAs). Although these are associated with improved cardiovascular risk (Pelkman *et al.*, 2004; Appel *et al.*, 2005), at high levels they can acutely impair endothelial function (Ong *et al.*, 1999). Recent reviews of the literature note that stimulation of insulin release by increased carbohydrates promotes adipogenesis, weight gain and atherogenesis, all associated with the metabolic syndrome (German and Dillard, 2004; Suter, 2005; Volek and Feinman, 2005). The biscuit-supplemented diet was higher in overall carbohydrate content than the high-fat diet, and it may be this, in combination with relatively high fat content, that accounts for its more detrimental effects. The relatively lower saturated fat and higher carbohydrate content may also be more palatable and account for the increased daily intake in the biscuit-fed animals, and, hence, epididymal adiposity and overall greater hyperlipidaemia. This would have to be more carefully analysed in future, however, by matching intake volume and caloric content between groups. An adipocentric view would suggest that the increased adiposity *per se* of biscuit-fed animals is critically linked with the worsened vascular defects in this group, with excessive fat mass in this depot resulting in fat cell dysfunction, which in turn contributes to the metabolic disorders that increase the risk of athersclerosis (Bays and Dujovne, 2006).

High-carbohydrate meals may also stimulate sympathetic nervous system activity *in vivo* (Marques-Lopes *et al.*, 2003), and counteract insulin-induced vasodilation. Although fasting insulin levels were similar in the three groups studied, it is possible that differences in post-prandial insulin (which would be expected to be higher in rats fed a carbohydrate-enriched diet) and triglycerides may account for the differences observed. Exactly which dietary factors are directly responsible for atherosclerosis and how, is the subject of ongoing debate and discovery, but triglycerides appear to play an important role in both humans and animals (Naderali and Williams, 2001b; German and Dillard, 2004; Barbagallo *et al.*, 2006) in that hypertriglyceridaemia is a recognized atherogenic risk factor (for review, see

German 2005). Indeed, this is even the case in the absence of insulin resistance (Naderali and Williams, 2001b) and in subjects asymptomatic for atherosclerosis (Corrado *et al.*, 2005), emphasising once again the insidious nature of the disorder.

Finally, although % energy derived from protein was lower in both biscuit-supplemented and high-fat diets compared to chow, being lowest in the former, the total average daily protein intake was similar (67 g and 78 g/day, respectively; biscuit-supplemented *vs* high-fat: $p>0.05$). Hence, although very low protein diets are known to be associated with vascular dysfunction (Koumentaki *et al.*, 2002), we consider it unlikely that protein deficiency explains the differences in vascular function seen in this study. Differences in antioxidant levels in the diets may also play a role (Kuno *et al.*, 1998; Sato *et al.*, 2002), but it is not possible to comment further on these as they were not measured in this study.

In conclusion, this study shows that high-energy diets of varying compositions can induce vascular dysfunction in the rat over a protracted period prior to the onset of obesity. They do this by distinct mechanisms involving different layers of the blood vessel wall, and it appears that combining atherogenic dietary components is particularly damaging, possibly through increased hyperlipidaemia. This clarifies to some extent our understanding of the nature of the vascular pathology of high-energy diets blamed for the current obesity epidemic. Clinicians now must be watchful not only of overtly obese patients, but also of those who display body weight lying persistently in the overweight category. A detailed review of dietary intake is thus important regardless of body type or individual phenotypic response to high-energy diets.

Chapter 4

Results: The effect of high-energy diet supplementation on changes in metabolic parameters and vascular reactivity in male Wistar rats.

4.1 INTRODUCTION

Acceleration in the rate of diet-induced obesity throughout the world is becoming a major health problem. Excess energy intake in the form of refined carbohydrates and fatty acids is one of the main causes of the obesity in human. Obesity is a major risk factor for cardiovascular disorders, which include hypertension, atherosclerosis and ultimately myocardial infarction (Andres, 1980; Lakka *et al.*, 2002). Arterial function and structural abnormalities which include alterations of the release of vasoactive substances from endothelial cells as well as changes in arterial wall elasticity have been seen in obese human subjects and certain animal models that reflect some aspects of human obesity(Cowan *et al.*, 1991; Dobrian *et al.*, 2000). Moreover, human and animal studies have shown various degrees of endothelial dysfunction, namely a reduction to vasorelaxants, in obesity (Steinberg *et al.*, 1996; Abram, 1997; Perticone *et al.*, 2001; Naderali and Williams, 2001b). Furthermore, diet rich in fat and refined carbohydrate induces insulin resistance (Abram, 1997; Barnard *et al.*, 1998), endothelial dysfunction, and oxidant/antioxidant imbalance (Roberts *et al.*, 2005), which may increase morbidity and premature mortality.

Increasing reports suggest that diet and its variant contents and/or components plays important roles in arterial function. Studies have shown that consumption of a specific diet of high fat or low protein causes endothelial abnormalities in humans and animals, while a low fat diet may produce a protective effect on vascular reactivity as shown in chapter three and other reports (Vogel *et al.*, 1997; Hannum and Erdman, 2000; Verhamme *et al.*, 2002; Serafini *et al.*, 2003), hight fat diet have detrimental effects on vascular function (Vogel *et al.*, 1997; Naderali and Williams, 2001b; Naderali, 2003). Moreover, pharmacological lowering of plasma lipids by fenofibrate a PPAR-α agonist, retards atheroma developments (Lee *et al.*, 1995; Auwerx *et al.*, 1996), perhaps by stimulating the expression of mitochondrial enzymes involved in fatty acid catabolism as well as induction of mitochondrial and peroxisomal fatty acid β-oxidation pathways (Aoyama *et al.*, 1998). Therefore, the aim of this study was to investigate the effects of supplementation of high-fat, refined carbohydrate diet (Galaxy creamy milk chocolate supplemented) in the

absence or presence of short-term (7 days) administration of fenofibrate on the genesis of obesity, metabolic parameters, vascular functions in the rat.

4.2 METHODS

4.2.1 Experimental protocol

Adult male Wistar rats (n=21) were randomized and assigned to three groups which were fed on either a standard pelletted laboratory chow (which provided 60% of energy as carbohydrate, 30% as protein and 10% as fat) throughout (n=7; control, providing total energy content of 1310 KJ/100 g), or given chow plus chocolate (Galaxy; which provided 42.2% of energy as carbohydrate, 5.1% as protein and 52.7% as fat) (n=14; chocolate supplemented, providing total energy content of 2256 KJ/100 g). After 14 weeks, half of the chocolate supplemented group was given fenofibrate (50 mg/kg/day) and the remainder were given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight) by oral gavage daily for 7 days, before being sacrificed. All animals had free access to their respective diets and water, and were singly housed under controlled environmental conditions, and a 12-h light/dark cycle. Daily energy-intake was determined in all three groups for the last 7 days of the study. The rats were killed by CO_2 inhalation, and the epididymal and perirenal fat pads and the gastrocnemius and cardiac muscles were dissected and weighed. Blood was collected for haematocrite studies and for the measurement of glucose, insulin, leptin, non-esterified fatty acids (NEFA) and triglyceride (TG).

4.2.2 Assessment of vascular function

Six third-order mesenteric arteries were carefully dissected from each animal and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK). A Pair of arteries were incubated in a 5-ml organ bath containing physiological salt solution (PSS; composition [in mM]: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5) gassed with 95% O_2 and 5% CO_2 at 37 °C.

After 30 min equilibration, the length-tension characteristics for each vessel were determined. Arteries were allowed a further 30 min to equilibrate before being

depolarised twice with high-potassium physiological salt solution (KPSS) in which NaCl in normal PSS was replaced by an equimolar concentration of KCl (125 mM). Cumulative concentration-response curves to either KCl (10-125 mM) or noradrenaline (NA, 0.5-6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to KCl (125 mM) was discarded. The effects of selected endothelium- dependent vasodilator, carbamylcholine (CCh), and endothelium-independent vasodilator, sodium nitroprusside (SNP) on arteries preconstricted with 8 μM NA, were also measured.

4.2.3 Data interpretation and statistical analyses

KCl- and NA-induced vasoconstriction responses are expressed as absolute force generated. Relaxation in response to CCh, and SNP are calculated as the percentage reduction from the maximal tension generated in response to a supramaximal concentration of NA (8 μM).

Data are means ± S.E.M. Statistical significance was tested using repeated-measures ANOVA or the Mann-Whitney test, as appropriate. Differences were considered statistically significant at the $p<0.05$ levels.

4.3. RESULTS

4.3.1 Metabolic data

Supplementation of chocolate did not alter total body weight gain. In fact throughout the experiment there were no significant differences in total body weight at any given time between chow-fed control and chocolate supplemented test groups (Fig. 4.1). Furthermore, the epididymal and perirenal fat pad masses as well as gastrocnemius muscle mass were comparable in all three groups, indicating the absence of any obesity (Figs. 4.2 A, B, & C). Analysis of food intake during the last 7 days showed that compared with control chow-fed rats, total weekly energy intake were slightly increased in chocolate supplemented group, but this increased were not statistically significant (Fig. 4.3), whereas, fenofibrate-treated rats, had significantly ($p<0.05$) higher energy intake than control chow-fed group (Fig. 4.3).

While supplementation of chocolate had no significant effect on cardiac muscle mass or haematocrit values, fenofibrate treated animals had significantly ($p<0.01$) higher cardiac muscle mass and reduced haematocrit values (Fig. 4.4 A, & B). At the end of the experiment, fasting plasma glucose, insulin levels were comparable between control chow-fed and chocolate supplemented groups, whereas, fenofibrate treated animals had significantly ($p<0.01$) lower insulin levels than their control counterparts (Figs. 4.5 A, & B). Furthermore, chocolate supplemented animals had significantly higher fasting concentrations of plasma leptin (Fig. 4.6), TG, and NEFA (Figs. 4.7 A, & B), than chow-fed controls. These raises were almost completely reversed by fenofibrate-treatment.

4.3.2 Agonist-induced contractile responses

KCl (10-125 mM)- and NA (0.5-6 μM)-induced vasoconstriction in arteries from all three groups produced a characteristic sigmoid relationship. The contractile responses to KCl and NA in arteries from chocolate supplemented animals were significantly ($p<0.001$) increased in comparison to control groups (Figs. 4.8 & 4.9). The maximal KCl-induced contractions were 5.78 ± 0.54 control, 7.14 ± 0.57 chocolate supplemented, and 7.07 ± 0.52 mN/mm artery fenofibrate-treated, respectively (Fig. 4.8). A similar picture was also seen with NA-induced vasoconstriction giving maximal contraction of 7.68 ± 1.02 control, 10.91 ± 0.96 supplemented, and 10.34 ± 0.66 mN/mm artery fenofibrate-treated, respectively (Fig. 4.9).

4.3.3 Endothelium-dependent and -independent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM-100 μM), achieving a maximum of 87 ± 2% at a CCh concentration of 100 μM. Arteries from test and fenofibrate-treated groups that were similarly exposed to CCh displayed similar sigmoid responses with maximal relaxation of 72 ± 6% and 92 ± 2% (Fig. 4.10). However, arteries from chocolate supplemented rats had significantly ($p<0.01$) higher EC_{50} than control chow-fed animals (EC_{50}: 1.86 ± 0.02 chocolate supplemented, vs EC_{50}: 0.28 ± 0.01 μM Chow-fed). Fenofibrate-treatment significantly ($p<0.05$) improved

EC_{50} compared with chocolate supplemented group (EC_{50}: 0.68 ± 0.01 μM fenofibrate-treatment *vs* EC_{50}: 1.86 ± 0.02 μM chocolate supplemented).

Endothelium-independent vasodilator (SNP; 10 nM-100 μM) also induced concentration dependent vasorelaxation of arteries from all three groups with no significant differences in arterial vasorelaxation between the three groups achieving a maximal relaxation of 85 ± 3 control, 82 ± 2 chocolate supplemented, and 84 ± 3% fenofibrate-treated groups, respectively (Fig. 4.11).

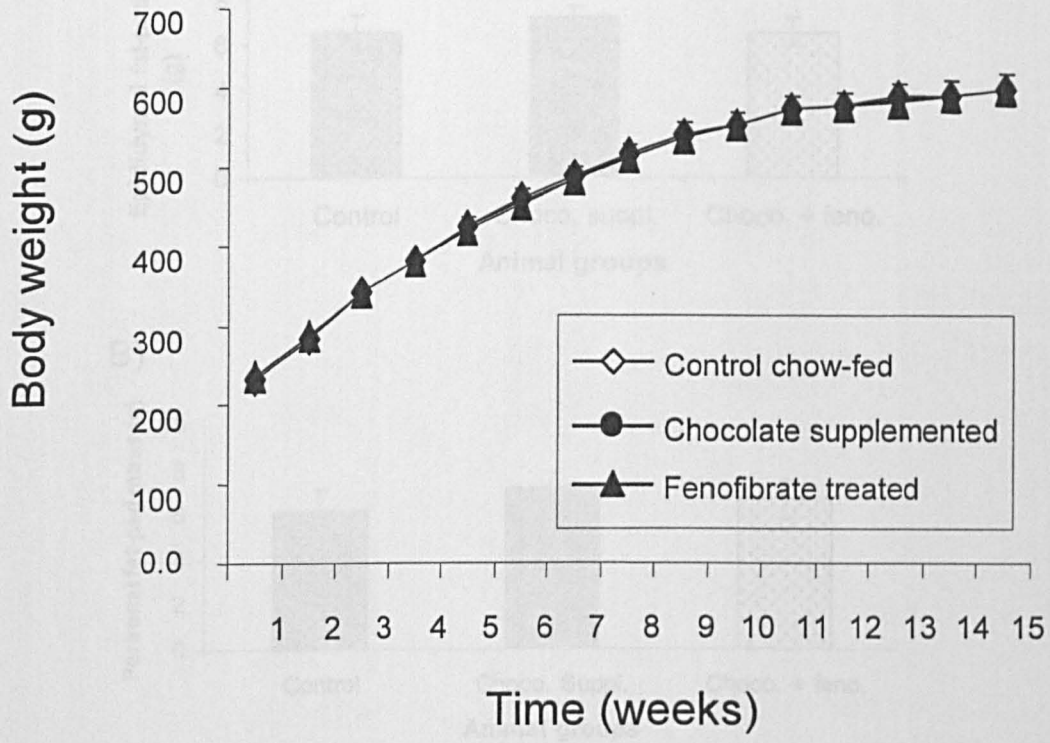


Figure 4.1. The effect of chocolate supplementation and fenofibrate treatment on total body weight in the male wistar rats. Control animals were fed standard chow, while chocolate supplemented groups was given chow plus chocolate (galaxy) for 15 weeks. Fenofibrate-treated rats were given fenofibrate (50 mg/kg/day) daily for 7 days. At the end of 15 week, there were no significant changes in total body weight between the three groups. In fact weight increase in all three groups were superimposable. Data are expressed as mean ± S.E.M.

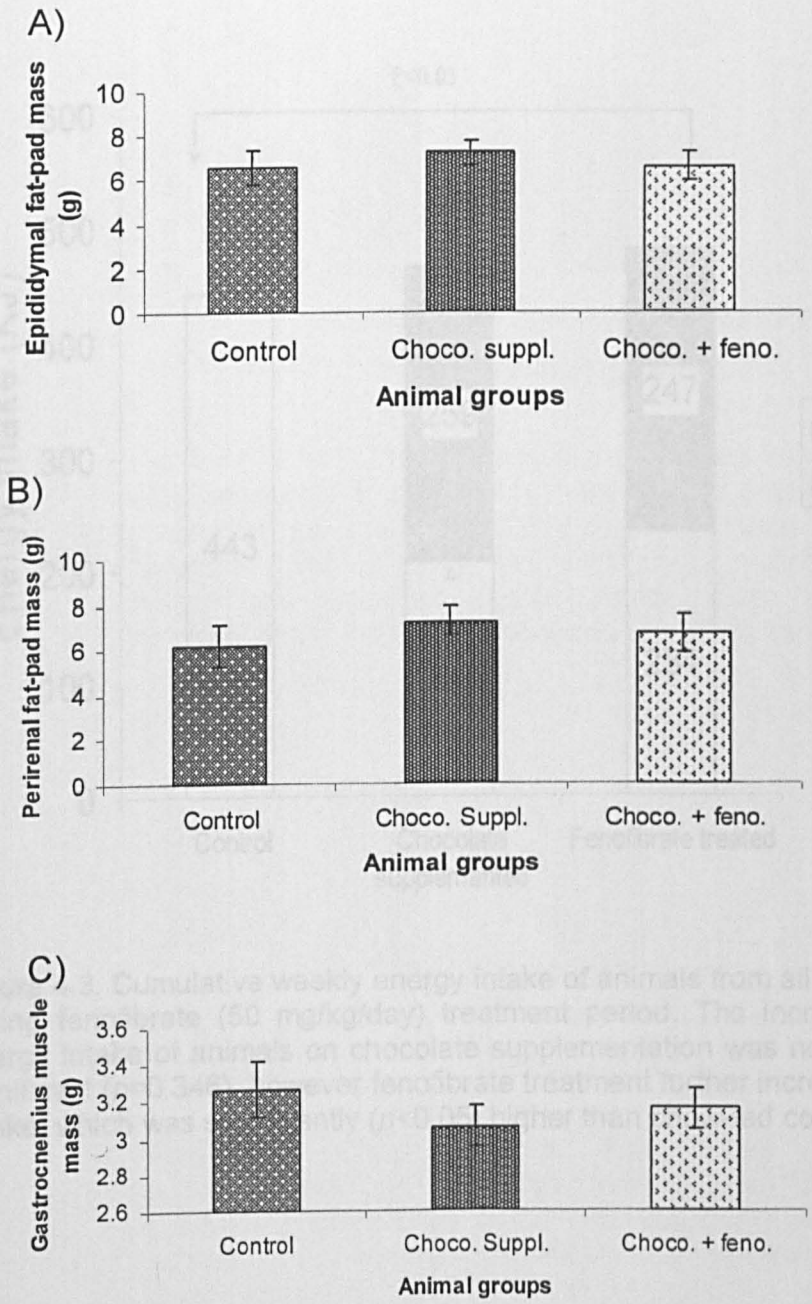


Fig. 4.2. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on A) epididymal fat-pad, B) perirenal fat-pad mass, and C) gastrocnemius muscle mass. Data are presented as mean ± S.E.M. No significant differences were seen between all three groups. Choco. suppl. = chocolate supplemented, and Choco. + feno. = Chocolate supplemented + Fenofibrate.

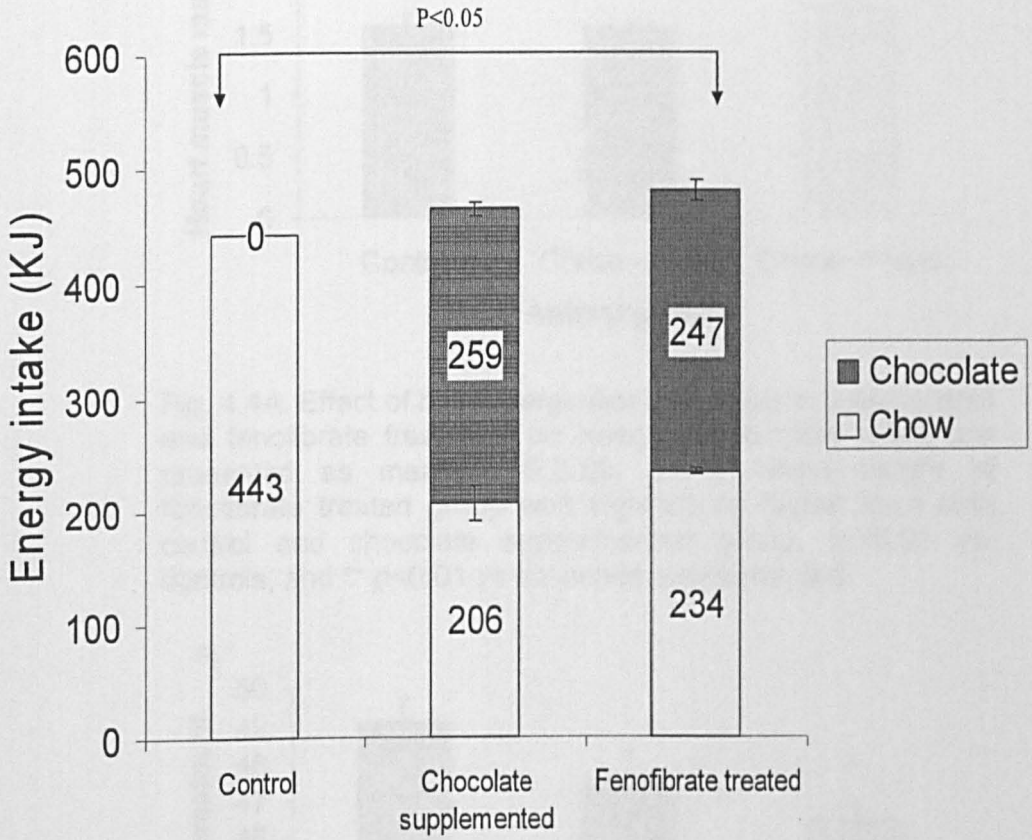


Figure 4.3. Cumulative weekly energy intake of animals from all three groups during fenofibrate (50 mg/kg/day) treatment period. The increase in total energy intake of animals on chocolate supplementation was not statistically significant (p=0.346), however fenofibrate treatment further increased energy intake, which was significantly (p<0.05) higher than chow-fed controls.

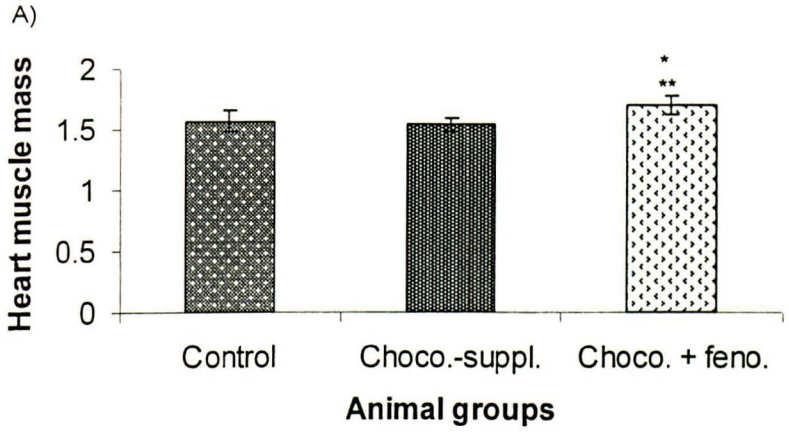


Fig. 4.4A. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on heart muscle mass. Data are presented as mean ± S.E.M., (n=7). Heart weight of fenofibrate treated group was significantly higher than both control and chocolate supplemented group. *p<0.01 vs. controls, and ** p<0.01 *vs* chocolate supplemented.

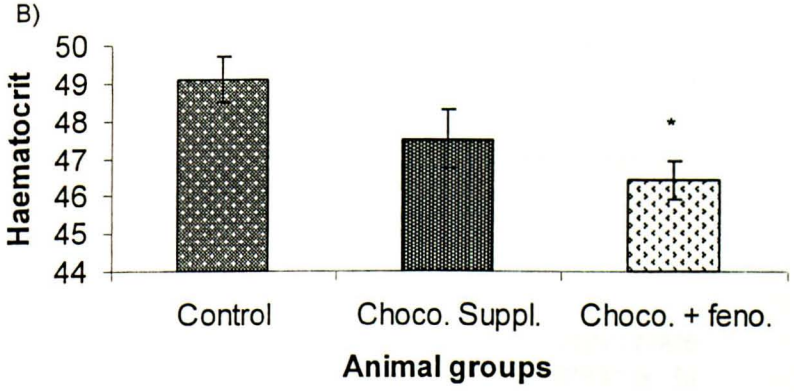


Fig. 4.4B. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on haematocrit. Data are presented as mean ± S.E.M. (n=7). Fenofibrate treated group had significantly lower haematocrit than chow-fed control. * p<0.01 *vs* control.

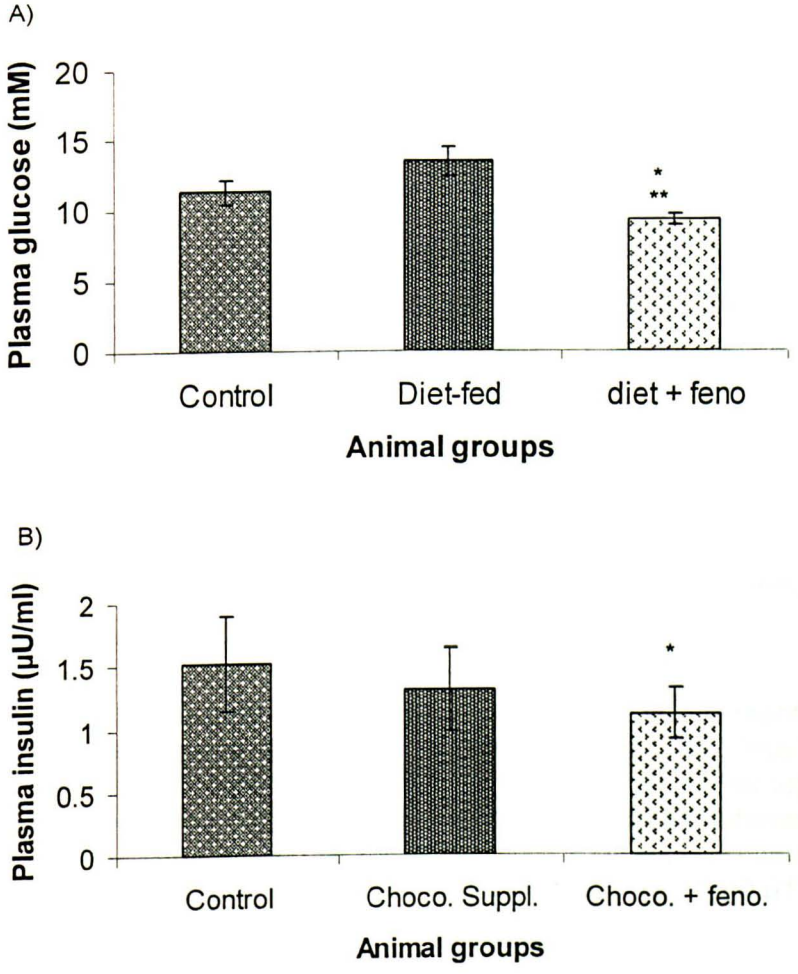


Fig. 4.5. The effects of high energy diet (chocolate supplementation) and fenofibrate-treatment on plasma A) glucose, B) insulin levels. Fenofibrate-treated animals had significantly lower plasma glucose and insulin than their chow-fed counterparts. Data are presented as means ± S.E.M. *$p<0.01$ *vs* control, **$p<0.01$ *vs* chocolate supplementation.

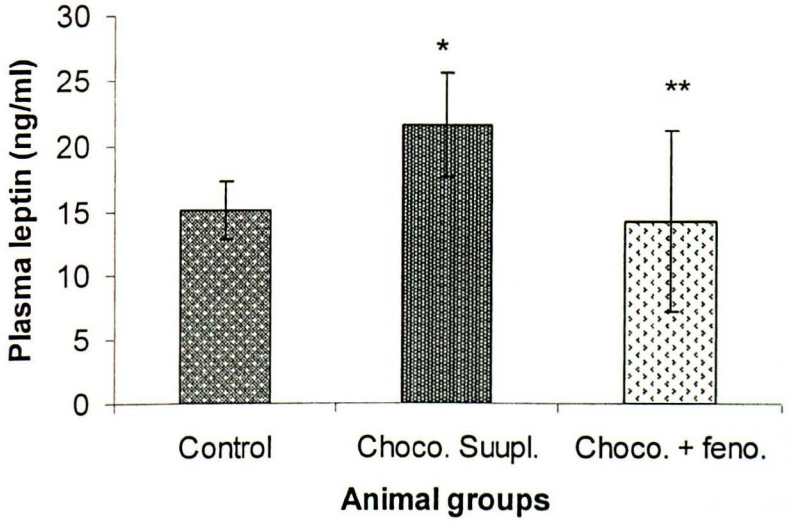


Fig. 4.6. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on plasma leptin. The level of chocolate supplemented group had significantly higher leptin than control, while there was no significant difference between control and fenofibrate groups. Data are presented as mean ± S.E.M. *p<0.01 *vs* controls, and **p<0.01 *vs* chocolate supplemented.

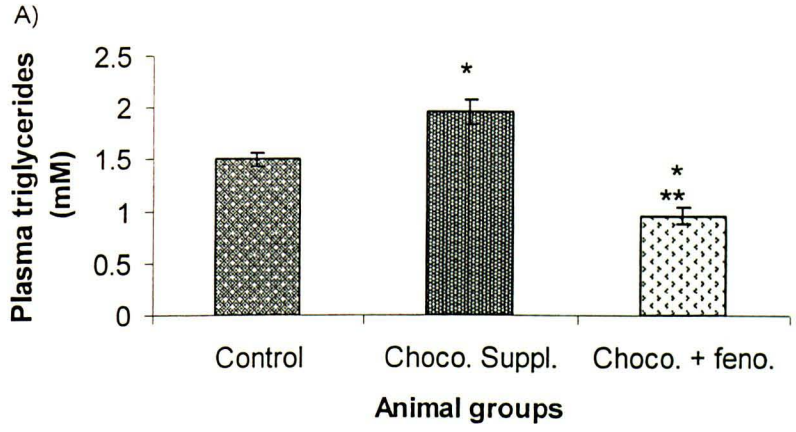


Fig. 4.7A. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on plasma triglycerides. The level of chocolate supplemented group was significantly higher than control, while fenofibrate treated group was significantly lower than both control and chocolate supplemented groups. Data are presented as mean ± S.E.M. *p<0.01 vs. controls, and **p<0.01 *vs* chocolate supplemented.

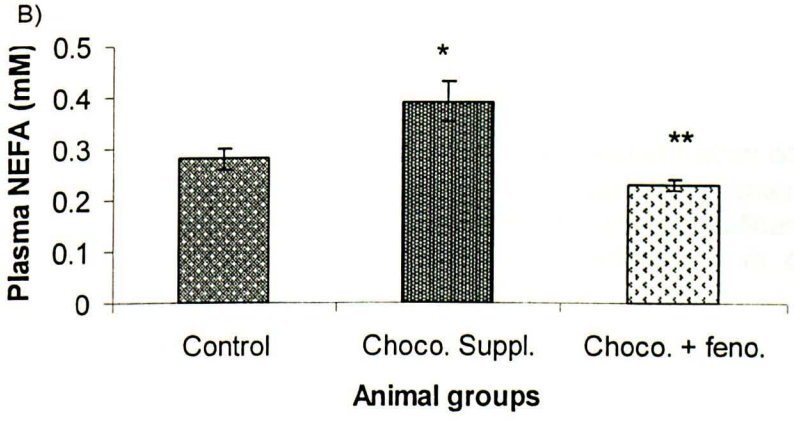


Fig. 4.7B. Effect of high energy diet (chocolate supplemented) and fenofibrate treatment on plasma NEFA. The level of chocolate supplemented group was significantly higher than control, while there was no significant difference between control and fenofibrate groups. Data are presented as mean ± S.E.M. *p<0.01 *vs* controls, and **p<0.01 *vs* chocolate supplemented.

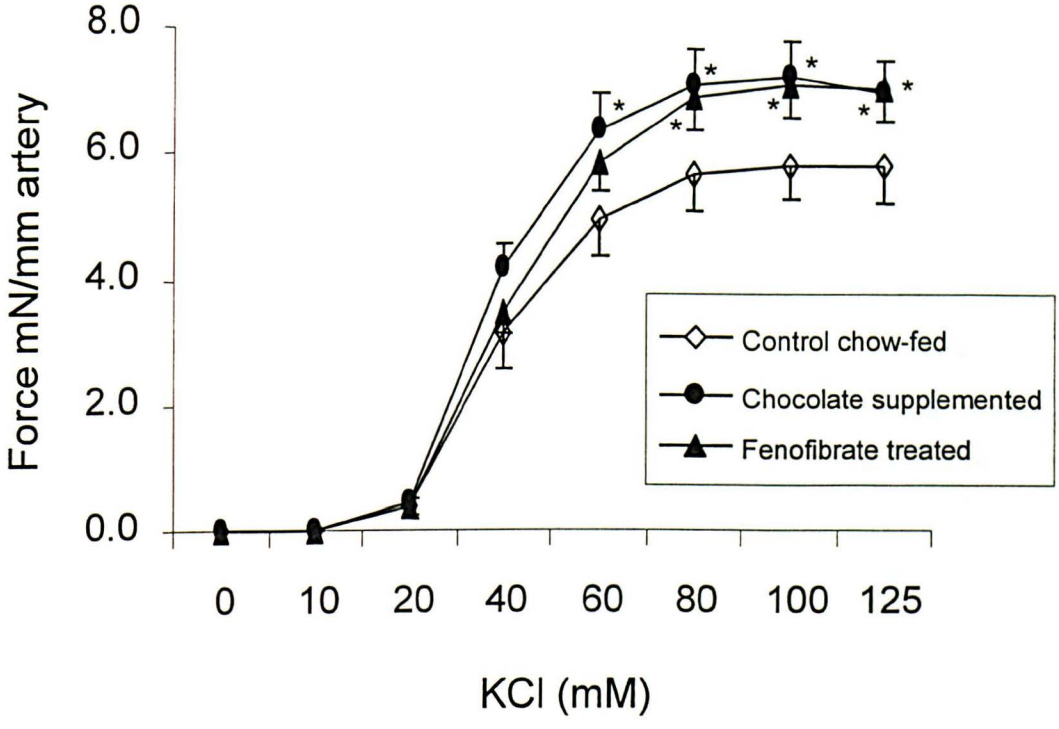


Figure 4.8. Vasoconstriction responses to cumulative concentration of KCl (10-125 mM) in arteries from chow-fed control, chocolate supplemented and fenofibrate-treated animals. KCl-induced contractions were significantly (n=7; ANOVA (Bonferroni test), *p<0.001) augmented in arteries from chocolate supplemented animals. Data represent mean ± S.E.M.

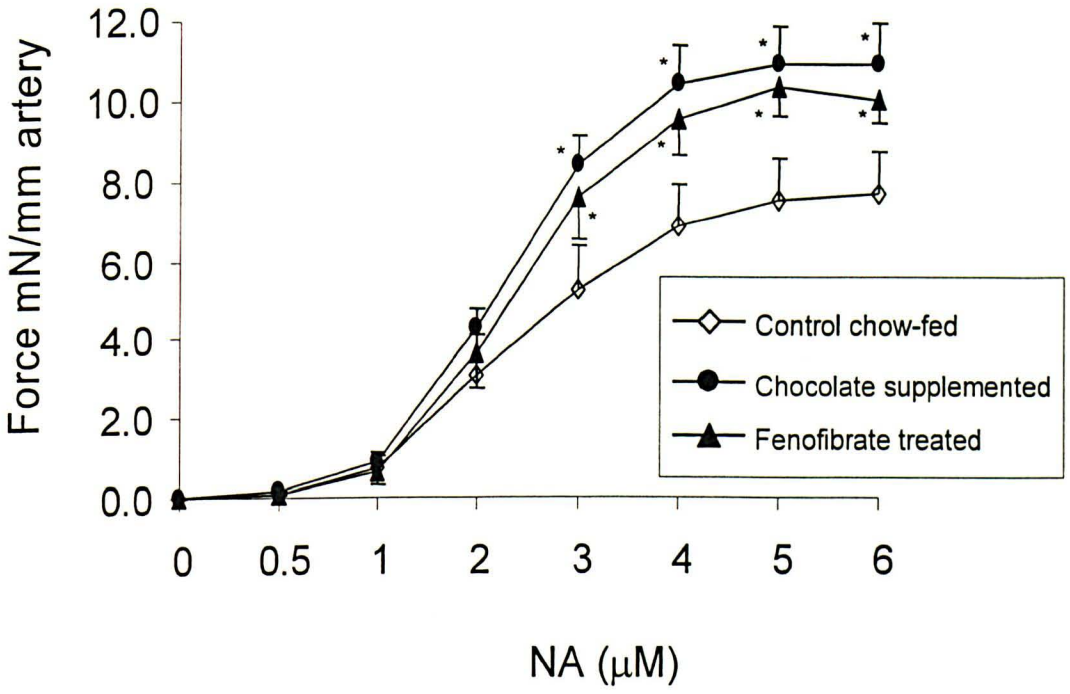


Figure 4.9. Vasoconstriction responses to cumulative concentration of noradrenaline (NA; 0.5-6 μM) in arteries from chow-fed control, chocolate supplemented and fenofibrate-treated animals. NA-induced contractions were significantly (n=7; ANOVA (Bonferroni test), *p<0.01) augmented in arteries from chocolate supplemented animals. Data represent mean ± S.E.M.

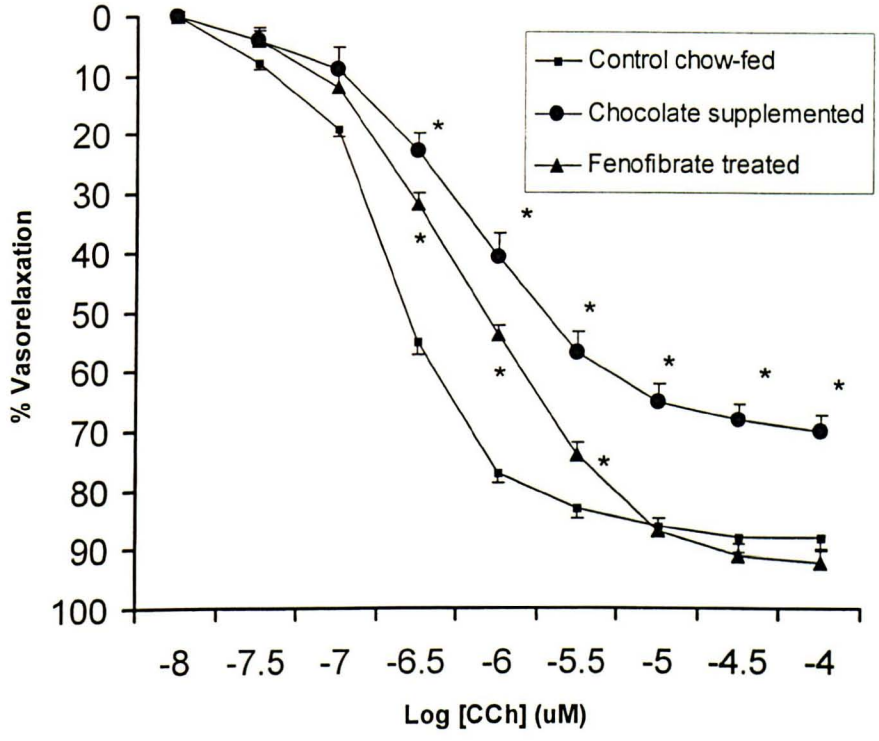


Figure 4.10. The effects of carbamylcholine on NA-precontracted (8μM) arteries from three groups of rats. When contraction reached a plateau after 2 minutes, concentration-response curves to carbamylcholine (CCh) were carried out. Data represent mean ± S.E.M. The concentration-response curves to CCh, were significantly reduced in chocolate supplemented group (n=7; ANOVA (Bonferroni test), $p<0.01$), while fenofibrate-treatment significantly ($p<0.01$) improved vasorelaxation in chocolate supplemented animals.

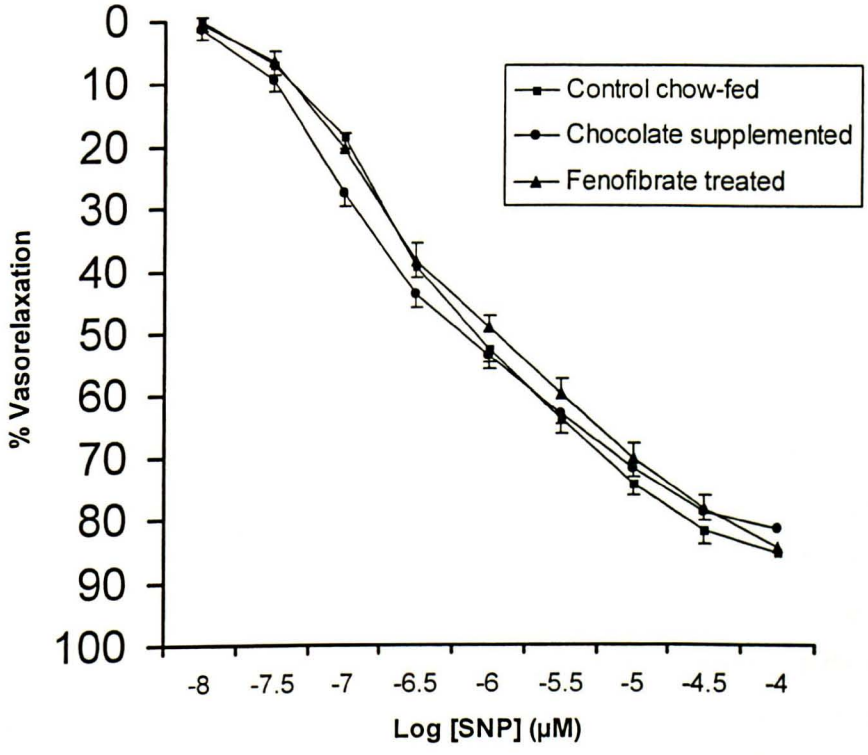


Figure 4.11. The effects of sodium nitroprusside (SNP) on NA-precontracted (8μM) arteries from three groups of rats. When contraction reached a plateau after 2 minutes, concentration-response curves to SNP were carried out. Data represent mean ± S.E.M. The concentration-response curves to SNP, were not significantly different in both chocolate supplemented and fenofibrate-treatment groups, when campared with control chow-fed.

4.4 DISCUSSION

Excessive availability of high-energy diet in many industrialised societies, is producing a health hazard, namely obesity and obesity related secondary disorders, which ultimately leads to the deterioration of quality of life, and in many cases to premature and sudden death. Numerous experimental studies have reported severe detrimental outcome of excessive high-energy diets on well being. For example, high-fat diet impairs endothelium-dependent vasorelaxation (Steinberg *et al.*, 1996; Vogel *et al.*, 1997; Naderali and Williams, 2001b), while removal of high-energy diet provides some protection against obesity-induced metabolic and cardiovascular abnormalities (Sasaki *et al.*, 2002; Verhamme *et al.*, 2002). In hypertensive obese patients, ACh-induced forearm blood flow (FBF) is significantly lower than healthy individuals. Caloric restriction decreases body weight and the mean blood pressure, fasting plasma insulin, total cholesterol, triglycerides, low-density lipoproteins (LDL) as well as enhancing ACh-induced FBF (Sasaki *et al.*, 2002). Moreover, animal studies have shown that withdrawal of high-fat, high-palatable diet significantly reduces plasma lipid profile and markedly improves vascular reactivity to acetylcholine (Verhamme *et al.*, 2002). Furthermore, Pharmacological lowering of circulating plasma lipids has also been shown to reduce the risk for coronary events, cardiovascular morbidity and mortality in a broad range of patients (Brown *et al.*, 1993 ; Bucher *et al.*, 1999). In this study, supplemental feeding of animals with chocolate did not resulted in obesity (characterised with an excessive increase in total body weight and fat pad mass), however, there were significant metabolic abnormalities, namely higher plasma lipids of TG and NEFA. Higher plasma lipids seen in this study were similar to those achieved by chronic consumption of high-fat, high-energy diets and obesity, as shown in chapter three, and other reports (Steinberg *et al.*, 1996; Perticone *et al.*, 2001; Naderali *et al.*, 2001c; Verhamme *et al.*, 2002), indicating that adipose accumulation is not a pre-requisite for abnormal lipid profile (Vogel *et al.*, 1997; Naderali and Williams, 2003). Fenofibrate treatment in the presence of chocolate diet completely reversed lipid profile, indicating beneficial effects of fenofibrate in correcting abnormal lipid profile. Moreover, treatment of animals with fenofibrate corrected all metabolic changes associated with diet and obesity.

Examination of contractile and relaxation properties of arteries showed striking differences in responses to various stimuli between the three groups. In contrast to the results reported in chapter three and previous studies (Walker *et al.*, 1997; Fontes *et al.*, 1998; Naderali and Williams, 2001b), contractile responses to KCl and NA were significantly augmented in chocolate supplemented diet groups (with or without fenofibrate administration). Although from this study it is not possible to contemplate the mechanism of hyperreactivity to KCl and NA, nonetheless, possibilities are that a) long-term consumption of chocolate may have a direct effect on the activity of the contractile apparatus, or b) it may have increased/eased the availability of the calcium for contraction required by KCl and NA. The latter hypothesis is more plausible as chocolate has been shown to markedly increase calciuria (Nguyen *et al.*, 1994). However, these hypothesis merits further investigation.

Results in chapter three and other numerous studies have shown that excessive high–energy diet impairs endothelium-dependent arterial function (Brown *et al.*, 1993 ; Walker *et al.*, 1997; Fontes *et al.*, 1998; Bucher *et al.*, 1999; Naderali *et al.*, 2001c; Sasaki *et al.*, 2002). Moreover, defect in vasorelaxation can also be seen with short-term consumption of high-energy diet in the absence of any obesity (Vogel *et al.*, 1997; Naderali and Williams, 2001b; Naderali and Williams, 2003). In agreement with previous reports, in this study there was a marked attenuation of endothelial mediated CCh-induced vasorelaxation in the absence of any obesity, suggesting a significant defect in either CCh-induced NO production via eNOS activity (Boulanger, 1999) or the release of endothelium-derived hyperpolarizing factor (EDHF) (Gerber *et al.*, 1998). On the other hand, vasorelaxant effect of SNP is due to its direct stimulatory action on cGMP and thereby induction of NO generation (Azula *et al.*, 1996) in vascular smooth muscle cells. In present study, chocolate supplementation did not alter SNP-induced vasorelaxation, suggesting that there were no changes in arterial smooth muscle activity.

A number of various hypothesises have been put forward as the mechanism(s) of action of high-energy diet and obesity related vascular dysfunction, which include a rise in plasma NEFA (Steinberg *et al.*, 1997) hypertriglyceridaemia (Tka *et al.*,

1997; Lewis *et al.*, 1999; Naderali *et al.*, 2001c). This rise in circulating plasma lipids has been, at least partly, attributed to inhibition of eNOS activity and thereby reduction in NO production (Liao *et al.*, 1995), ultimately resulting in a reduced CCh-induced vasorelaxation. Raised NEFA and TG levels in chocolate supplemented animals seen in this study, argue for the role of plasma NEFA and TG as the possible agents affecting vasorelaxation. However, previous reports have failed to show any relationship with raised plasma lipids and increased vasoconstriction (Fontes *et al.*, 1998; Naderali and Williams, 2001b; Naderali *et al.*, 2001c; Naderali and Williams, 2003), therefore, it is unlikely that augmented responses seen to KCl and NA in this study, might have been as a result of changes in circulating plasma lipids. In this study, despite no obesity or weight gain plasma leptin levels were significantly augmented in chocolate supplemented group. Moreover, Leptin has been shown to induce oxidative stress in human endothelial cells (Bouloumie *et al.*, 1998), resulting in activation of atherogenic process, which may contribute to the development of vascular pathology. In addition, leptin has been shown to induce vasoconstriction (Mark *et al.*, 1999 ; Correia *et al.*, 2001; Hall *et al.*, 2001) and exacerbate arterial contractility (Shek *et al.*, 1998). Therefore, raised leptin levels might participate an increase in contractility of arteries in response to NA and KCl.

In this study, although fenofibrate had very little or no effects on KCl- and NA-induced vasoconstriction, but improved CCh-induced responses, indicating improvement of endothelial function in fenofibrate-treated animals. The mechanism of action of fenofibrate on vascular function is not fully known. However, it is possible that reduction in circulating TG may play a role in fenofibrate-induced improvement of endothelial function.

Although, the component of excess energy content of high-fat, high-carbohydrate diet well known to increase weight gain and cause obesity (Matsui *et al.*, 2005), in the present study this component did not induce weight gain or obesity, and this could be related to the action of elevated plasma leptin levels, which may increase thermogenesis and suppress appetite (Pelleymounter *et al.*, 1995), leading to reduced body fat mass (Schwartz *et al.*, 2000).

Overall, this study indicates that consumption of high-energy diet (high-fat, refined carbohydrate diet, milk chocolate supplemented) attenuates arterial relaxation in the absence of any obesity in the rat. The arterial dysfunction may be due to an induction of hyperlipidaemia in general or hypertriglyceridaemia in particular. Lowering circulating plasma NEFA and/or TG by fenofibrate improved endothelial function but not increased vasoconstriction. Furthermore, increases in contractile responses of arteries from chocolate supplemented groups may not be due to the changes in circulating lipid profile

Chapter 5

Results: Palatable diet-induced obesity: changes in metabolic and vascular functions.

5.1 INTRODUCTION

Throughout the world, consumption of high-energy diets together with a general reduction in levels of physical activity causing an alarming health problem, namely obesity. Obesity is a major cardiovascular risk factor and the prevalence of atherosclerotic disease in obese human has been recognised for more than two decades (Andres, 1980; Barret-Conner, 1985). An association has been described between obesity, arterial hypertension, insulin resistance and dyslipidaemia, which comprise core features of the 'metabolic syndrome' or Syndrome X (Reaven, 1988). Many obese patients accomplish weight loss with diet, exercise and lifestyle modification, achieving substantial reductions in central obesity and associated disturbances corresponding to a significant decrease in cardiac events and mortality (Singh *et al.*, 1996; Jen *et al.*, 2002; Gutin *et al.*, 2002).

Obesity induces abnormalities of arterial function and structure in human and in certain animal models that reflect some aspects of human obesity (Cowan *et al.*, 1991; Singh *et al.*, 1996; Naderali *et al.*, 2001; Sasaki *et al.*, 2002). These include alterations of the release of vasoactive substances from endothelial cells as well as changes in arterial wall elasticity. A low-caloric diet improves (Sasaki *et al.*, 2002), while elevating plasma lipid profile further deteriorate endothelium-dependent vasodilataion in obese subjects (Steinberg *et al.*, 1997), and in patients with coronary artery disease (Zhao *et al.*, 2001). It has been shown that, acute consumption of a palatable diet causes endothelial dysfunction long before development of any significant obesity (Naderali and Williams, 2001b). Moreover, endothelial function was only partially corrected with short-term withdrawal of the palatable diet (Naderali and Williams, 2003). Thus, the aims of this study were to a) investigate the effects of long term highly-palatable diet on the genesis of obesity, metabolic abnormalities, and vascular dysfunction, b) to determine whether lifestyle modification (chronic withdrawal of highly-palatable diet) can improve the palatable diet-induced vascular and metabolic abnormalities.

5.2 METHODS

5.2.1 Experimental protocol

Adult male Wistar rats (n=24) were randomised and assigned to a control group (n=8) and a test group (n=16). All animals had free access to water and were housed individually under controlled environmental conditions (19-22 °C; 30-40% humidity) and a 12-hour light/dark cycle (lights on at 07:00h). Controls were fed a standard laboratory pelleted diet (chow-fed), while test group had free access to a highly-palatable diet as described in chapter 2, section 2.1.1. Chow-fed controls remained on their prospective diet for 15 weeks, while after 7 weeks, palatable-diet fed animals were subdivided into two subgroups. Palatable diet was removed from one group and the standard chow diet was reintroduced (diet-to-chow) for further 8 weeks, while the remaining subgroup was maintained on the palatable diet (diet-fed) for another 8 weeks. After 15 weeks from the start of the study, rats were killed by CO_2 inhalation after 2 hours of fasting, and the total body fat mass was measured by Bioimpedence Method using the TOBEC® equipment (Biotech Instrument Ltd.).

5.2.2 Biochemical analysis

Blood was withdrawn by cardiac puncture and removed into cold heparinised tubes and the gonadal and perirenal fat pads, as well as the gastrocnemius muscles, heart, liver, and lung were dissected and weighed. The plasma was immediately separated by centrifugation before being frozen for later measurements of blood analytes (glucose, insulin, leptin, TNF-α, CRP, nonesterified free fatty acids (NEFA) and triglycerides (TG). Plasma glucose concentration was determined using a glucose oxidase method, and NEFA and TG concentrations were measured using commercial diagnostic kits (Boehringer Mannheim, and Sigma Diagnostics.). insulin and leptin concentrations were measured by radioimmunoassay (RIA) kits, While, TNF-α and CRP levels were measured using Enzyme-immunoassay kits.

5.2.3 Assessment of vascular contractility

Six third-order mesenteric arteries (>200 μm diameter, 2 mm lengths) were carefully dissected from each animal. Each artery was freed of fat and connective tissue and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion Ltd). The vessels (in duplicate) were incubated in a 5-ml organ bath containing physiological salt solution. Cumulative concentration-response curves to either KCl (10-125 mM) or noradrenaline (NA, 0.5-6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded.

5.2.4 Assessment of vascular relaxation

To investigate defects in endothelial-dependent and –independent vasorelaxation, relaxation to endothelium-dependent (carbamylcholine; CCh) or endothelium-independent (sodium nitroprusside; SNP) vasodilators were measured in NA-preconstricted arteries. To eliminate interference of vasoactive prostanoids, 10 mM indomethacine was added to the organ bath prior to contraction with NA (8 μM). When contraction reached a plateau after 2 minutes, concentration-response curves were carried out to either CCh or SNP (for both, 10 nM-100 μM).

5.2.5 Homeostasis model assessment (HOMA)

HOMA, an index of insulin resistance, which employs measures of fasting plasma concentrations of glucose and insulin, was calculated according to the method described previously (Matthews *et al.*, 1985).

5.2.6 Data interpretation and statistical analysis

Vasoconstriction in response to NA and KCl were expressed as absolute force generated. Relaxation in response to CCh and SNP was calculated as the percentage reduction from the maximal tension generated in response to the supramaximal concentration of NA (8 μM). Data are expressed as mean ± SD or where stated as SEM for more clarity. An average response for all the vessels from a given animal was determined before group analysis. Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student

't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p<0.05$ levels. Univariate correlation between maximal CCh-induced vasorelaxation and fasting terminal insulin, leptin, TNF-α, CRP, and TG levels were determined by linear regression followed by two-tailed analyses, using ARCUS PRO-STAT (version 3.23).

5.3 RESULTS

5.3.1 Metabolic data

Animals giving palatable diet progressively gained more weight than their chow-fed counterparts. A significant difference in total body weight was observed after 5th week of feeding and further increased after 8th week where diet-fed animals had significantly (>10%, p=0.0059) higher total body weight than chow-fed controls (Fig. 5.1). Following the next 7 weeks, average weekly weight gain of diet-fed animals was 1.8-fold greater than chow-fed controls (18.43 ± 0.50 *vs* 10.34 ± 0.23 g control), while average weekly weight gain of diet-to-chow animals was significantly lower ($p<0.001$, 10.04 ± 0.06 *vs* 18.43 ± 0.50 g diet-fed) than their diet-fed counterparts (Fig. 5.1). However, at the end of the experiment, total body weight of diet-to-chow animals was still significantly ($p=0.043$) higher than control chow-fed but was markedly ($p<0.006$) lower than the diet-fed animals (Fig. 5.1).

At the end of the experiment, Bioimpedence measurements showed that diet-fed animals had 70 and 27% more total body fat than chow-fed and diet-to-chow groups, respectively (Figs. 5.2 C & D). Diet-fed animals had significantly higher epididymal and perirenal fat pad mass than both chow-fed (by >190%, $p<0.0001$) and diet-to-chow (by >165%, $p<0.0002$) groups (Fig. 5.2 C). Diet-fed rats had significantly (for both, $p<0.023$) higher heart weights than both chow-fed and diet-to-chow rats (Fig. 5.2 A). There were no significant differences between fat pad depots and heart weights of diet-to-chow and chow-fed groups, respectively; nor there were any significant differences in gastrocnemius muscle weights between the three groups (Fig. 5.2 B). However, diet-fed animals had significantly heavier (122%, $p<0.01$) liver weight than their chow-fed counterparts. Furthermore, liver weights of diet-to-chow animals showed marked reduction (18%, $p<0.01$)

compared to diet-fed animals with no difference with that of chow-fed animals (Fig. 5.3 A). Lung weights were also comparable in all three groups with no significant difference between them (Fig. 5.3 B).

While fasting plasma glucose levels were comparable in all three groups (12.42 ± 1.12 chow-fed; 14.40 ± 0.362 diet-fed; 14.43 ± 0.42 mM diet-to-chow) (Fig. 5.4 A), diet-fed rats had significantly higher fasting terminal levels of plasma insulin than both chow-fed and diet-to chow group (2.83 ± 0.18 diet-fed *vs* 1.22 ± 0.18 chow-fed and 1.22 ± 0.22 mM diet-to-chow; for all $p<0.009$) (Fig. 5.4 B). As a result, diet-fed rats had significantly (for both, $p<0.001$) higher HOMA index, indicating development of insulin resistance in these animals. Moreover, diet-fed rats had significantly higher plasma leptin levels than both chow-fed control and diet-to-chow groups (for both $p<0.001$; 9.35 ± 0.25 diet-fed *vs* 7.53 ± 0.52 chow-fed and 6.90 ± 0.24 mM diet-to chow) (Fig. 5.4 C).

Fasting plasma levels of NEFA were comparable in all there groups (0.45 ± 0.24 chow-fed; 0.48 ± 0.41 diet fed and 0.40 ± 0.24 mM diet-to-chow), while diet-fed rats had significantly (>2-fold, $p<0.001$ for both) higher levels of fasting TG than both chow-fed and diet-to chow groups (Fig. 5.5 A & B). Compared with chow-fed group, plasma levels of TNF-α in both diet-fed and diet-to-chow groups were significantly elevated (72% and 66% respectively, $p<0.05$ for both). There was no significant difference between diet-fed and diet-to-chow groups. Similarly, plasma levels of CRP in diet-fed animals were significantly higher than chow-fed group (> 3 fold, $p<0.05$), an effect which was not seen in diet-to-chow group (Fig. 5.6 A & B).

5.3.2 Vascular data

There were no significant differences in vessel diameter between the three groups (189 ± 20 chow-fed; 200 ± 15 diet-fed and 200 ± 10 μm diet-to-chow; $p>0.5$ for all).

5.3.3 Agonist-induced vasoconstriction responses

Cumulative concentration response curves (CCRC) to KCl (10-125 mM) and NA (0.5-6 μM) in arteries from all three groups produced characteristic sigmoid

relationship. KCl-induced contractions were similar in all three groups such that CCRC were virtually superimposable, giving maximum contractions of 5.90 ± 0.41 chow-fed, 6.16 ± 0.31 diet-fed and 6.32 ± 0.26 mN/mm artery for diet-to-chow groups (Fig. 5.7), with no significant differences between the groups, either overall (by ANOVA) or at any given KCl concentration. However, arteries from diet-fed group had significantly (>30%; $p<0.01$ for all) greater contractile responses to 3 and 4 μM NA than chow-fed and diet-to-chow groups. This difference was not seen at lower NA concentrations and was abolished at higher NA concentrations (Fig. 5.8).

5.3.4 Endothelium-dependent and -independent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM- 100 μM), achieving a maximum of 90 ± 2% at a CCh concentration of 100 μM, with EC_{50} values of 0.14 ± 0.02 μM. Arteries from diet fed rats that were similarly exposed to CCh displayed a significant rightward shift of CCRC compared with chow-fed control arteries (EC_{50} diet-fed: 0.75 ± 0.03, $p<0.002$ *vs* chow-fed) (Fig. 5.9). Maximal vasorelaxation to 100 μM CCh was reduced by 23% in diet-fed rats ($p<0.004$ *vs* chow-fed). However, CCh-induced vasorelaxation curves in arteries from diet-to-chow group were virtually identical to those in chow-fed group, achieving maximal relaxation of 95 ± 1% at 100 μM CCh, with EC_{50} values of 0.19 ± 0.04 μM.

SNP (10 nM-100 μM) also induced concentration dependent vasorelaxation of arteries from all three groups. Concentration-relaxation response curves were similar and superimposable in all three groups (Fig. 5.10), with no statistically significant differences between the groups, either overall (by ANOVA) or at any given SNP concentration.

5.3.5 Metabolic determinants of vascular dysfunction

Of the various blood analytes measured, insulin, leptin, TNF-α, CRP, and TG showed significant increases in diet-fed animals. Therefore, the relationship between endothelial dysfunction (measured as the percentage reduction of 100 μM CCh-induced vasorelaxation) and terminal plasma levels of insulin, leptin, TNF-α, CRP, and TG were examined. In each case, univariate correlation analysis showed an inverse correlation between fasting terminal levels of TG (r^2 = 0.4787; p = 0.0013), insulin (r^2 = 0.4237; p = 0.0023) and leptin (r^2 = 0.609; p = 0.0001) and maximal CCh-induced vasorelaxation.

The relationship of adipokines levels TNF-α and CRP with maximum CCh-induced vasorelaxation was also measured, and no siginificant correlations were found for both adipokines (r^2 = 0.0108; $p \geq 0.05$, and r^2 = 0.1402; $p \geq 0.05$, respectively). We also analysed the relationship between total body weight and total fat pad mass with vascular reactivity. Univariate correlation analysis once again indicated an inverse correlation relationship between total body weight (r^2 = 0.3751; p = 0.0043) and fat pad mass (r^2 = 0.3565; p = 0.0048) and maximum carbamylcholine-induced vasorelaxation. Furthermore, analysis of blood analytes showed significant positive correlations between fasting plasma insulin (r^2 = 0.3043; p = 0.0025) and leptin (r^2 = 0.4189; p = 0.0020) with that of circulating triglycerides.

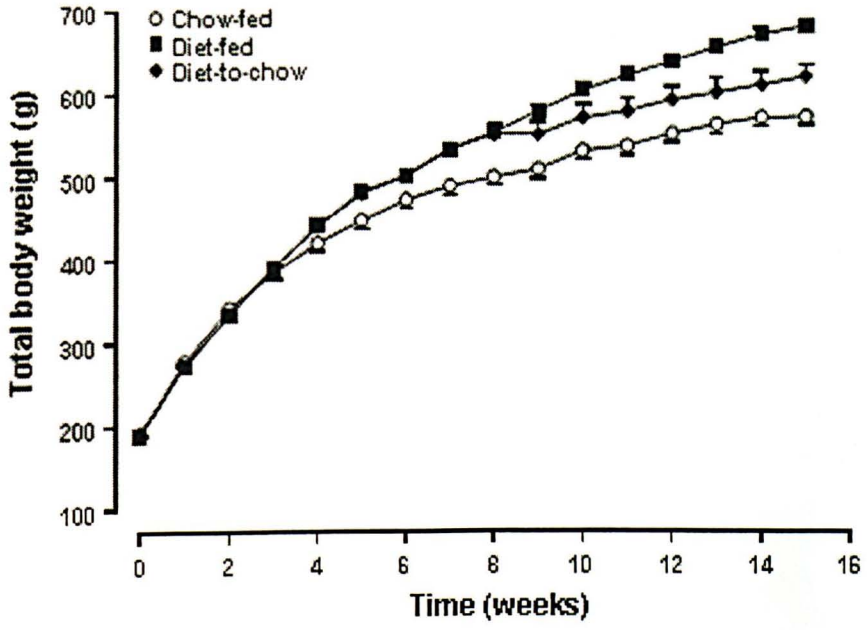


Fig. 5.1. The effect of a highly palatable-diet and its removal on total body weight in Wistar rats. Animals were fed either standard chow (chow-fed) or a palatable diet (diet-fed) for 15 weeks, or fed the palatable diet for 8 weeks and then returned to standard chow for further 7 weeks (diet-to-chow). Diet-fed animals were significantly ($p<0.00001$) heavier than both chow-fed and diet-to-chow groups. Diet-to-chow group was significantly heavier ($p<0.04$) than chow-fed but lighter ($p<0.006$) than diet-fed groups. Data are expressed as mean ± S.E.M.

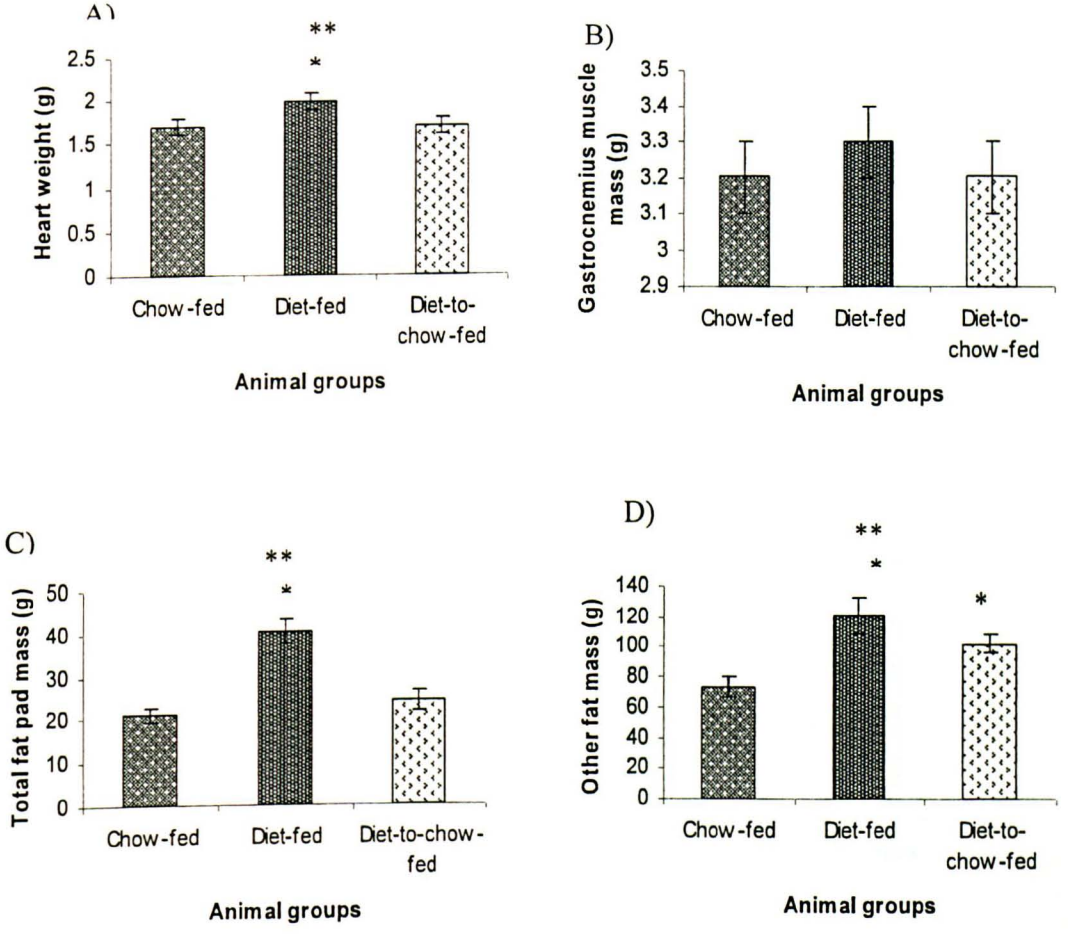


Fig. 5.2. The physiological characteristics: A) heart weight, B) gastrocnemius muscle mass, C) total fat pad mass, and D) other fat mass, of the three experimental groups at the end of the experiment. Animals were either fed standard chow (chow-fed) or a palatable diet (diet-fed) for 15 weeks, or fed the palatable diet for 7 weeks and then returned to standard chow for further 8 weeks (diet-to-chow). Data are expressed as mean ± S.D. $p<0.001$ for all. *Diet-fed or diet-to-chow *vs* chow-fed. **diet-fed *vs* diet-to-chow-fed.

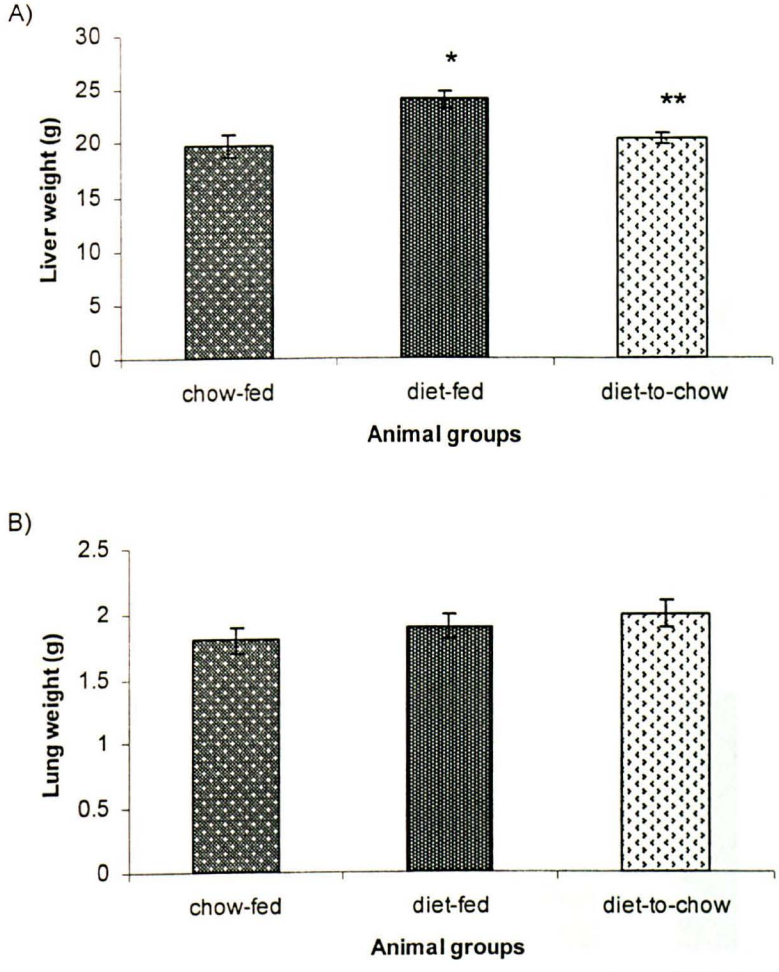


Fig. 5.3. The effect of diet-induced obesity and chronic withdrawal of the palatable diet on the weights of A) liver, and B) lung, in male Wistar rats. Lean control animals were fed a standard laboratory chow, while the second group was given high-palatable diet for 15 weeks (diet-fed). In the third group, the palatable-diet was removed after 7 weeks and replaced by standard laboratory chow for the last 8 weeks (diet-to-chow). Data are expressed as mean ± S.E.M. *$p<0.008$ *vs* chow-fed, and **$p<0.05$ *vs* diet-fed.

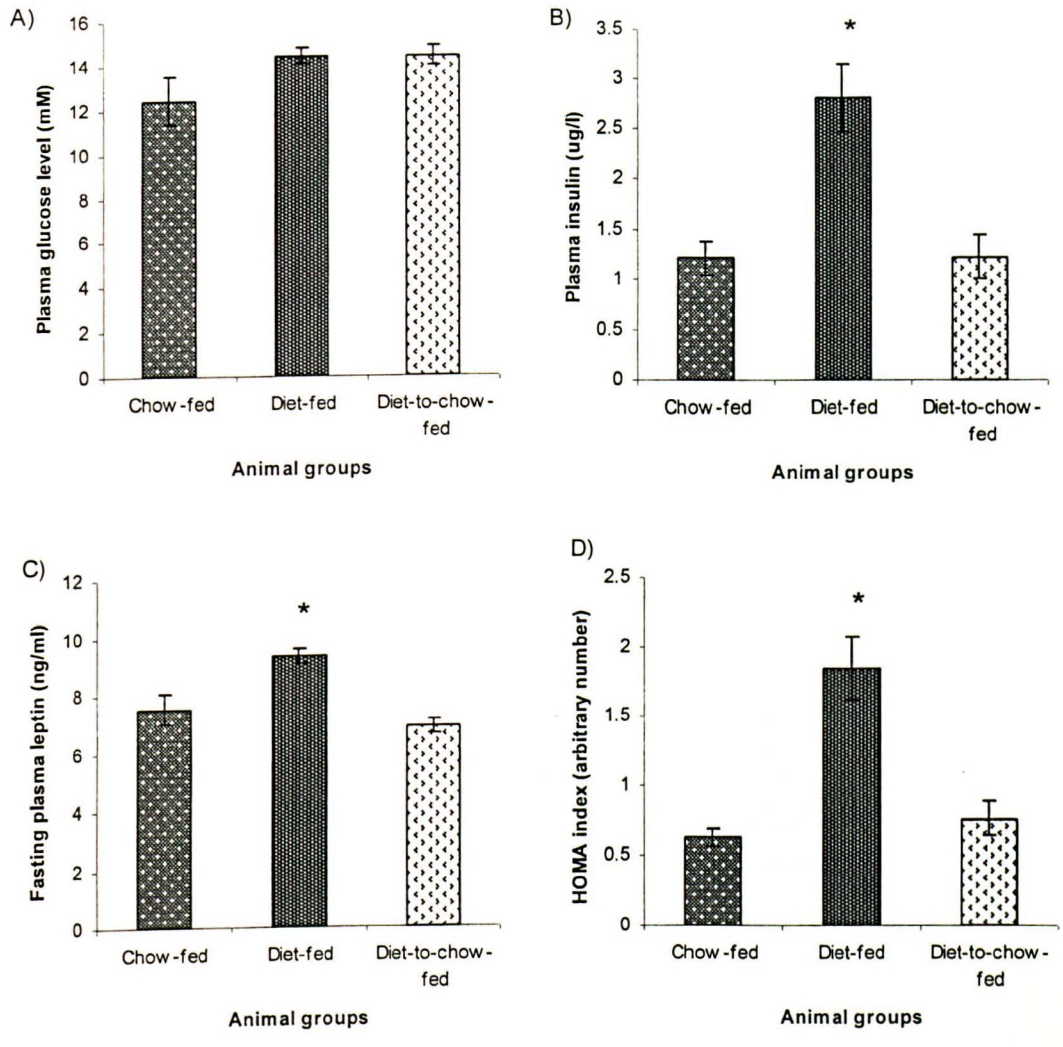


Fig. 5.4. Effects of highly-palatable diet feeding and its withdrawal on fasting plasma levels of A) glucose, B) insulin, C) leptin, and D) HOMA index in three experimental animals. There were no significant changes in glucose levels in three groups. Diet-fed rats had significantly (for all $p<0.01$) higher fasting insulin, and leptin levels than both chow-fed and diet-to-chow groups. Diet-fed animals were also insulin resistance, indicated by a higher HOMA index. Fasting insulin and leptin levels returned to normal levels by long-term removal of the highly-palatable diet and insulin resistance was corrected subsequently. Data are presented as mean ± S.E.M. *$p<0.01$ *vs* chow-fed.

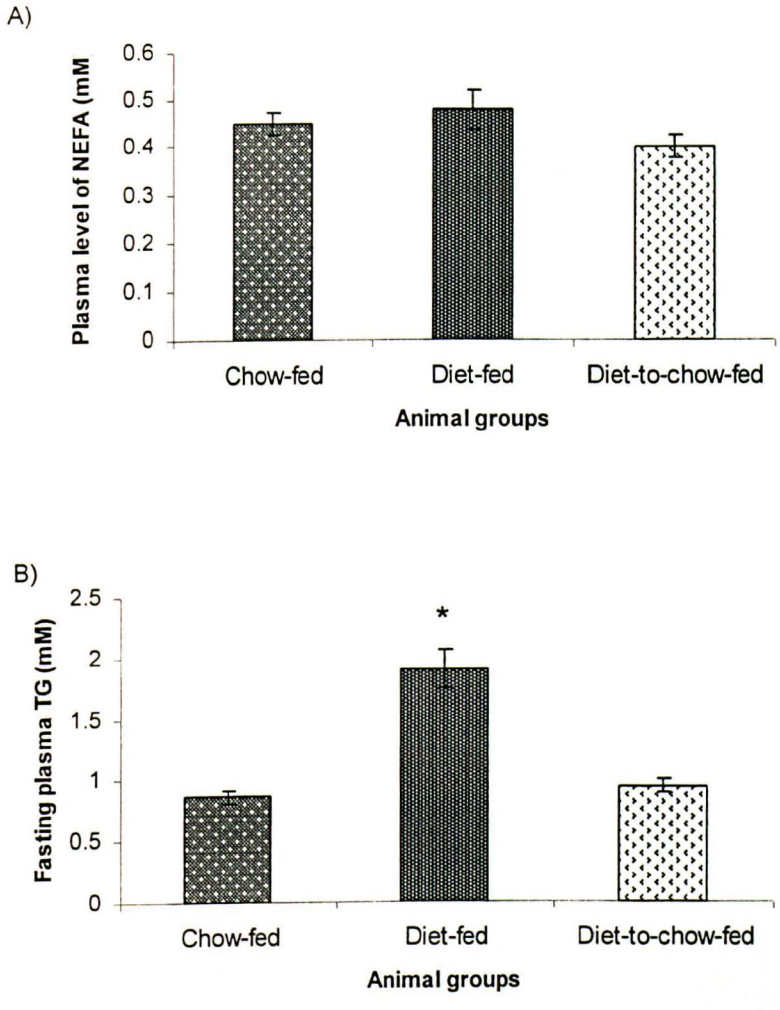
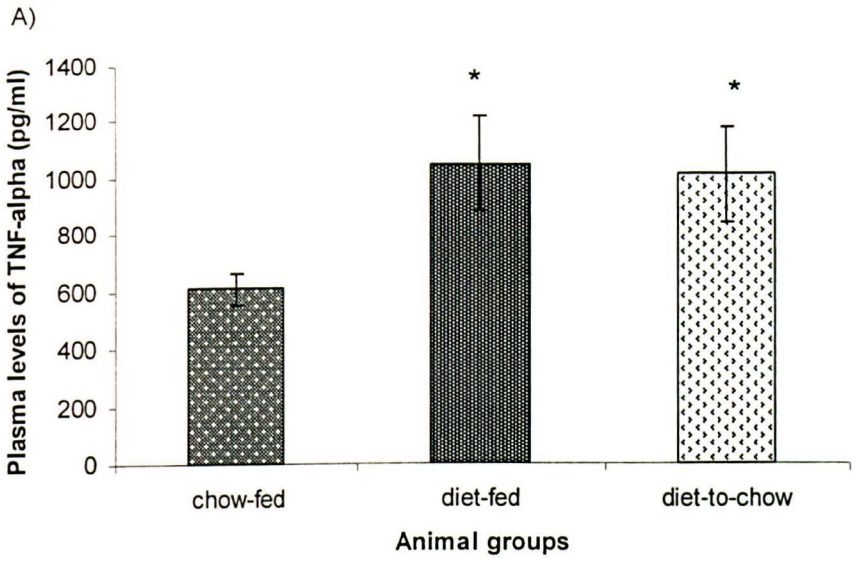


Fig. 5.5. Effects of highly-palatable diet feeding and its withdrawal on fasting plasma levels of A) Non-esterified fatty acid (NEFA), and B) triglyceride (TG) in rats. Levels of NEFA were comparable in all three groups. Diet-fed rats had significantly (for both, *p<0.001) higher fasting TG levels than both chow-fed and diet-to-chow groups. Data are expressed as mean ± S.E.M.



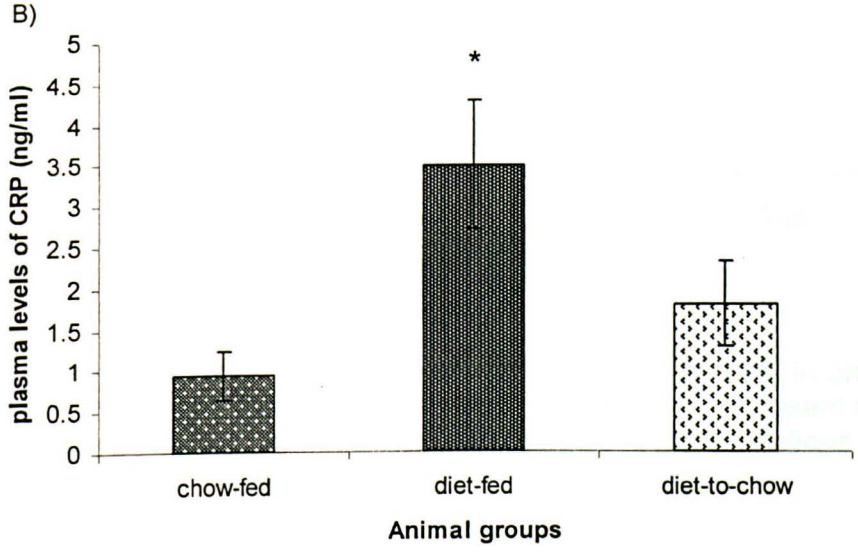


Fig. 5.6. the effects of diet-induced obesity and chronic withdrawal of the palatable diet on plasma levels of A) TNF-α, and B) C-reactive protein (CRP) in male Wistar rats. Lean control animals were fed a standard laboratory chow, while the second group was given high-palatable diet for 15 weeks (diet-fed). In the third group, the palatable-diet was removed after 7 weeks and replaced by standard laboratory chow for the last 8 weeks (diet-to-chow). Data are expressed as mean ± S.E.M. *$p<0.05$ *vs* chow-fed.

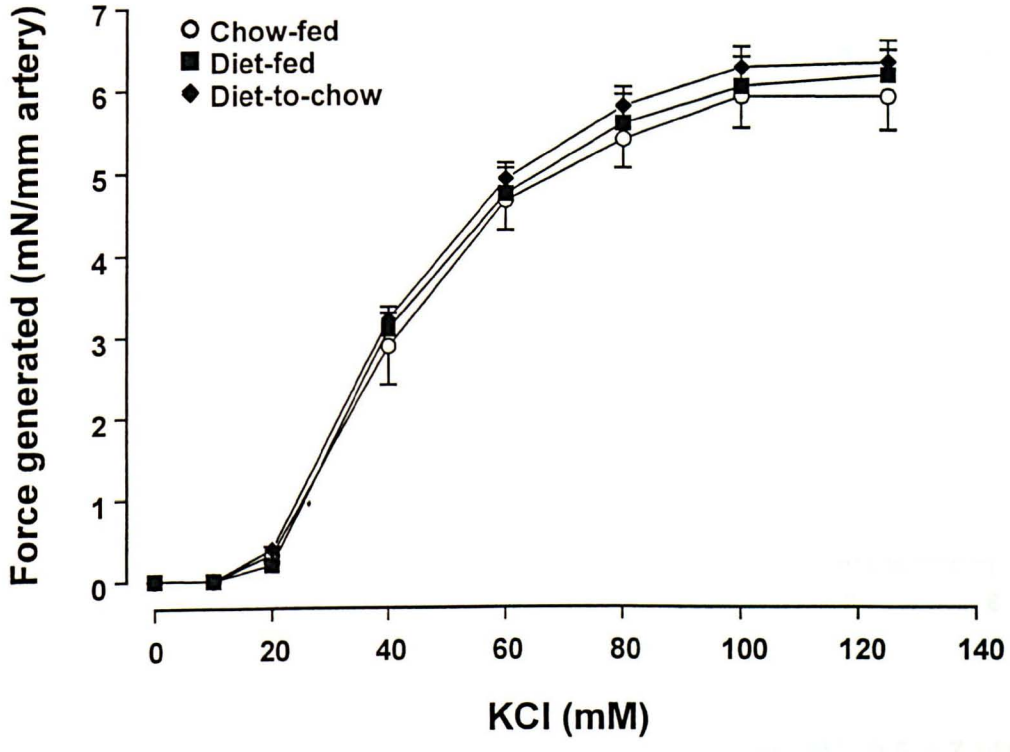


Fig. 5.7. Concentration-response curves for KCl (10 – 125 mM) in arteries from chow-fed, diet-fed and diet-to-chow-fed animals. Data represent mean ± S.E.M for 6 animals in each group. KCl-induced vasoconstrictions were similar between all three groups.

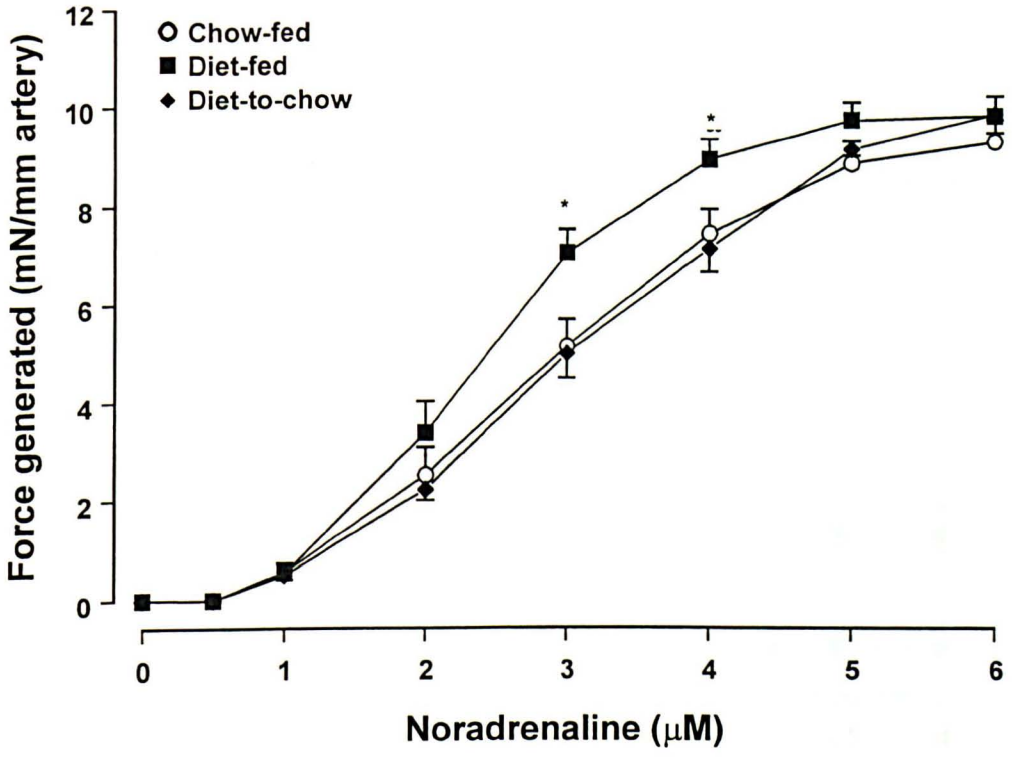


Fig. 5.8. Concentration-response curves for noradrenaline (NA; 0.5 – 7 μM) in arteries from chow-fed, diet-fed and diet-to-chow-fed animals. Data represent mean ± S.E.M. for 6 animals in each group. However, 3 and 4 μM NA-induced contractions significantly (*p<0.01; ANOVA, Bonferroni test) were greater than both chow-fed and diet-to-chow-fed groups.

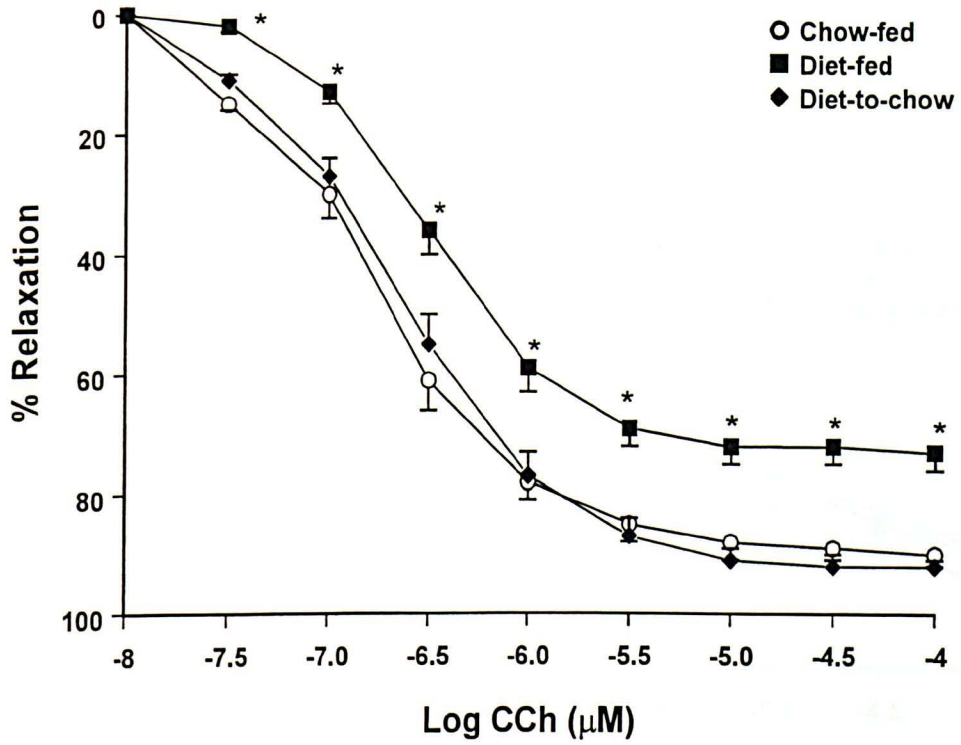


Fig. 5.9. Relaxation response curves (mean ± S.E.M) of carbamylcholine (CCh) in the 3 groups. Arteries were contracted with 8 μM Noradrenaline. When contractions reached a plateau cumulative concentration responses to CCh were carried out. CCh-induced vasorelaxation was significantly reduced in diet-fed animals (*p,0.001; ANOVA, Bonferroni test) in comparison to chow-fed group. This abnormality was completely abolished by the chronic removal of the palatable diet.

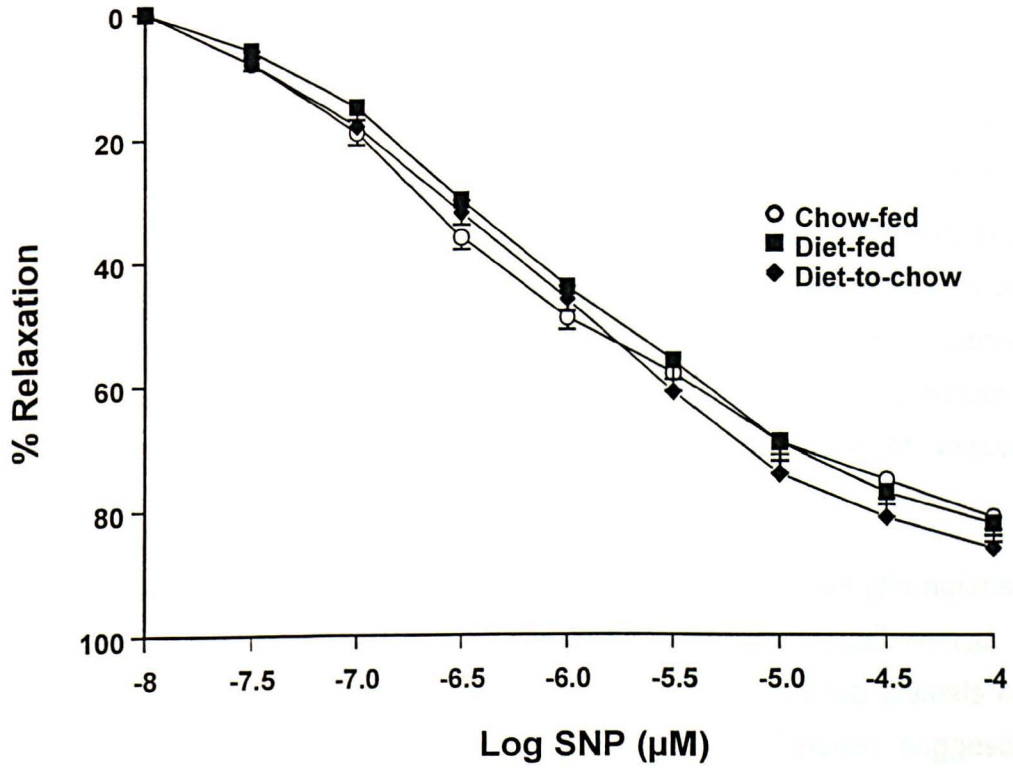


Fig. 5.10. Relaxation response curves (mean ± S.E.M) of sodium nitroprusside (SNP) in the 3 groups. Arteries were contracted with 8 µM Noradrenaline. When contractions reached a plateau cumulative concentration responses to SNP were carried out. SNP-induced vasorelaxations were similar in all three groups.

5.4 DISCUSSION

The present study, demonstrates for the first time that chronic removal of an obesity-inducing palatable diet corrects all the metabolic abnormalities, cardiac hypertrophy and vascular dysfunctions associated with obesity. It has been demonstrated previously that chronic consumption of a palatable diet induces significant level of obesity and markedly effects metabolic parameters and vascular reactivity in the absence of any visible insulin resistance (Naderali *et al.*, 2001a). Moreover, short-term feeding (2-days) of the same palatable diet significantly alters lipid profile and markedly attenuates endothelial function in the absence of any significant obesity (Naderali *et al.*, 2001c). These defects were only partially corrected by acute removal of the diet (Naderali and Williams, 2003), indicating that diet *per se* may have a major role in the development of vascular abnormalities in animals.

In this study, diet-fed animals developed marked obesity, which was characterised by more than 2-fold increase in total body fat content as determined by Bioimpedence methods and measurements of fat pad mass. Obese animals had significantly higher heart weight and fasting plasma levels of insulin, suggesting cardiac hypertrophy and development of insulin resistance in dietary-obese animals. These findings are similar to those seen in human obesity (Andres, 1980; Barret-Conner, 1985; Reaven, 1988; Singh *et al.*, 1996). In human, obesity leads to dyslipidaemia and type 2 diabetes and hypertension (Lind *et al.*, 1995; Carey *et al.*, 1997; Parker *et al.*, 2003), while changes in life style improves obesity-induced abnormalities to a certain degree (Singh *et al.*, 1996; Sasaki *et al.*, 2002; Gutin *et al.*, 2002).

A number of experimental studies have reported severe detrimental outcome of excessive high-energy diet on vascular reactivity. For example, high-fat diet impairs endothelium-dependent vasorelaxation (Vogel *et al.*, 1997; Naderali *et al.*, 2001a; Naderali and Williams, 2001b; Naderali *et al.*, 2001c), while antioxidant therapy improves endothelial function (Sato *et al.*, 2002; Plotnick *et al.*, 1997), suggesting presence of diet-induced oxidative stress in the vasculature. In obesity,

plasma antioxidant levels are decreased (Kuno *et al.*, 1998; Sato *et al.*, 2002; Plotnick *et al.*, 1997), whereas, myocardial oxidative stress is increased (Moor-de Burgos *et al.*, 1992; Vincent *et al.*, 1999). These changes may, at least in part, explain the increased risk of vascular disease and the abnormal endothelium-dependent vasorelaxation found in obese subjects and animals. In obese patients with hypertension, ACh-induced forearm blood flow (FBF) is significantly reduced compared to healthy individuals. Caloric restriction decreases body weight and the mean blood pressure, fasting plasma insulin, total cholesterol, triglycerides, low-density lipoproteins (LDL) as well as enhancing ACh-induced FBF, while nitric oxide synthase inhibitors mask increase in ACh-induced FBF, indicating that reduction in energy intake improves endothelial function possibly by increasing endogenous NO production in obese hypertensive patients (Sasaki *et al.*, 2002). Lipid lowering also reduces the risk for coronary events, cardiovascular morbidity and mortality in a broad range of patients (Bucher *et al.*, 1999). Similar observations have also been reported in animal studies. In miniature pigs, 6 to 9 month of cholesterol-rich diet induces LDL-hypercholesterolaemia and advanced coronary atherogenesis. Withdrawal of dietary cholesterol reverses hypercholesterolaemia and stabilises coronary plaque in miniture pigs (Verhamme *et al.*, 2002) and partially restores endothelial function in rats (Naderali and Williams, 2003).

In the present study, diet-fed obese rats had significant vascular abnormalities, in particular a marked endothelial dysfunction. The potential factors for vascular abnormalities are obesity *per se* (Andres, 1980), insulin resistance (Reaven, 1988), increased leptin levels (Wallace *et al.*, 2001), and higher level of plasma triglyceride (Naderali *et al.*, 2001a; Naderali and Williams, 2001b; Naderali and Williams, 2003). In fact, there were negative correlations between endothelial function and plasma levels of insulin, leptin and triglycerides. The findings that changes in vascular function occurs in the absence of obesity or rise in plasma leptin levels and insulin resistance (Naderali *et al.*, 2001c; Naderali and Williams, 2003) argue against major roles of obesity *per se*, insulin and leptin resistance in vascular dysfunction. Moreover, in this study diet-to-chow rats were significantly obese compared with chow-fed controls, yet their vascular function was similar to

that of chow-fed group, further arguing, at least in the rat, against the role of obesity *per se* in the genesis of vascular abnormalities. On the other hand, recent human and animal studies point to the role of circulating triglycerides in obesity-indcued vascular dysfunction (Tka *et al.*, 1997; Krauss, 1998; Zhao *et al.*, 2001; Naderali *et al.*, 2001a; Naderali and Williams, 2001b; Naderali and Williams, 2003). In this study, circulating triglyceride levels were raised more than 115% and a significant negative correlation was seen between fasting plasma triglyceride levels and endothelium-dependent vasorelaxation. The plasma TG levels of chow-fed and diet-to-chow groups were similar as were CCh-induced vasorelaxation between the two groups, suggesting that changing diet (i.e. removing palatable diet and replacing it with the standard chow) resulted in lowering plasma triglyceride levels and thereby improving vascular function.

The adipocytokines, TNF-α (Myers *et al.*, 1994; Bhagat and Valance, 1997; Locksley *et al.*, 2001; Wajant *et al.*, 2003) and CRP (Biasucci *et al.*, 1999; Buffon *et al.*, 1999; Verma *et al.*, 2002) have been documented to induce vascular and metabolic defects. TNF-α and CRP were significantly increased in diet-fed animals, suggesting possible role for these adipocytokines in obesity-induced vascular dysfunction.

Overall, this study indicates that prolonged consumption of a high-palatable, high-energy diet induces metabolic and cardiovascular disorders. Nonetheless, these abnormalities are completely reversible by chronic withdrawal of the palatable diet.

Chapter 6

Results: The effects of fenofibrate on adiposity, metabolic abnormalities, and endothelial dysfunction in dietary obese rats

6.1 INTRODUCTION

Obesity and type 2 diabetes are strong risk factors for the development of atherosclerosis. The underlying pathophysiological mechanisms are not fully understood, but may include hyperlipidaemia and increased oxidation of low-density lipoproteins (Renier *et al.*, 2000), hypertriglyceridaemia (Naderali *et al.*, 2001a), insulin resistance (Reaven, 1988) and hyperleptinaemia (Wallace *et al.*, 2001). Obesity, type 2 diabetes and the above risk factors are also associated with marked defects in vascular function. Endothelial dysfunction in particular is prominent and may contribute to atherogenesis (Renier *et al.*, 2000; Suwaidi *et al.*, 2000; Mancini *et al.*, 2001; Naderali *et al.*, 2001; Naderali *et al.*, 2001a; Naderali *et al.*, 2001c).

The lipid-lowering fibrates, such as fenofibrate and ciprofibrate have been shown to impede atheroma development. These drugs bind to peroxisome proliferator-activated receptor-α (PPAR-α), leading to enhanced lipid catabolism in the liver (Mancini *et al.*, 2001) and reductions in total body fat as well as the circulating plasma lipids. Fenofibrate has been reported to retard angiographic progression of coronary atherosclerosis in diabetic patients (Steiner *et al.*, 2001), and improve the microcirculation in patients with hyperlipidaemia (Liang *et al.*, 2000). The mechanism(s) of the beneficial effects of fenofibrate on vascular function has not been fully elucidated yet, but direct activation of PPAR-α in arterial wall (Fruchart *et al.*, 1999), correction of lipid abnormalities (Desprēs *et al.*, 2002; Capell *et al.*, 2003), and increasing the formation, availability and action of NO (Haak *et al.*, 1998; Playford *et al.*, 2002) have all been postulated.

Consequently, this study aimed to further evaluate the effect of fenofibrate on adiposity, metabolic, and vascular abnormalities seen with obesity-inducing diet in obese rats.

6.2 METHODS

6.2.1 Experimental protocol

Adult male Wistar rats (n=27) were randomised and assigned to a control group (n=9, 191 ± 3 g) and a test group (n=18, 190 ± 1 g) that was fed a highly palatable diet for 15 weeks.

All animals had free access to water and were housed individually under controlled environmental conditions (19-22 °C; 30-40% humidity) and a 12-hour light/dark cycle (lights on at 07:00h). Controls were fed a standard laboratory pelleted diet (chow-fed), The test group had free access to a highly-palatable diet. After 8 weeks, half of the test group was given fenofibrate (50 mg/kg/day) and the remainder were given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, Pool, UK) by oral gavage, daily for 7 weeks (for more details see chapter 2, section 2.3.2). On the day of experiment, the rats were killed by CO_2 inhalation after 2 hours of fasting. The total body fat mass was measured immediately by Bioimpedance Method using the TOBEC® equipment (Biotech Instruments Ltd, Kimpton, UK). The gonadal and perirenal fat pads, the gastrocnemius and soleus muscles, liver, heart, and lungs were dissected and weighed.

6.2.2 Biochemical analysis

Blood was removed by cardiac puncture into cold heparinised tubes. The plasma was immediately separated by centrifugation before being frozen for later measurements of glucose, insulin, leptin, TNF-α, CRP, NEFA and TG. Plasma glucose concentration was determined using a glucose oxidase method, and NEFA and TG concentrations using commercial diagnostic kits. Insulin and leptin concentrations were measured by RIA kits, while plasma TNF-α and CRP concentrations were measured using a commercially available ELISA kits.

6.2.3 Homeostasis Model Assessment (HOMA)

HOMA, an index of insulin resistance, which employs measures of fasting plasma concentrations of glucose and insulin, was calculated according to the method described previously (Matthews *et al.*, 1985).

6.2.4 Assessment of vascular function

Six third-order mesenteric arteries (<250 μm diameter, 2-mm lengths) were carefully dissected from each animal. Each artery was freed of fat and connective tissue and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK), based on the principle of the Mulvany myograph which measures isometric tension generated in response to various stimuli. Arterial segments (two at a time) were incubated in a 5-ml organ bath containing physiological salt solution gassed with 95% O_2 and 5% CO_2 at 37 °C.

Cumulative concentration-response curves to either KCl (10-125 mM) or noradrenaline (NA, 0.5-6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded (for more details see chapter 2).

6.2.5 Assessment of defect in vascular relaxation

Endothelial-dependent and –independent defects were investigated by exposing NA-precontracted arteries to CCh or SNP, respectively. To eliminate possible interference by vasoactive prostanoids, 10 mM indomethacin was added to the organ bath prior to contraction with NA (8 μM). When contraction reached a plateau (after 2 minutes) concentration-response curves were performed to either CCh or SNP (for both, 10 nM-100 μM).

6.2.6 Data interpretation and statistical analyses

Vasoconstriction in response to NA and KCl were expressed as absolute force generated (mN/mm artery). Relaxation in response to CCh and SNP was calculated as the percentage reduction from the maximal tension generated at the supramaximal concentration of NA (8 μM). The average response for all the

vessels from a given animal was used in the group analysis. EC_{50} values were determined to evaluate changes in agonist-induced responses.

Data showed a normal distribution (Shapiro-Wilkes W test) and are therefore expressed as mean ± S.E.M. Statistical significance was tested using Student 't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney test, as appropriate. Differences were considered statistically significant at the $p<0.05$ levels.

6.3 RESULTS

6.3.1 Effects of fenofibrate on body weight and adiposity

Animals fed palatable diet progressively gained more weight than chow-fed controls. This difference became significant after the third week of feeding and was maintained thereafter. The final body weight of the diet-fed animals was significantly higher than that of chow-fed controls (>21%; 695 ± 17 *vs* 572 ± 8 g, $p<0.0001$). Overall, the weekly weight gain of diet-fed animals was significantly greater than that of chow-fed controls (diet-fed: 20.1 ± 4.77 *vs* control: 11.98 ± 4.65 g, p=0.013) (Fig. 6.1). Administration of fenofibrate significantly reduced weekly weight gain of diet-fed animals and final total body weight (Fig. 6.1). After 7 weeks of treatment, total body weight of the diet-fed fenofibrate-treated animals was not significantly different from that of chow-fed controls (fenofibrate treated: 593 ± 16 *vs* chow-fed: 572 ± 8 g, $p<0.2$), but it was significantly lower ($p<0.0007$) than that of untreated diet-fed animals (Fig. 6.1).

After 15 weeks of study, gastrocnemius and soleus muscle masses were comparable between all three groups. However, diet-fed animals had significantly higher total white adipose tissue (sum of epididymal and perirenal fat pads) mass than both chow-fed (by >2-fold, $p<0.0002$) and fenofibrate-treated (by >1.5-fold, $p<0.01$) groups (Figs. 6.2 a, b, c & d). Fenofibrate-treated animals also had significantly (by >1.4-fold $p<0.02$) greater white adipose tissue than chow-fed controls. Bioimpedence measurements indicated higher percentage of body-fat in diet-fed animals compared with chow-fed and fenofibrate-treated groups. Conversely, diet-fed rats had significantly lower percentage of lean mass than

chow-fed and fenofibrate-treated animals (Fig. 6.2 e, & f). Liver weight increased significantly in fenofibrate treated group compared with chow-fed (by > 213%, $p<0.00001$) and diet-fed groups (by > 174%, $p<0.008$), while there was no significant difference between chow-fed and diet-fed groups. Moreover, heart weight also increased significantly in fenofibrate-treated group, compared with chow-fed group (by > 111%, $p<0.01$), whereas lungs weight was comparable in all three groups with no significant difference (Fig. 6.3 a, b, and c).

6.3.2 Effects of diet and fenofibrate on blood metabolites

Palatable diet-feeding did not effect fasting plasma glucose levels (11.7 ± 1.2 chow-fed; 14.3 ± 0.3 mM diet-fed; $p<0.06$) (Fig. 6.4 a). However, diet-fed rats had significantly higher fasting terminal insulin (3.8 ± 0.8 diet-fed *vs* 1.6 ± 0.3 mM chow-fed; $p<0.002$) than the chow-fed group (Fig.6.4 b), with a significantly higher HOMA index value (2.40 ± 0.52 diet-fed *vs* 0.73 ± 0.10 chow-fed; $p<0.008$) indicating development of insulin resistance in diet-fed animals (Fig.6.5 a). Fenofibrate treatment significantly lowered levels of both glucose (10.8 ± 0.3 mM; $p<0.0001$ *vs* untreated diet-fed group) and insulin (1.4 ± 0.2 mM; $p<0.001$ *vs* untreated diet-fed group) levels. Consequently, HOMA index was also reduced (0.67 ± 0.10; $p<0.007$ *vs* untreated diet-fed group), indicating correction of insulin resistance (Fig. 6.5 a).

Diet-fed rats had significantly higher plasma leptin levels than chow-fed control (9.4 ± 0.7 diet-fed *vs* 6.9 ± 0.5 ng/ml chow-fed; $p<0.0131$) (Fig. 6.5 b). The rise in letpin levels was not affected by fenofibrate treatment (8.5 ± 0.4 ng/ml; $p<0.03$ vs chow-fed). Fasting plasma levels of NEFA were comparable in diet-fed and chow-fed groups (0.44 ± 0.02 chow-fed; 0.45 ± 0.04 mM diet fed) (Fig. 6.6 a). However, diet-fed rats had significantly higher (>110%) levels of fasting TG than chow-fed (chow-fed: 0.79 ± 0.04; diet-fed: 1.68 ± 0.17 mM; $p<0.0003$) (Fig. 6.6 b). Fenofibrate treatment significantly lowered fasting plasma levels of both NEFA (0.31 ± 0.02 mM; $p< 0.001$ *vs* chow-fed and untreated diet-fed groups) and TG (0.80 ± 0.06 mM $p<0.001$ *vs* untreated diet-fed group) below those of chow-fed group.

6.3.3 Effects on TNF-α and CRP

Diet-fed animals had significantly ($p<0.05$) higher TNF-α than their chow-fed counterparts (608.6 ± 56 chow-fed *vs* 1046.9 ± 166.9 pg/ml diet-fed). Fenofibrate-treated animals had significantly ($p<0.04$) lower TNF-α (532 ± 91 pg/ml) than their obese untreated counterparts. There were no significant differences between TNF-α level of chow-fed and fenofibrate treated animals (Fig. 6.7 a). Similarly, compared to chow-fed group, CRP levels increased significantly ($p<0.05$) in diet-fed animals (3.5 ± 0.8 diet-fed *vs.* 0.9 ± 0.3 ng/ml chow-fed). Interestingly, CRP levels further increased significantly ($p<0.001$) by fenofibrate-treatment (6.3 ± 0.7 ng/ml) (Fig. 6.7 b).

6.3.4 Vascular data

Diet-induced obesity did not alter vessel diameter, and there were no significant differences in arterial diameter between the three groups (200 ± 15 in chow-fed; 205 ± 15 in diet-fed; 200 ± 10 μm in fenofibrate-treated).

6.3.5 Agonist-induced vasoconstriction responses

Cumulative concentration response curves to KCl (10-125 mM) and NA (0.5-6 μM) in arteries from all three groups showed the characteristic sigmoid shape. KCl-induced contraction was similar in all three groups (Fig. 6.8 a), and the concentration response curves were virtually superimposable, reaching maximum force of 5.68 ± 0.44 (chow-fed), 6.07 ± 0.33 (diet-fed) 5.35 ± 0.37 (fenofibrate-treated) mN/mm artery. There was no significant differences between the groups, either overall (by ANOVA) or at any given KCl concentration. A similar pattern was also seen with NA-induced contractions (Fig. 6.8 b), giving maximum force of 9.14 ± 0.63 (chow-fed), 9.94 ± 0.43 (diet-fed) and 9.70 ± 0.84 (fenofibrate-treated) mN/mm artery.

6.3.6 Endothelium-dependentand and -independent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM- 100 μM), achieving a maximum of 88 ± 2% at a CCh concentration of 100 μM (Fig. 6.9 a), with EC_{50} values of 0.28 ± 0.03 μM.

Arteries from diet fed rats that were similarly exposed to CCh displayed a significant rightward shift of the concentration-response curve, as compared with chow-fed control arteries (EC_{50} diet-fed: 0.94 ± 0.04 μM $p<0.01$ vs. chow-fed). Maximal vasorelaxation to 100 μM CCh was reduced by 17% in diet-fed rats ($p<0.005$ *vs.* chow-fed). Fenofibrate-treatment did not improve EC_{50} values (0.86 ± 0.04 μM, $p<0.002$ *vs.* chow-fed), but it enhanced maximal CCh-induced vasorelaxation at higher concentrations (>10 μM), achieving 86 ± 3% relaxation at 100 μM CCh (Fig. 6.9 a).

Feeding the high-palatable diet did not effect concentration-dependent vasorelaxation induced by SNP (10 nM-100 μM) (Fig. 6.9 b). The concentration-relaxation response curves were identical and virtually superimposable in all three groups, with EC_{50} values of 2.08 ± 0.07 (chow-fed); 1.73 ± 0.12 (untreated obese), and 1.46 ± 0.10 μM (fenofibrate-treated). There were no statistically significant differences between all three groups, either overall (by ANOVA) or at any given SNP concentration.

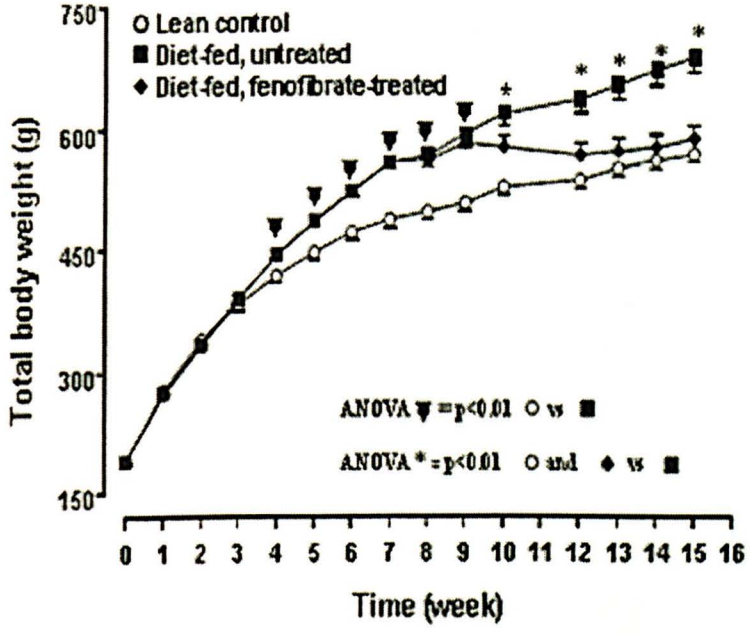


Fig. 6.1. The effects of highly-palatable-diet and fenofibrate on total body weight in male Wistar rats. Lean control animals were fed a standard laboratory chow, while diet-fed group was given a palatable diet for 15 weeks. Fenofibrate-treated animals were given fenofibrate (50 mg/kg/day) daily for the last 8 weeks of the experiment. At the end of 15 weeks, untreated diet-fed animals were significantly (p<0.0001) heavier than lean control and fenofibrate-treated groups. Data are expressed as mean ± S.E.M.

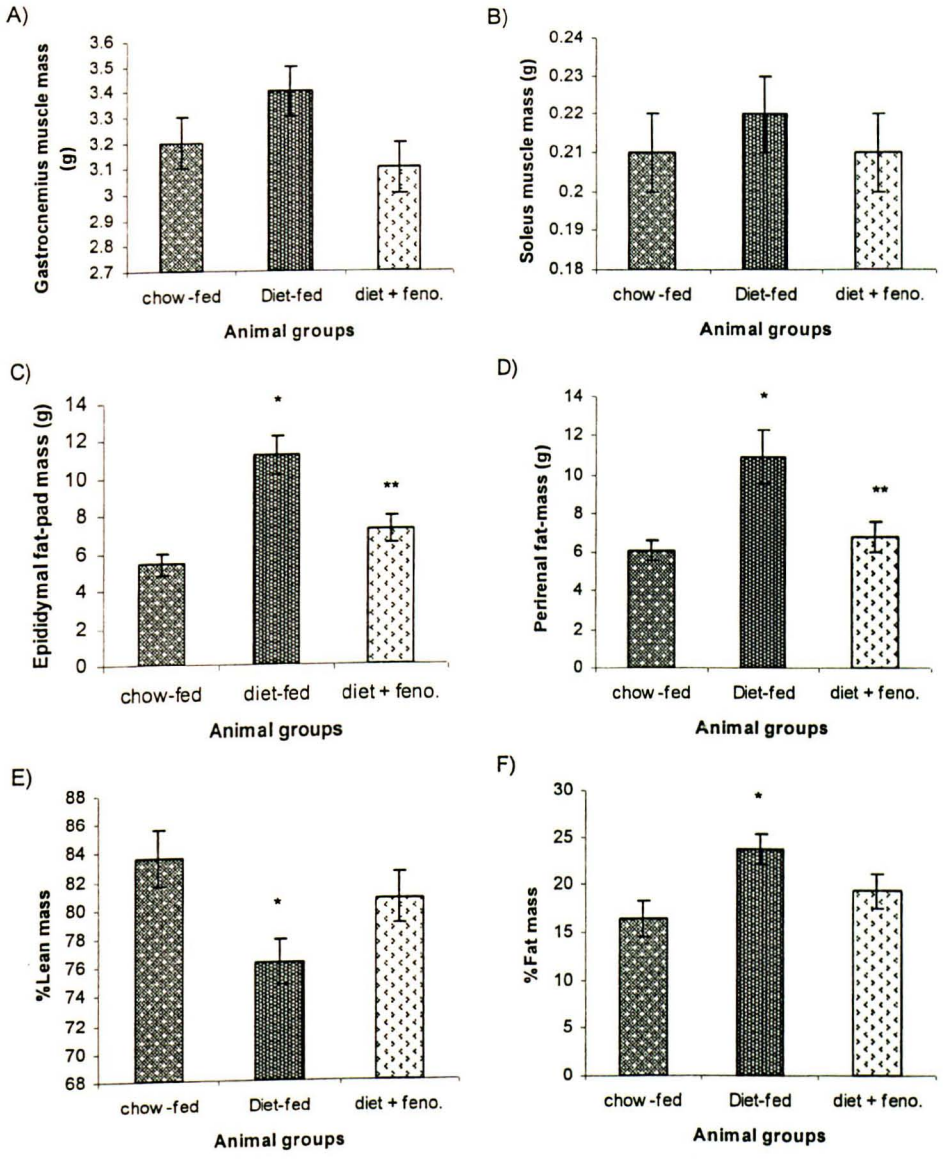


Fig. 6.2. The effects of fenofibrate on the weight of: A) Gastrocnemius muscle mass, B) Soleus muscle mass, C) Epididymal fat-pad mass (*p<0.001 *vs* chow-fed, **p<0.001 *vs* diet-fed), D) Perirenal fat-mass (*p<0.001 *vs* chow-fed, **p<0.001 vs. diet-fed), E) % Lean mass (*p<0.01 *vs* chow-fed and diet + fenofibrate treated groups), and F) % fat mass **p<0.01 *vs* chow-fed and diet + fenofibrate treated groups), in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow while the two diet-fed groups were given a palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are mean ± S.E.M.

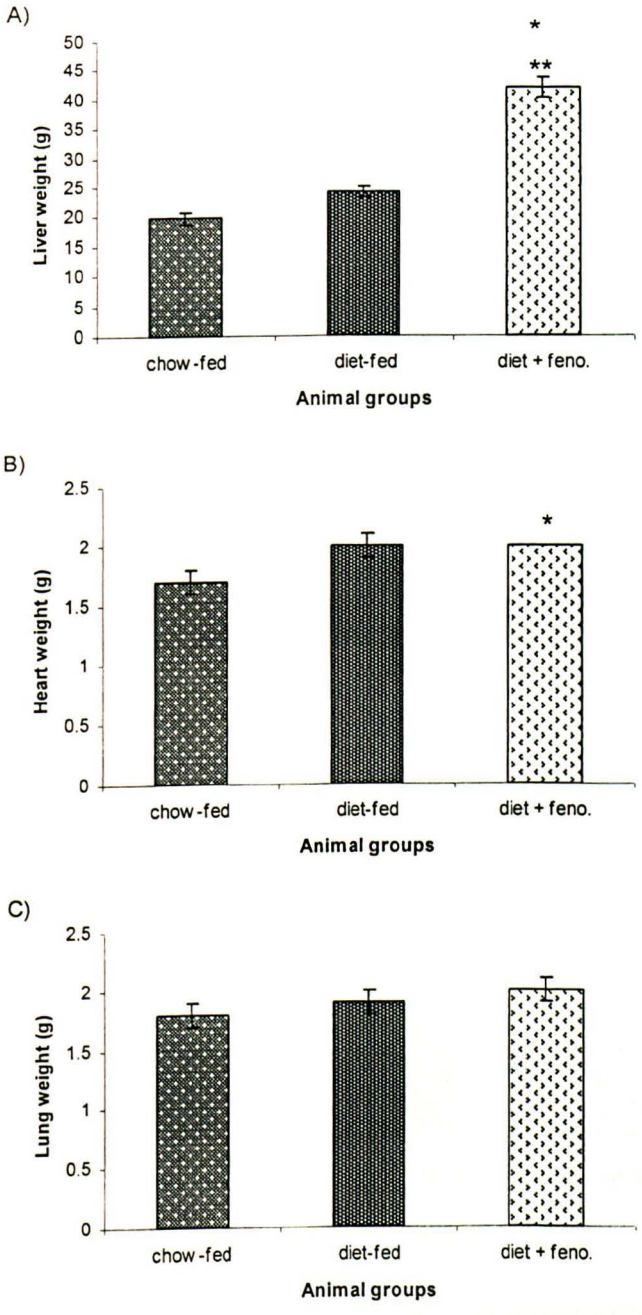


Fig. 6.3. The effect of fenofibrate treatment on the weights of: A) liver, (*p<0.0001 vs chow-fed, and **p<0.0001 vs diet-fed), B) heart, (*p<0.02 vs chow-fed) and C) lung in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow, while the two diet-fed groups were given a highly-palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are expressed as mean ± S.E.M.

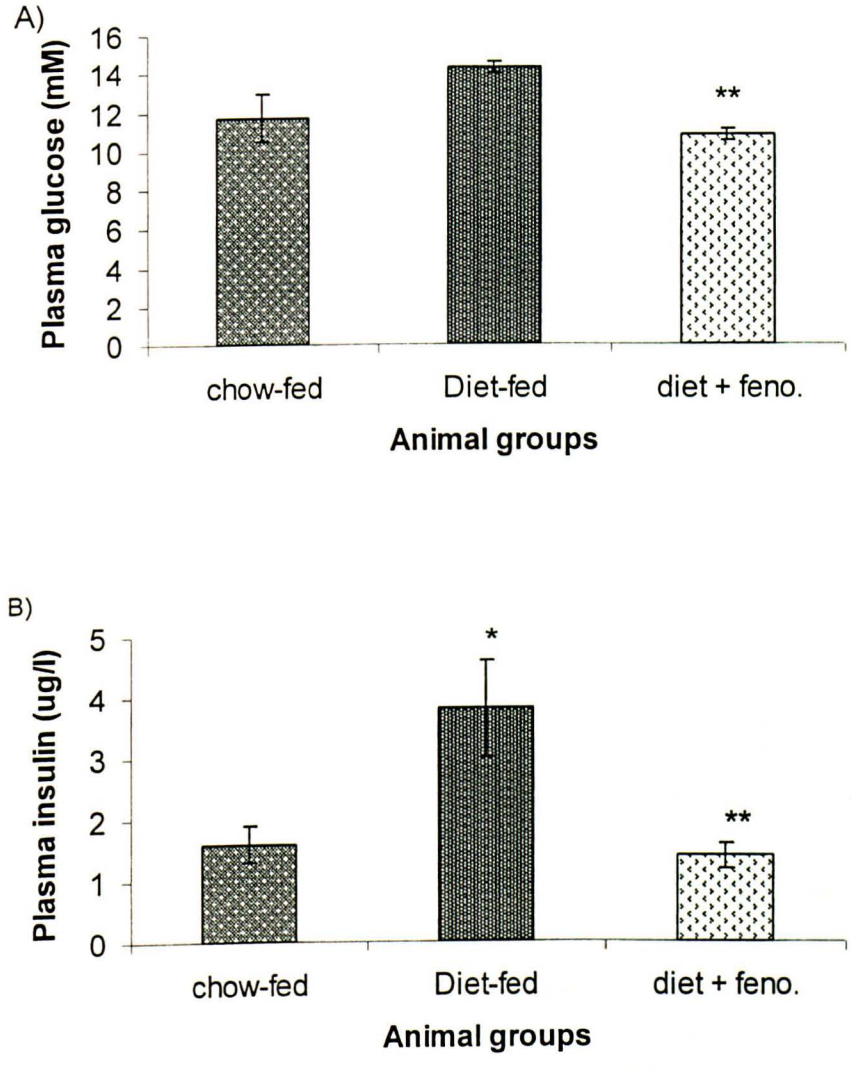


Fig. 6.4. The effects of fenofibrate on the concentration of: A) Plasma glucose, and B) Plasma insulin, in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow while the two diet-fed groups were given a palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are mean ± S.E.M. *$p<0.001$ *vs* chow-fed, and **$p<0.001$ *vs* diet-fed.

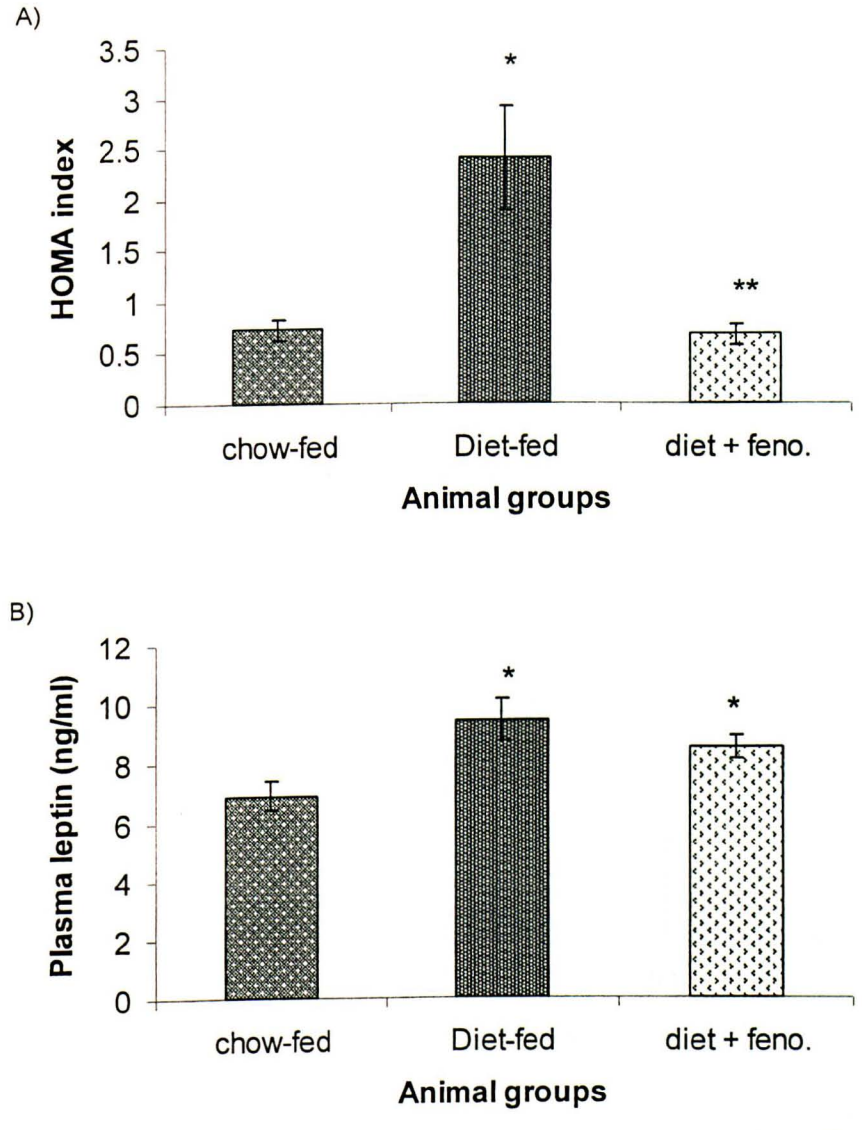


Fig. 6.5. The effects of fenofibrate on: A) HOMA index, and B) the concentration of Plasma leptin, in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow, while the two diet-fed groups were given a palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are mean ± S.E.M. *p<0.001 *vs* chow-fed, and **p<0.001 *vs* diet-fed.

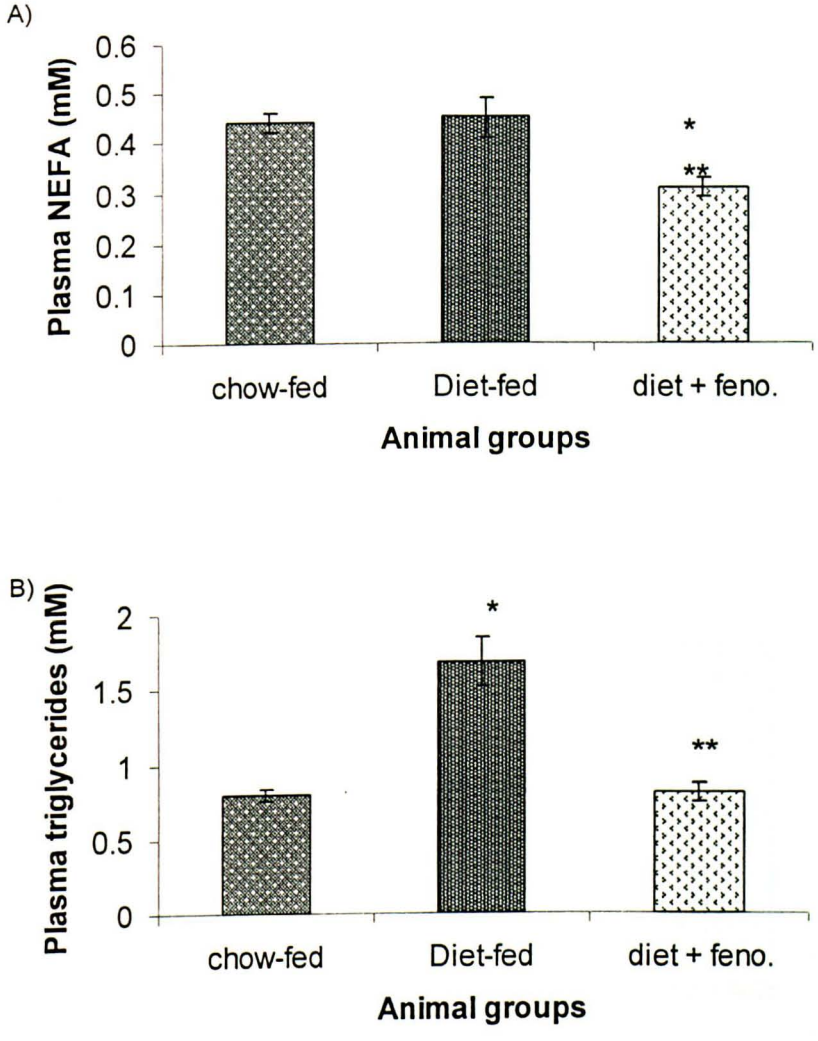


Fig. 6.6. The effects of fenofibrate on the concentration of: A) Plasma NEFA, and B) Plasma triglycerides, in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow while the two diet-fed groups were given a palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are mean ± S.E.M. *p<0.001 *vs* chow-fed, and **p<0.001 *vs* diet-fed.

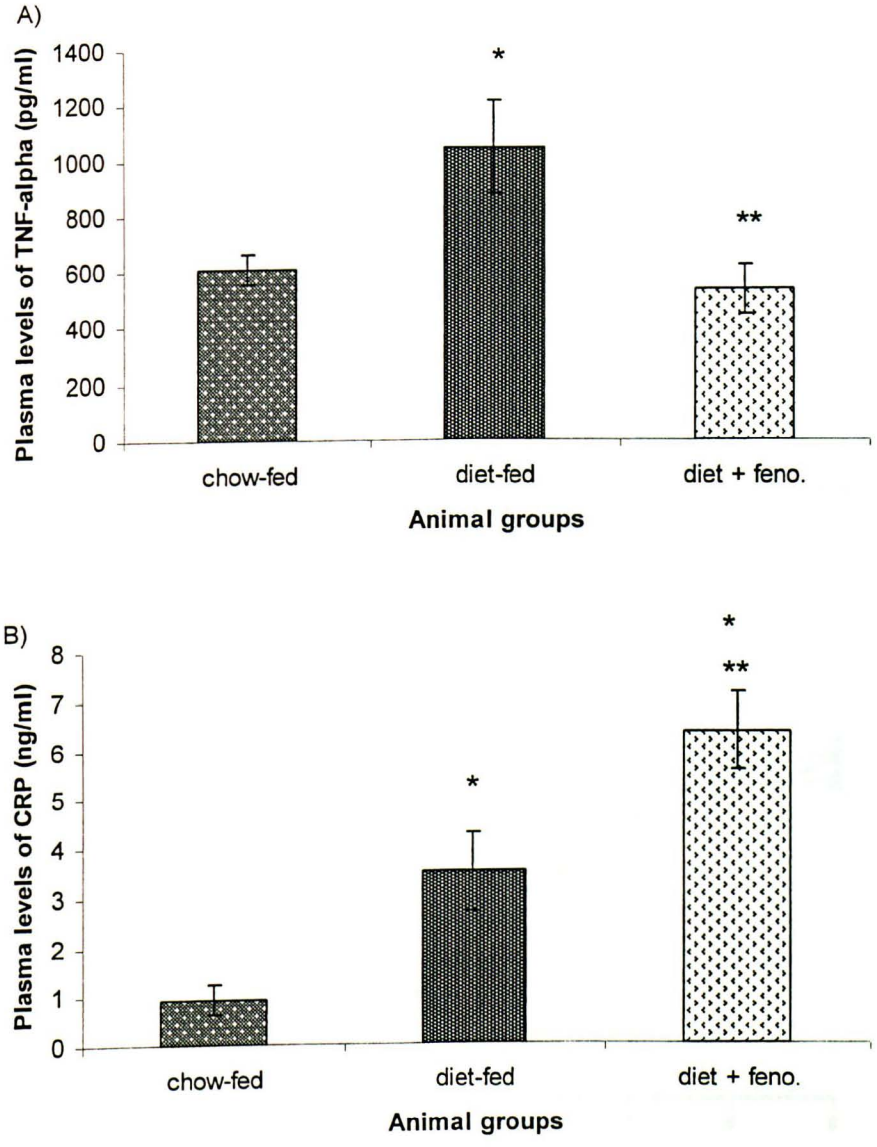
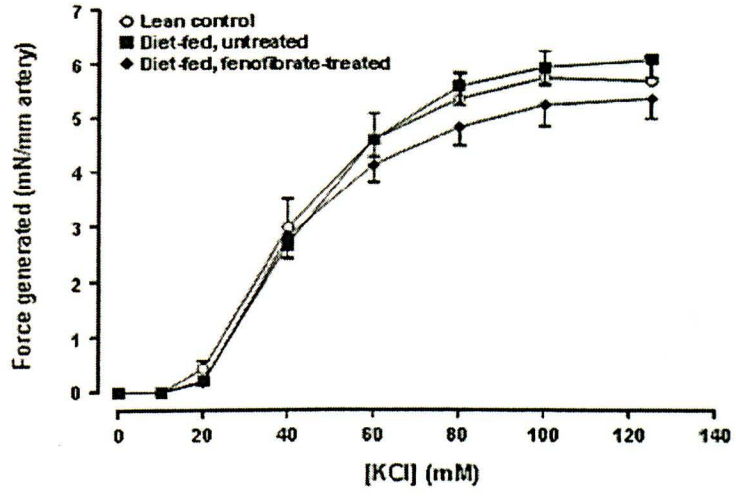


Fig. 6.7. The effect of fenofibrate treatment on plasma levels of A) TNF-α, (*p<0.05 vs chow-fed and **p<0.05 vs diet-fed) and B) C-reactive protein (CRP) (*p<0.0001 vs chow-fed and **p<0.02 vs diet-fed) in dietary-induced obese rats. Lean control animals were fed a standard laboratory chow while the two diet-fed groups were given a palatable diet for 15 weeks. Fenofibrate was given to one of the diet groups (50 mg/kg/day) daily for the last 8 weeks. Data are expressed as mean ± S.E.M.

a)



b)

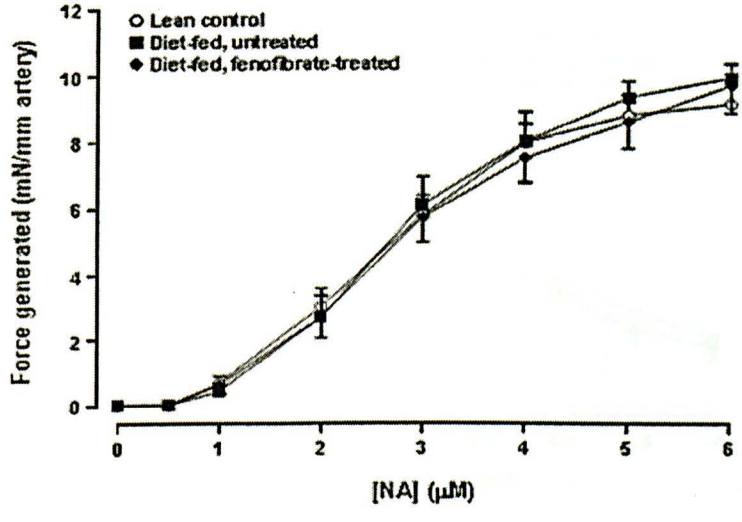


Fig. 6.8. Vasoconstriction responses to cumulative concentration of: a) KCl (10 – 125 mM) and b) noradrenaline (0.5 – 7 μM) in arteries from lean control, untreated diet-fed and fenofibrate-treated diet-fed animals. Data represent mean ± S.E.M for 9 animals in each group. There were no significant differences in agonist-induced vasoconstriction between the three groups.

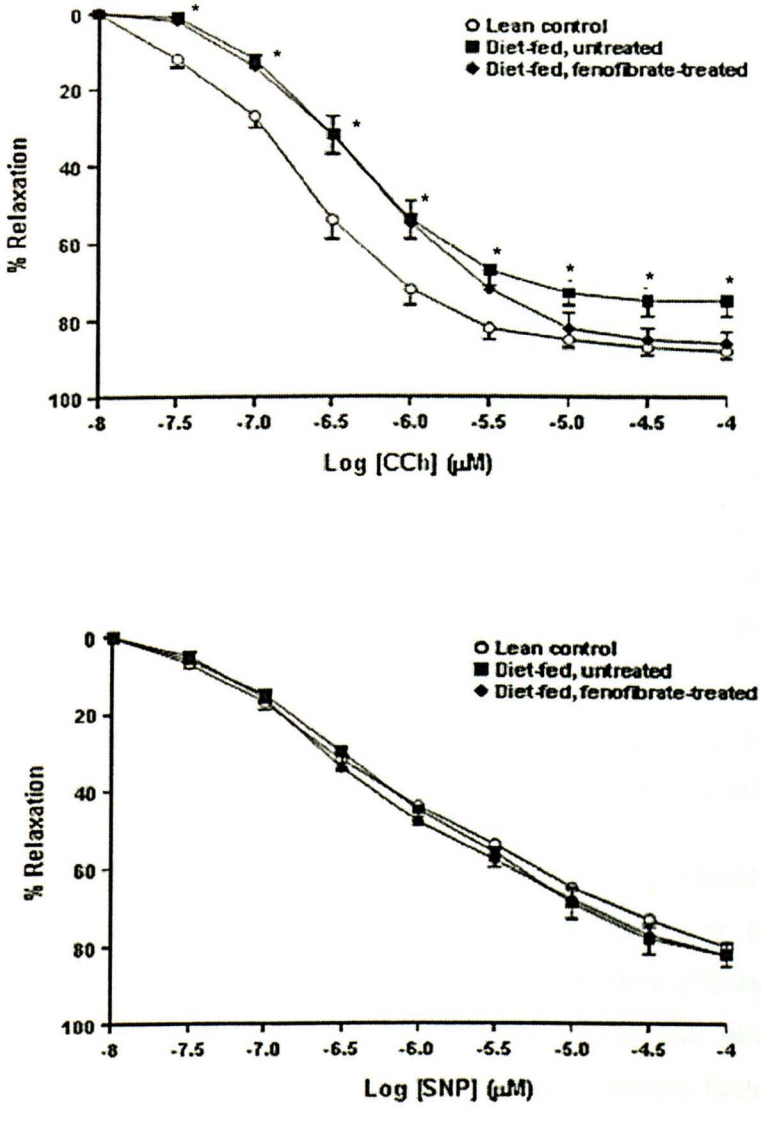


Fig. 6.9. The effects of (a) carbamylcholine (CCh), (b) sodium nitroprusside (SNP) on arteries from lean control, untreated diet-fed and fenofibrate-treated diet-fed rats. Noradrenaline (8 μM) preconstricted arteries challenged with cumulative concentrations of CCh or SNP. CCh-induced vasorelaxation was significantly reduced in diet-fed animals (*p<0.001; ANOVA, Bonferroni test) in comparison to lean control group. Moreover, vasorelaxation curves were shifted to the right in both diet-fed groups. Fenofibrate-treatment only partially restored CCh-induced vasorelaxation. Data represent mean ± S.E.M for 9 animals in each group.

6.4 DISCUSSION

Various studies in animal models and in subjects with diabetes have shown that PPAR-α activation reduces diet-induced weight gain and visceral fat mass and improves insulin resistance (Guerre-Millo *et al.*, 2000; Lee *et al.*, 2002). The precise mechanism(s) by which PPAR-α improves insulin resistance is not fully known yet, but increasing fatty acid catabolism in liver, decreasing skeletal muscle TG content or decreasing production of cytokines associated with insulin resistance pathways (Wang *et al.*, 2001), counteracting hypertriglyceridaemia by increasing hepatic oxidation of fatty acids and reduced synthesis and secretion of TG (Staels *et al.*, 1998) have all been postulated as possible mechanisms. Similarly, the mechanism of PPAR-α-induced reduction in weight gain also remains controversial. There is a suggestion that a reduction in fat mass and consequential decrease in leptin production may ultimately induce hypophagia and the resultant decrease in weight gain (Kubota *et al.*, 1999), while another study reported lack of any association between plasma levels of leptin, food intake and inhibition of weight gain by PPAR-α agonist, fenofibrate treatment (Guerre-Millo *et al.*, 2000).

The present study and other reports have shown that dietary obesity not only causes metabolic abnormalities, it also markedly affects vascular function, in particular endothelium-dependent agonist-induced vasorelaxation (Steinberg *et al.*, 1996; Naderali *et al.*, 2001a; Naderali *et al.*, 2001c). The vascular abnormalities induced by dietary obesity are complex and multifactorial. Possible factors include fat mass *per se*, insulin resistance (Reaven, 1988), increased in NEFA levels (Steinberg *et al.*, 1996) hypertriglyceridaemia (Naderali *et al.*, 2001a) decrease in plasma HLD-c levels (Desprēs *et al.*, 2002), and hyperleptinaemia (Wallace *et al.*, 2001). The key players could, in theory be identified through interventions that aim to correct these abnormalities and findings which leads to the amelioration of vascular dysfunction.

In this study, in agreement with previous reports (Furuhashi *et al.*, 2002) 8 weeks treatment of fenofibrate reduced total body weight, weekly weight gain and adiposity. Moreover, fenofibrate-treatment completely corrected dietary-induced insulin-resistance, hyperinsulinaemia, and hypertriglyceridaemia but not

hyperletinaemia. By contrast, the beneficial effects of fenofibrate on endothelial function were relatively minor and only appeared at higher concentration of carbamylcholine (>31.6 μM CCh). The failure of fenofibrate to completely correct dietary-induced endothelial dysfunction seen in this study is in agreement with previous reports. Studies by others have not shown any significant changes in endothelial function after acute treatment with 250 mg fenofibrate (Bae *et al.*, 2001), nor were there any beneficial effects on blood pressure or progression of atherosclerosis process (Wilmink *et al.*, 2001; Furuhashi *et al.*, 2002), despite improving insulin sensitivity and hypertriglyceridaemia. However, others have shown significant improvement of endothelial function after fenofibrate-treatment in human subjects (Idzior-Walus *et al.*, 2000; Malik *et al.*, 2001; Capell *et al.*, 2003). These contrasting findings could be explained by differences in experimental conditions. For example, subjects in Idzior-Walus and colleagues' (Idzior-Walus *et al.*, 2000) study had been under lipid management for at least 12 months, establishing a steady state of weight and lipid parameters and those in Malik and colleagues (Malik *et al.*, 2001) study did not include obese or insulin resistance subjects. Another recent study (Capell *et al.*, 2003) reported that fenofibrate treatment (2 weeks) significantly reduced fasting TG levels and improved both endothelium-dependent and –independent vasorelaxation. Once again, these patients underwent specific dieting for at least 2 weeks before any vascular analysies were carried out. Taken together, these observations, it is possible to hypothesise that withdrawal of a fat-enriched diet attenuated vascular dysfunction in these subjects and that this may have further improved by administration of fenofibrate, as seen in the present study. Consistent with an effect of diet *per* se, it has been reported recently that acute withdrawal of an obesity-inducing diet partially restores normal endothelial function (Naderali and Williams, 2003). While, data in previous chapter (chapter 5) have demonstrated that chronic withdrawal completely corrects dietary-obesity-induced vascular dysfunction, indicating that diet *per se* rather than dietary-induced obesity plays a key role in vascular abnormalities. However, it is also possible to argue that hyperleptinaemia (Wallace *et al.*, 2001) seen in diet-fed rats (both vehicle- and fenofibrate-treated rats) may at

least in part, explain the limited beneficial effects of fenofibrate on vascular function.

The present study, identifies for the first time the possible causative factors and mechanisms which might be involved in "the limited" beneficial effects of fenofibrate treatment on the vascular abnormalities caused by diet-induced obesity (Fig. 6.10). In this study, fenofibrate was administrated in the presence of high palatable diet feeding, and was associated with elevated levels of plasma CRP and leptin. A recent study has demonstrated that hyperleptinaemia is associated with increased CRP, and that a significant correlation between plasma leptin and CRP is evident, suggesting involvement of leptin and CRP in inflammatory and cardiovascular disease processes (Shamsuzzaman *et al.*, 2004). Various studies reported that highly-palatable diet enhances leptin production well before a significant weight increase (Harrold *et al.*, 2000; Archer *et al.*, 2005). Similarly, in the present study increase in plasma leptin could be attributed to diet-induced obesity and not to fenofibrate-treatment, since fenofibrate administration (in the absence of high palatable diet feeding) induces significant reduction in plasma levels of leptin (Damci *et al.*, 2003).

This study also hypothesises that the increased concentration of plasma CRP seen in fenofibrate treated group might be induced by hyperleptinaemia. In fact physiological concentrations of leptin stimulates expression of CRP in human primary hepatocytes (Chen *et al.*, 2006). Various studies have demonstrated that CRP not only has pro-inflammatory effects (Leinonen *et al.*, 2003; Lin *et al.*, 2004; Nesto, 2004; Garanty-Bogacka *et al.*, 2005), it also causes insulin resistance, endothelial dysfunction and involved in the pathogenesis of atherosclerosis (Lin *et al.*, 2004). Incubation of cultured human coronary artery smooth muscle cells with CRP resulted in reactive oxygen species (ROS) generation, suggesting that CRP might play a crucial role in plaque instability and in the pathogenesis of acute coronary syndrome (Kobayashi *et al.*, 2003). Therefore, the vascular abnormalities seen in this study could be caused by the elevated levels of plasma CRP *via* its pro-inflammatory, atherogenic, and pro-oxidative effects.

Leptin affects vascular system, promoting cardiovascular disease (Wallace *et al.*, 2001). Leptin increases CRP production which, in turn, leads to induction of leptin-resistance (Chen *et al.*, 2006), and subsequent cardiac hypertrophy (Barouch *et al.*, 2003). Similarly, in this study diet-induced obese animals had higher leptin levels, reduced vascular function, and increased cardiac mass, suggesting involvement of leptin-CRP axis in cardiovascular pathology. Both leptin and CRP have direct and indirect detrimental effects on vascular function. In the presence of highly-palatable diet, production of leptin and CRP continued such that beneficial effects of fenofibrate failed to counter their negative effects on vasculature.

Fenofibrate protects vascular function and improves insulin resistance (Badiou *et al.*, 2004; Koh *et al.*, 2005), by lowering lipid profile (Damci *et al.*, 2003; Empen *et al.*, 2003; Badiou *et al.*, 2004), total body weight and adiposity (Filippatos *et al.*, 2005; Ji *et al.*, 2005). This in turn would result in reduction of adipocytokines such as TNF-α (Skoog *et al.*, 2002 ; Yamaguchi *et al.*, 2003; Rask-Madsen *et al.*, 2003 ; Matsuda and Shimomura, 2004; Recasens *et al.*, 2004). In the present study, fenofibrate reduced plasma concentration of TNFα to levels similar to chow-fed groups, indicating anti-inflammatory effects of fenofibrate (Lin *et al.*, 2004). However, despite complete improvement of systemic insulin resistance, fenofibrate only partly restored obesity-induced vascular abnormalities (Fig. 6.10).

In conclusion, this study indicates that fenofibarte treatment in the presence of obesity-inducing diet, decreases adiposity, lowers plasma TG and NEFA levels and improves insulin resistance in dietary-obese animals, but has little effects on vascular function. The lack of marked beneficial effects of fenofibrate on vascular function may, at least in part, be due to the continuous presence of detrimental factors in obesity-inducing diet, of which high sucrose and/or fats may play important roles. in particular, the diet-induced elevated levels of CRP might play a crucial role in the development of vascular abnormalities. Furthermore, findings from this study, indicate that pharmacological attenuation of adiposity alone does not alleviate obesity-induced endothelial dysfunction, and that removal of obesity-inducing diet (as shown in chapter five) is an important step in correcting vascular dysfunction.

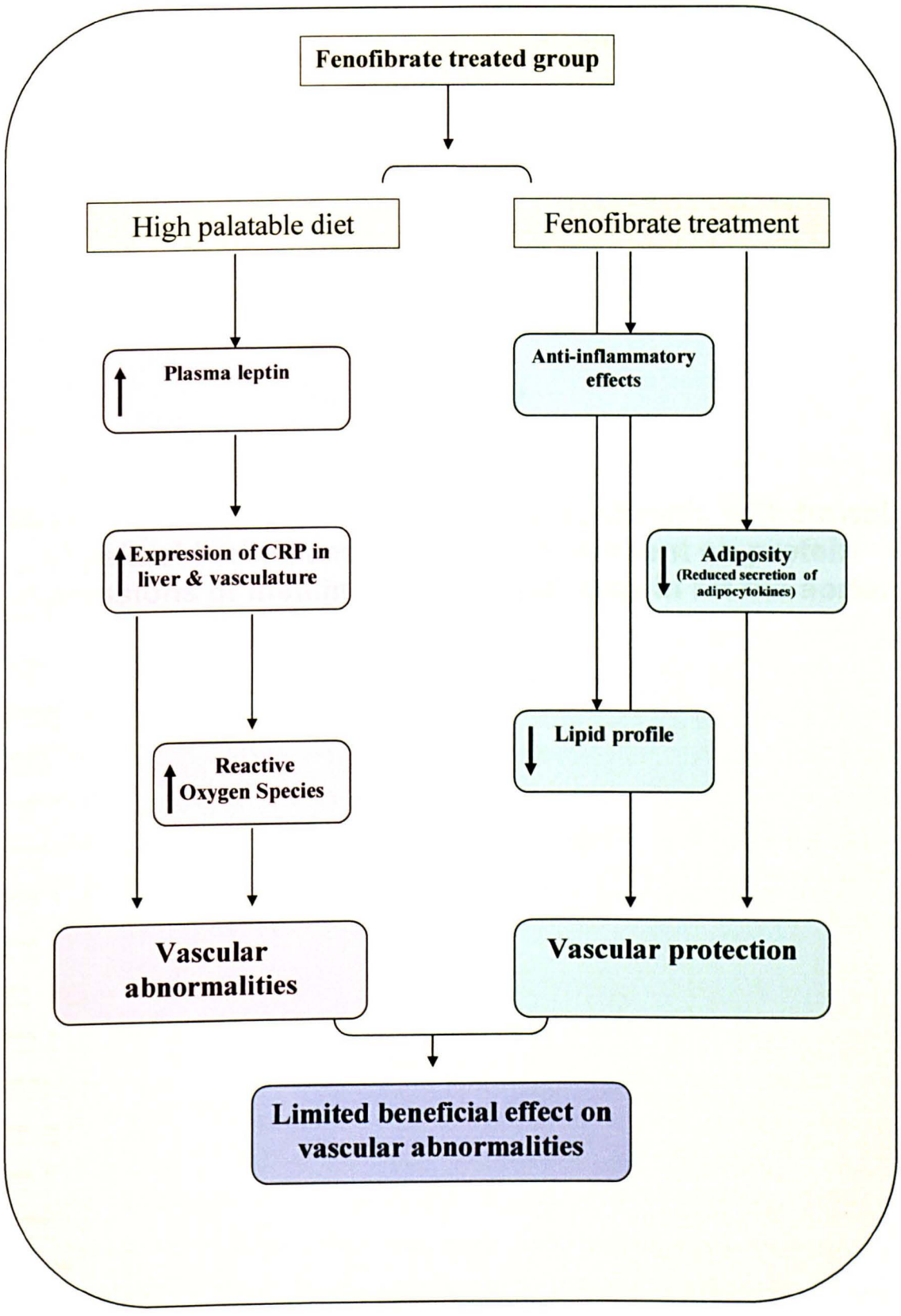


Fig. 6.10. Analysis for the vascular effects of fenofibrate treatment in diet-induced obesity "in the present study".

Chapter 7

Results: the effects of dietary obesity, chronic withdrawal of palatable diet, and fenofibrate treatment on protein expressions of insulin signalling pathway in rat the aorta.

7.1 INTRODUCTION

Obesity, characterised by excess adipose tissue is now becoming a worldwide epidemic (Staels *et al.*, 1998; James *et al.*, 2001). Various studies have suggested that obesity *per se* is an independent cardiovascular risk factor (Duflou *et al.*, 1995), as well as predisposing to type 2 diabetes, hypertension and dyslipidaemia (Reaven, 1988). Furthermore, obesity induces insulin resistance which is associated with development of cardiovascular diseases that include hypertension (Reaven, 1993), and reduced endothelial function (Steinberg, 1996). Insulin has a protective role in vascular function. It stimulates nitric oxide (Mancini *et al.*, 2001) production, leading to vasorelaxation. Insulin-induced NO-dependent vasorelaxation is markedly decreased in obesity (Walker *et al.*, 1997), however, the mechanism(s) of decrease in insulin-induced vasorelaxation is not fully understood. Insulin has other major physiological roles that include facilitation and increase of amino acid transport, glycogen synthesis, DNA synthesis and gene expression (Obata, 1996). Moreover, it specifically enhances release of nitric oxide (Zeng and Quon M, 1996; Mancini *et al.*, 2001), regulates mRNA matrix proteins (Tamaroglio and Lo, 1994) and constitutive endothelial NO synthase (Kuboki, 1998) activity in vasculature.

In vascular cells, the effects of insulin are initiated through binding to the insulin receptor alpha subunit (IR-α), which activates the intrinsic receptor tyrosine kinase (Kasuga *et al.*, 1982), resulting in autophosphorylation of insulin receptor beta subunit and tyrosine phosphorylation of intracellular adaptor proteins - insulin receptor substrates (IRS-1 and IRS-2) (Sun, 1991) and Shc (Skolink, 1993). Tyrosine phosphorylated IRS-1 or IRS-2 then binds to src-homology 2 (SH2) domains of intracellular proteins, including the p85 regulatory subunit of phosphatidyl-inositol (PI) 3-kinase (Myers and Jr., 1992). The interactions of IRS and p85 subunit of PI 3-kinase results in the activation of p110 catalytic subunit of PI 3-kinase. Activation of PI 3-kinase increases serine phosphorylation of Akt, which in turn, directly phosphorylates eNOS on serine 1177 and activates the enzyme, leading to increased NO production and thus providing vascular protection (Dimmeler *et al.*, 1999). Tyrosine phosphorylated Shc and IRS proteins

can also bind to SH2 domain of Growth factor Receptor-protein Bound 2 (GRB2), leading to the activation of the Ras-Raf-MAP kinase signal pathway that is associated with gene expression and cell growth (Skolink, 1993). PI 3-kinase expression and activation has been linked to NO production, whereas, Raf-MAP kinase pathway is associated with the growth of vascular cells and the expression of extracellular matrix proteins (Zeng and Quon M, 1996). Furthermore, various studies (Tamaroglio and Lo, 1994; Obata, 1996; Zeng and Quon M, 1996; Walker *et al.*, 1997; Kuboki, 1998; Mancini *et al.*, 2001) have shown attenuation of insulin-induced vasorelaxation in obesity, however, there is little information on the mechanism(s) of diet-induced changes in insulin signalling pathway in vasculature. Therefore, it is possible that changes in any component of insulin signalling pathway may alter vasorelaxant property of insulin. Furthermore, the level of protein expression of these kinases could fluctuate and respond differently to any pathological, physiological, or pharmacological conditions, such as dietary obesity and its treatment. Therefore, investigating the protein expression of these kinases under certain obesity-related experimental conditions could provide vital information about obesity-induced insulin resistance in vascular tissues. Consequently, this study aimed to investigate the protein expression of insulin signalling components in aorta of four different experimental groups, namely, 1) control chow-fed lean animals, 2) untreated diet-induced obese animals, 3) obese animals following chronic withdrawn of palatable diet, and 4) obese animals treated with fenofibrate. Fenofibrate has been shown to improve lipid profiles, reduce adiposity, and may have a role in correction of vascular function in obesity.

7.2 METHODS

7.2.1 Experimental protocol

Adult male Wistar rats (n = 28, 190 ± 3 g) were randomised and assigned to a control group (n = 10) and a test group (n = 20). Controls were fed standard laboratory pelleted diet (Chow-fed), while test group had free access to a highly-palatable diet. Chow-fed controls remained on their prospective diet for 15 weeks, while after 8 weeks palatable-diet-fed animals were subdivided into three subgroups (each group 7 animals). In the first subgroup, palatable diet was removed and the standard chow diet was re-introduced (diet-to-chow), while the

second subgroup remind on palatable diet and were given fenofibrate (fenofibrate-treated, 50 mg/kg/day) by oral gavage for further 7 weeks and the third subgroup (diet-fed) was given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, UK), by oral gavage daily for 7 weeks. On the day of experiment (after 15 weeks), the rats were killed by CO_2 inhalation after 2 hours of fasting, the animal body was weighed, and the aorta was dissected and snap frozen in liquid nitrogen.

7.2.2 Molecular analysis of Aorta

Western-immunoblotting technique was then carried out (as described in chapter 2) on the four experimental groups (chow-fed, vehicle, diet-to-chow, and fenofibrate treated groups) for measuring protein expressions of insulin signaling pathway in the aorta (IR-β, IRS-1, IRS-2, PI 3-kinase, AKt, eNOS, SHC, and ERK1/2). The method which described in the previous paper by Iartey et.al. (2006) retrospectively should have been used for comparing the blotting data statistically.

7.2.3 Data interpretation and statistical analysis

Changes in body weights of each group were collected weekly and are expressed as absolute total body weight, respectively. For Western blotting, the data from chow-fed (control) animals were expressed as 100% response, and the results from other three groups were normalized and subsequently expressed as the percentage of their respective controls. Data are expressed as mean ± S.E.M. Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student 't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p<0.05$ levels.

7.3 RESULTS

7.3.1 Changes in total body weight

Animals given palatable diet progressively gained more weight than their chow-fed counterparts. Untreated diet-fed animals were significantly heavier than chow-fed (>21%; 695 ± 17 versus 572 ± 8 g, $p<0.0001$); diet-to-chow (>11%; 695 ± 17 versus 625 ± 16 g, $p<0.01$) and fenofibrate treated (>17%; 695 ± 17 versus 593 ± 17 g, $p<0.001$) animals. However, at the end of the experiment, body weight of

diet-to-chow and fenofibrate treated animals were significantly ($p<0.01$) lower than untreated diet-fed animals (Fig. 7.1).

7.3.2 Protein expression studies

IR-β. There were no significant differences in IR-β protein levels in aorta from chow-fed, untreated diet-fed and diet-to-chow animals, while aorta from fenofibrate-treated animals had significantly (up to 30%, $p<0.001$) higher IR-β protein levels than chow-fed group (Fig. 7.2).

IRS-1. and IRS-2. As with IR-β, the IRS-1 and IRS-2 levels in chow-fed, untreated diet-fed and diet-to-chow fed groups were comparable, while aorta from fenofibrate-treated animals had significantly higher levels of both IRS-1 (26%, $p<0.05$) and IRS-2 (25 %, $p<0.05$) compared to chow-fed group (Fig. 7.3 & 7.4).

PI 3-kinase. Compared with chow-fed animals, there was a marked reduction in PI 3-kinase levels in untreated diet-fed animals (59%, $p<0.001$). PI 3-kinase levels were further reduced by fenofibrate-treatment (92%, $p<0.0001$), while removal of palatable diet completely reversed the reduction in PI 3-kinase levels seen in diet-fed group (Fig. 7.5).

Akt. The protein levels of Akt were similar in chow-fed, untreated diet-fed and fenofibrate-treated animals, while aorta from diet-to-chow group had significantly lower (13%, $p<0.01$) levels of Akt protein (Fig. 7.6).

eNOS. There was a significant (25%, $p<0.001$) elevation of eNOS levels in aorta from untreated diet-fed animals compared with that of chow-fed control, while removal of diet or fenofibrate-treatment markedly reduced the elevation of eNOS protein concentration seen in untreated diet-fed animals (Fig. 7.7).

Shc. Although, Shc protein levels were not significantly altered by diet feeding but removal of the diet or fenofibrate-treatment significantly (by up to 20%, $p<0.05$) attenuated Shc levels in comparison to that of chow-fed controls (Fig. 7.8).

ERK1/2. Immunoblotting analysis of ERK1/2 (MAP kinase pathway) protein in all four groups was remarkably similar to each other. Palatable-diet feeding in the presence or absence of fenofibrate had no effect on ERK1/2 levels nor did the removal of the palatable diet altered ERK1/2 levels in aorta (Fig. 7.9).

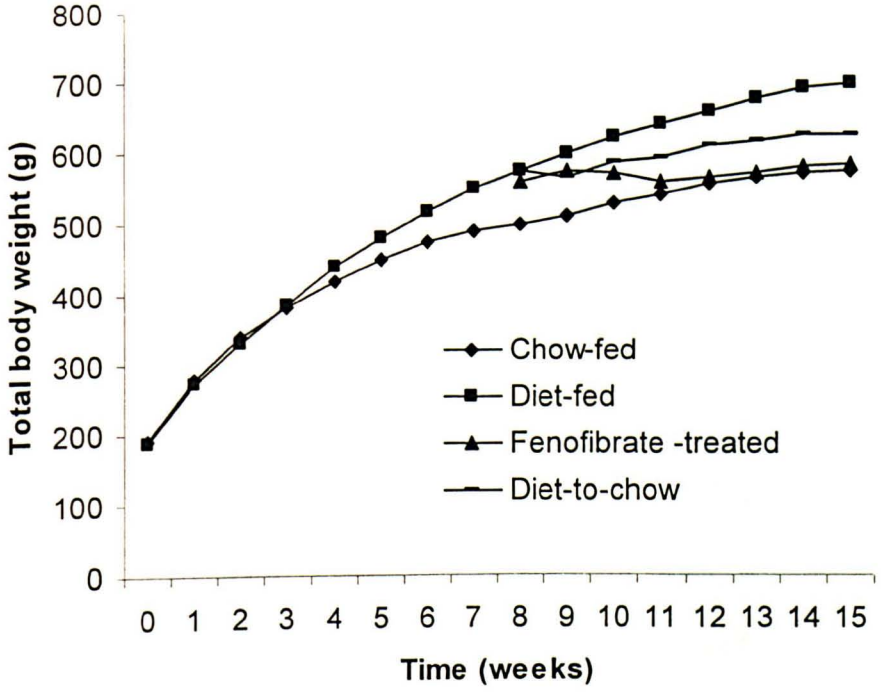


Fig. 7.1. The effects of palatable-diet, its removal, and fenofibrate treatment on total body weight in the rat. Animals were fed either standard chow (chow-fed) or a palatable diet (diet-fed) for 15 weeks. Fenofibrate treated group was fed palatable diet for 15 weeks and received fenofibrate (50 mg/kg/day) for the last 7 weeks, while diet-to-chow group was fed palatable diet for the first 8 weeks and chow for the last 7 weeks of the experiment. At the end of the experiment (15 weeks) diet-fed animals weighed significantly greater than the chow-fed, fenofibrate-treated, and diet-to-chow groups ($p<0.0002$, 0.002, and 0.02 respectively). The body weight of diet-to-chow group was significantly greater ($p<0.02$) than the chow-fed group, while that of the fenofibrate-treated group was not significantly ($p<0.66$) different from the chow-fed group. Data are expressed as mean ± S.E.M.

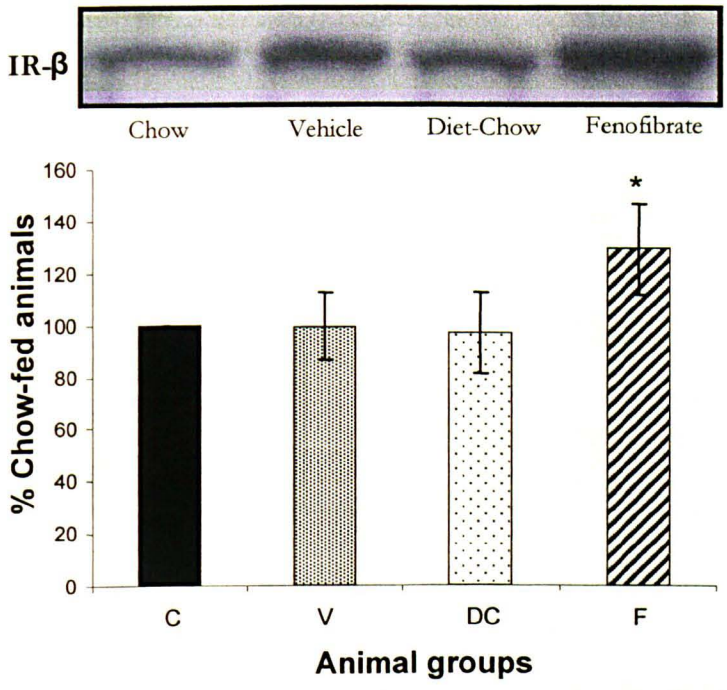


Fig. 7.2. Protein expression of IR-β in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with IR-β antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p< 0.05$ *vs* chow fed.

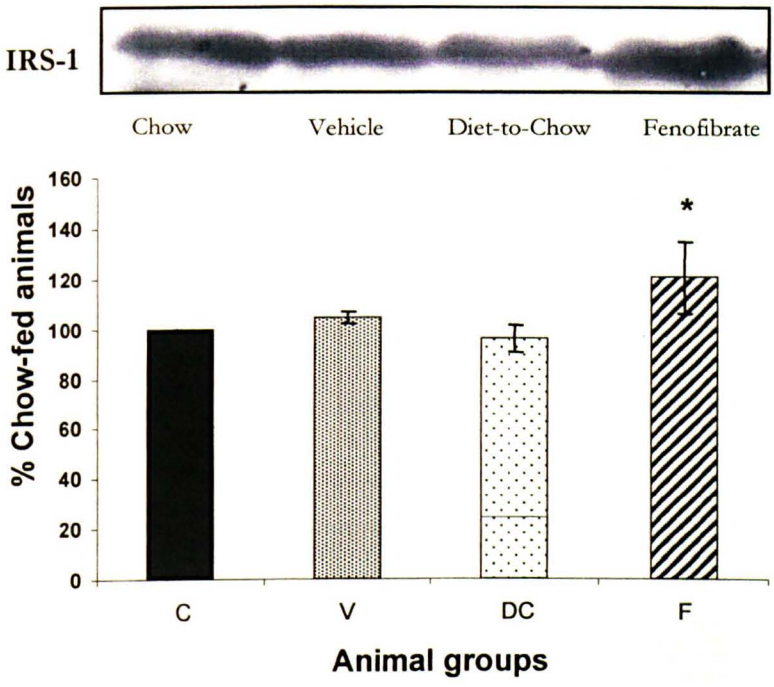


Fig. 7.3. Protein expression of IRS-1 in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with IRS-1 antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p< 0.05$ *vs* chow fed.

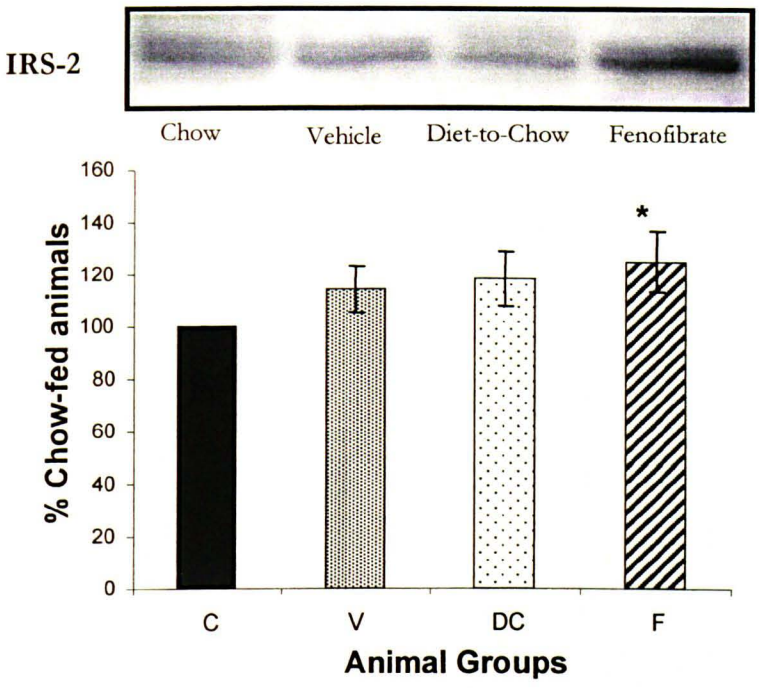


Fig. 7.4. Protein expression of IRS-2 in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with IRS-2 antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

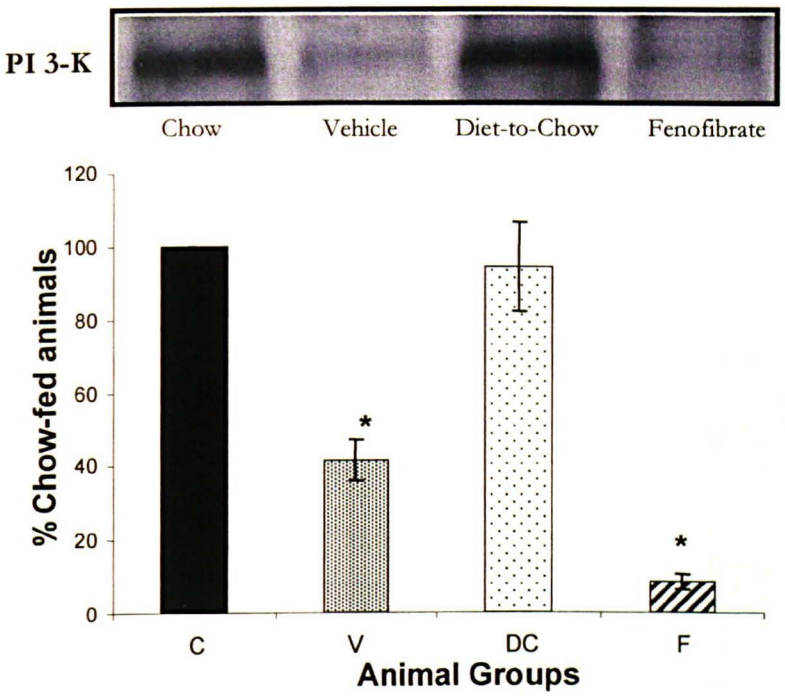


Fig. 7.5. Protein expression of PI 3-K in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with PI 3-kinase antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p< 0.0001$ *vs* chow fed.

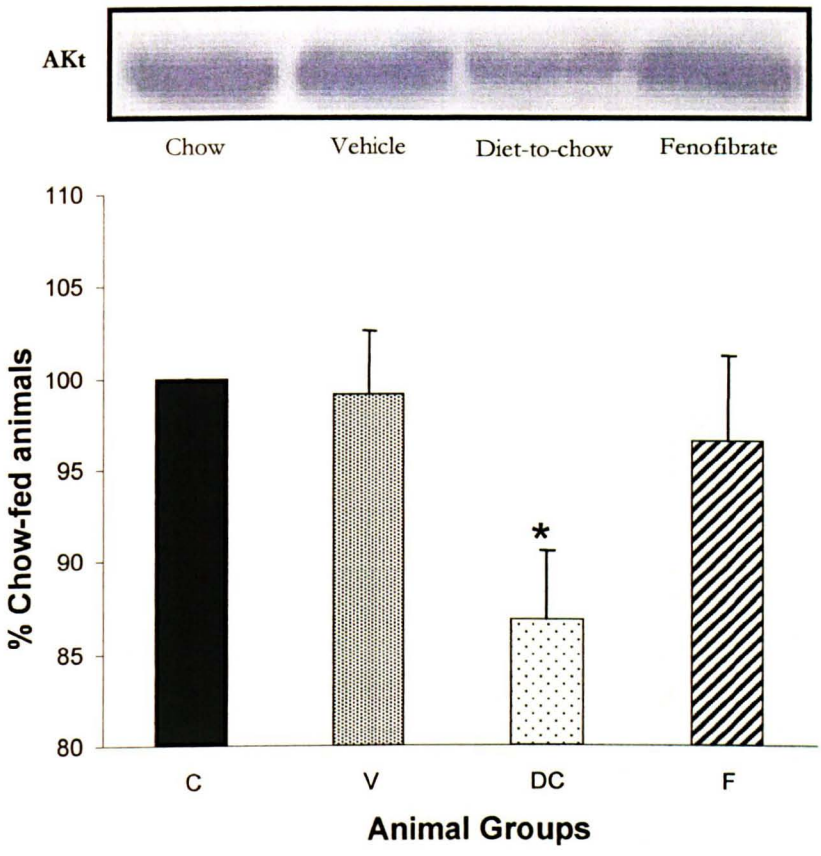


Fig. 7.6. Protein expression of Akt in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with Akt antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p< 0.05$ *vs* chow fed.

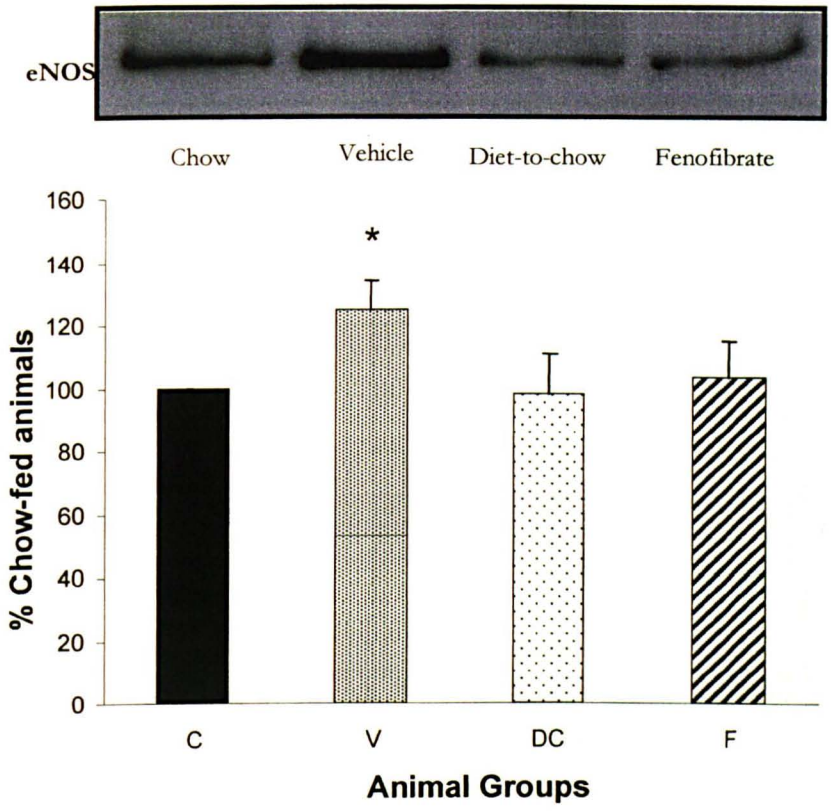


Fig. 7.7. Protein expression of eNOS in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with eNOS antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. *$p< 0.05$ *vs* chow fed.

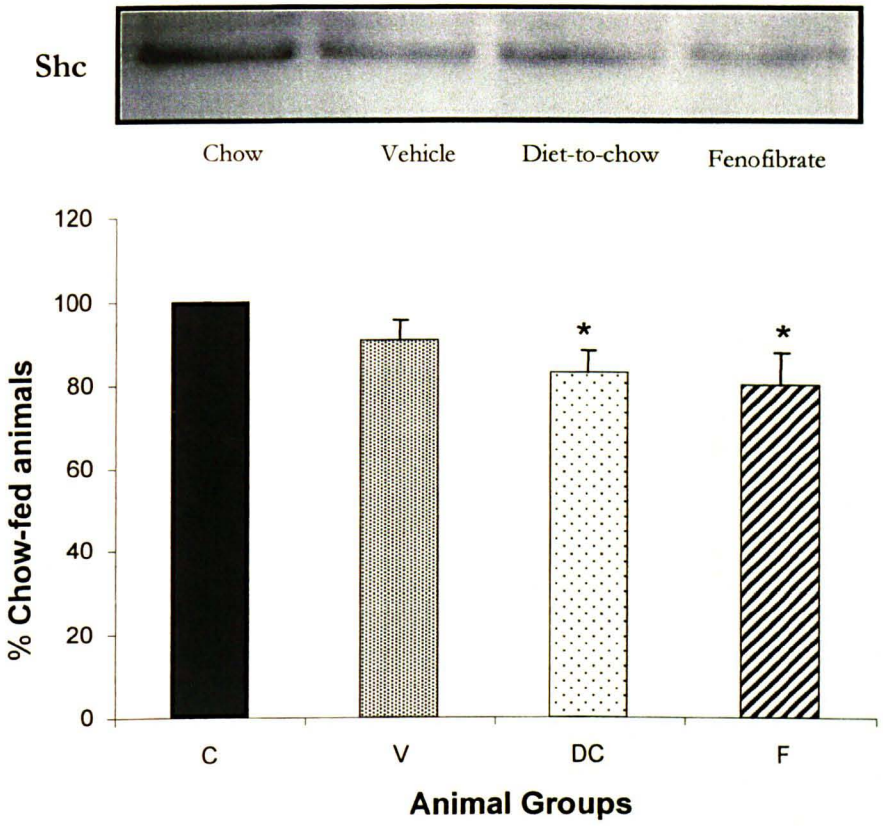


Fig. 7.8. Protein expression of Shc in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with Shc antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p< 0.05$ *vs* chow fed.

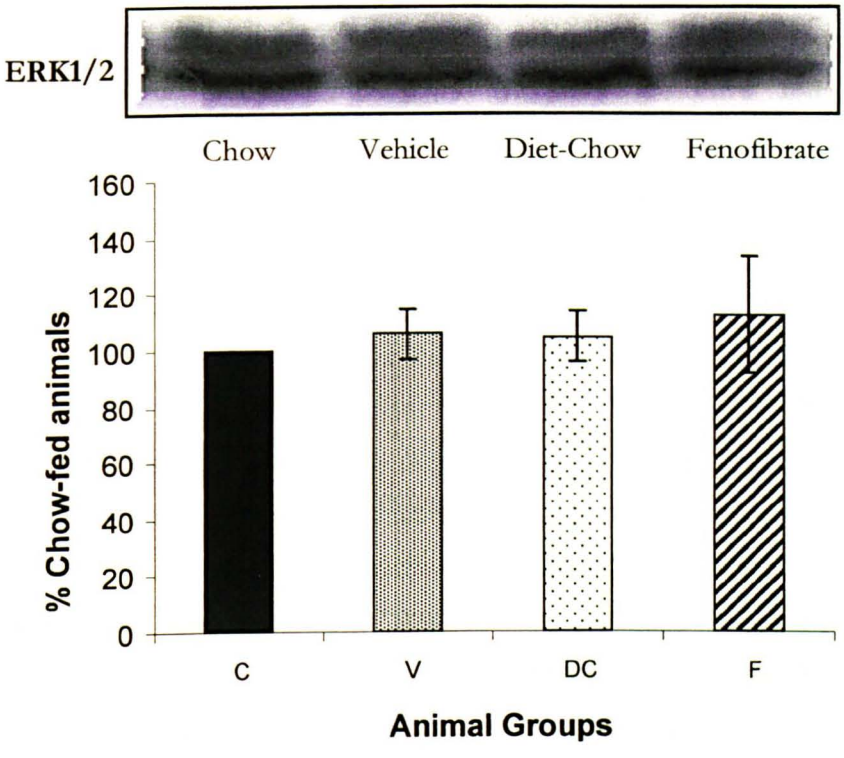


Fig. 7.9. Protein expression of ERK1/2 in the rat aorta. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with PERK1/2 antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ±S.E.M; n=7) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats.

7.4 DISCUSSION

Interactions between insulin, IR-β, IRS-1, and IRS-2 activates PI 3-kinase pathway resulting in vasorelaxation. Therefore, the integrity of protein levels and activation of the cascade in insulin signaling pathway in vasculature is a crucial factor in mediating normal vascular functions. In the present study, dietary-obesity did not adversely alter the concentrations of IR-β, IRS-1, and IRS-2, suggesting that reduced vasorelaxation to insulin seen in dietary-obesity (Walker *et al.*, 1997; Naderali *et al.*, 2001a), may not be due to the changes in protein levels of IR-β, IRS-1, and IRS-2. This raises possibility that attenuated insulin responses seen in obesity (Walker *et al.*, 1997; Naderali *et al.*, 2001a) may be due to changes in protein levels beyond membrane receptors. In fact, the present study indicates a marked decrease in PI 3-kinase levels in aorta from dietary-obese rats, suggesting that dietary-obesity adversely affects PI3-kinase protein level and thereby reducing insulin-induced vasorelaxation. Studies on genetically obese animals have reported similar observation. Jiang and colleagues have shown reductions in IRS-1, and IRS-2 but not IRS-β protein levels in obese Zucker rats in comparison with lean animals (Jiang *et al.*, 1999). Moreover, the same study reported a marked inhibition of PI 3-kinase activation in aorta of Fatty-Zucker rats. Although, the present study did not measure insulin-induced phosphorylation of signalling components, however, low level of PI 3-kinase seen in this study together with reduced functionality of PI 3-kinase reported on Zucker rats (Jiang *et al.*, 1999) may play an important role in integrity of endothelial function. A similar finding has also been reported in human umbilical vein endothelial cells where inhibition of PI 3-kinase markedly attenuated insulin-stimulated NO production (Zeng *et al.*, 2000), further arguing for a significant role of PI 3-kinase-changes in endothelial dependent insulin-induced vasorelaxation.

Earlier experiments (chapter five) indicated that in obese animals, removal of obesity-inducing diet completely restores endothelial function, suggesting that reversal of adiposity might be of benefit in correcting obesity-induced attenuation of PI 3-kinase. In fact, in this experiment, PI 3-kinase levels were restored in diet-to-chow group, further strengthening the hypothesis that PI 3-kinase levels may

determine the magnitude of endothelial function in obesity. Therefore, it is possible that any deficiency in the concentrations of PI 3-kinase may participate in inducing insulin resistance in vascular system accompanied with reduced NO production, leading to endothelial dysfunction.

Peroxisome proliferator-activated receptor-α (PPAR-α) plays a crucial role in the control of mitochondrial β-oxidation of fatty acids (Pyorala *et al.*, 2000; Eringa *et al.*, 2004). PPAR-α expressed in the vascular tissue, mainly the endothelial cells (Marx *et al.*, 1999). Hence, it is plausible to assume that the increased intracellular fatty acids and the presence of abundant amount of (PPAR)-α agonist (fenofibrate) may enhance fatty acid oxidation leading to increase in the intracellular fatty acid metabolites. Fatty acid metabolites such as fatty acyl-CoA, diacylglycerols, and ceramides shown to alter insulin signaling and induce insulin resistance (Schmitz-peiffer, 2002) thus causing reduction in PI3 kinase levels in these animals. Therefore, it is reasonable to assume that further reduction of PI 3-kinase levels seen in fenofibrate-treated animals may be the consequence of increased fatty-acid oxidation in these animals. However, this hypothesis merits further investigation. Fenofibrate-treated group had deficient protein expression of PI 3-kinase in vasculature, "the key controlling step of insulin signalling pathway", possibly contributing to insulin resistance, and subsequent endothelial dysfunction (chapter six).

Functionally active PI 3-kinase stimulates Akt, which in turn activates eNOS, leading to production of NO and subsequent vasorelaxation (Zeng *et al.*, 2000). In this study, protein levels of Akt were similar between dietary-obese and lean aorta, while removal of palatable diet caused a marked decrease in Akt levels. Surprisingly, on the other hand, there was a significant increase of eNOS levels in the obese group, whereas removal of palatable diet or fenofibrate treatment restored eNOS levels to that of lean control group. Although, increased eNOS level in this study is suggestive of augmented endothelial-dependent vasorelaxation, but results in chapter five in addition to numerous human and animal studies have shown attenuated endothelial function in obesity (Steinberg, 1996; Naderali *et al.*, 2001; Naderali *et al.*, 2001a). There are several possibilities on mechanisms of

increased level of eNOS in obese animals. One possible explanation, is activation of a compensatory mechanism to overcome the decrease in NO production or increase inactivation of NO seen in arteries of dietary obese rats as a result of increased oxidative stress (Katakam *et al.*, 2005). Moreover, in obese animals there is an elevation of insulin concentration and endothelial dysfunction (Naderali *et al.*, 2004a). Furthermore, insulin is a potent enhancer of reactive oxygen species (ROS) synthesis in endothelial and vascular smooth muscle cells, and superoxide is known to reduce NO and subsequently increased vasoconstriction (Cai and Harrison, 2000; Griendling and FitzGerald, 2003), thus, the elevation of eNOS could compensate this reduction. Insulin is also shown to induce eNOS expression in endothelial cells (Aljada and Dandona, 2000; Mather *et al.*, 2001) and therefore, insulinaemia which observed in obese rats could directly be responsible for the enhancement of eNOS expression. Similar results and hypothesis have also been reported by others indicating an increase in eNOS levels in Zucker obese coronary (Katakam *et al.*, 2005) and cerebral (Erdos *et al.*, 2004) arteries. However, the function of eNOS maybe regulated by PI 3-kinase or MAP-kinase pathway (Park *et al.*, 2002), and thus, decrease in PI 3-kinase seen in untreated obese rats may cancel any beneficial effects of increased eNOS. Furthermore, activated vascular endothelial growth factor (VEGF) stimulates eNOS expression at both mRNA and protein levels in a dose-dependent manner (Park *et al.*, 2002), suggesting that elevated levels of eNOS in dietary-obese group seen in the present study maybe due to an increased level of VEGF through MAP-kinase pathway, correlating positively with the dietary-obese subjects (Miyazawa-Hoshimoto *et al.*, 2003). Therefore, restoration (i.e. reduction) of total body fat seen in diet-to-chow and fenofibrate-treated animals, may have resulted in reduced effect of VEGF on eNOS expression (Miyazawa- Hoshimoto *et al.*, 2003) and thus improved vascular function (Naderali *et al.*, 2004a; Naderali *et al.*, 2004b). Inhibition of PI 3-kinase pathway enhances the mitogenic actions of insulin through MAP kinase pathway (Montagnani *et al.*, 2002). Hence, it is possible that inhibition of PI 3-kinase pathway seen in both untreated dietary-obese and fenofibrate treated groups may cause over activation of other signalling pathway such as MAP kinase. This result is compatible with the result in chapter five where endothelial

dysfunction in untreated-diet fed group was evident, indicating that deficient PI 3-kinase pathway in this group and over-activated MAP kinase pathway may result in endothelial dysfunction.

In conclusion, the present study demonstrates for the first time that long term feeding (15 weeks) of animals with an obesity-inducing palatable diet causes selective changes in protein levels of PI 3-kinase-dependent signalling pathway in aorta. Chronic withdrawal of obesity-inducing diet causes a complete normalization of PI 3-kinase, while fenofibrate treatment failed to improve PI 3-kinase concentrations in dietary-obese animals. A reduction in PI 3-kinase levels may have a role in inducing insulin resistance in vasculature, contributing to increased incidence of cardiovascular events seen in obese subjects. Furthermore, it can be postulate that, the inhibition of PI 3-kinase pathway may result in increased activation of MAP kinase pathway leading to an increased proliferation and migration of endothelial cells, thereby increasing the risk of cardiovascular events. However, this hypothesis merits further investigation. Furthermore, the adverse effects of dietary obesity on insulin transduction in vasculature are post receptor, on the level of PI3-kinase and downstream, and reversible, mainly by removal of obesity-inducing diet, which may have a role in combating diet-induced obesity-related cardiovascular dysfunction

Chapter 8

Results: effects of diet-induced obesity on liver: alternations in insulin signaling pathway and formation of non-alcoholic fatty liver

8.1 INTRODUCTION

Insulin is the primary anabolic hormone promoting the storage of energy in the fed state. In response to nutrient secretagogues, insulin is secreted from pancreatic β cells directly into the portal circulation. The resulting high portal insulin levels prime the liver for rapid alterations in hepatic carbohydrate and lipid homeostasis, such as stimulation of glycogen synthesis, lipogenesis, lipoprotein synthesis and suppression of gluconeogenesis / glycogenolysis and VLDL secretion (Cherrington *et al.*, 1978; Sparks and Sparks, 1994; Zammit, 2000). In fact, the liver is also a major depot for glucose uptake and storage, and may account for disposal of up to one third of an oral glucose load (pagliassotti and Horton, 1994).

Obesity has been associated with glucose intolerance, insulin resistance, dyslipidemia, and cardiovascular disease (Evans *et al.*, 1984; Golay *et al.*, 1990; DeFronzo and Ferrannini, 1991). The liver has specifically been implicated as a primary site of insulin resistance observed with visceral obesity. The mechanism(s) relating visceral fat accumulation and hepatic insulin resistance are not well understood yet, but several possible factors have been implicated. For example, increased release of adipokines such as TNF-α, resistin, and adiponectin have been implicated in the development of hepatic insulin resistance (Hotamisligil, 1999; Steppan *et al.*, 2001). Alternativly "portal hypothesis" posits a high rate of lipolysis of visceral adipose tissue leading to increased delivery of free fatty acids to the liver *via* portal vein, thus contributing to increased fat accumulation and liver insulin resistance (Bjorntorp, 1990; Boden, 1997; Bergman and Ader, 2000).

Chronic exposure of the liver to elevated FFA can promote liver gluconeogenesis (Williamson, 1966), deplete enzymes involved in FFA oxidation, and increase hepatic lipogenesis (Xu *et al.*, 1999; Clarke, 2000). In addition, elevated FFA are known to increase liver triglyceride content (Oakes *et al.*, 1997), and decrease insulin clearance (Mittelman *et al.*, 2000), both of which are associated with insulin resistance. Thus, insulin signalling in liver is critical in regulating glucose homeostasis and maintaining normal hepatic function and insulin resistance in hepatocytes leads to sever systemic insulin resistance and progressive hepatic

dysfunction (Michael *et al.*, 2000). It is therefore, possible that defective levels in protein expression of insulin signalling components in liver and/or inhibition of their activation will participate in insulin resistance.

A complex interaction of insulin resistance, deranged lipid profile, and vascular dysfunction is known as metabolic syndrome X (Zeman *et al.*, 2005). Patients with metabolic syndrome develop non-alcoholic fatty liver disease (NAFLD). As obesity is closely associated with NAFLD (Marchesini and Babini, 2006), it is expected that its incidence and prevalence would become more common. NAFLD is characterized by fatty liver and hepatitis, thus it is a spectrum of disorders that range from simple hepatic steatosis to steatohepatitis, cirrhosis, and hepatocellular carcinoma (Ludwig *et al.*, 1980; Luyckx, 2000). Various studies have pointed to hyperinsulinaemia and insulin resistance as pathogenic factors in NAFLD. Using the homeostasis model assessment (HOMA) method (Matthews *et al.*, 1985), Marchesini et al. (Marchesini *et al.*, 1999) shows that insulin resistance was the laboratory finding most closely associated with the presence of NAFLD in large series of patients, irrespective of BMI, fat distribution, or glucose tolerance. The strong association of NAFLD with other features of the metabolic syndrome (obesity, central fat, distribution, diabetes, dyslipidaemia, hypertension, and atherosclerotic cardiovascular disease) supports the hypothesis (Cortez-Pinto *et al.*, 1999) that NAFLD might represent another feature of the metabolic syndrome, with decreased insulin sensitivity being the common factor (DeFronzo and Ferrannini, 1991).

The pathogenesis of NAFLD is not fully understood, and the 'the two-hit' hypothesis proposed by Day et al. remains the prevailing theory (Day and James, 1998).

First hits: insulin resistance and fatty acid accumulation. It is believed that, resistance leads to the accumulation of triglycerides in hepatocytes. As more fatty acids being synthesised, more free fatty acids being delivered to the liver, less fatty acids being degraded, and less triglycerides being released from the liver. Lipid accumulation in hepatocytes (steatosis) is a characteristic histological feature of

NAFLD. This link is supported by findings that many patients with NAFLD have hyperinsulinaemia, insulin resistance, and the metabolic syndrome. (Marchesini, 2001; Chitturi *et al.*, 2002). There are two major mechanisms of fatty acid toxicity in the pathogenesis of NAFLD, namely the "direct" and "indirect" mechanisms. The "direct" mechanism involves direct toxicity such as membrane disruption at very high concentrations (detergent effects), apoptosis, and necrosis, while the "indirect" mechanism involves cytotoxic effects of lipid peroxidation of fatty acids.

Second hits: Oxidative stress, cytokines. A variety of second hits could account for the progression from simple steatosis to steatohepatitis. Oxidative stress occurs when more oxidant substances are produced than antioxidant processes of the liver can handle. Oxidative stress can cause lipid preoxidation, leading to activation of hepatic stellate cells and hepatocyte death contributing to hepatocellular injury and fibrosis. Sources of oxidative stress in steatohepatitis include reactive oxygen species that leak from the mitochondria during oxidation of fatty acids, cytochrome P450 enzymes, and hepatic iron (George *et al.*, 1998; Younossi *et al.*, 1999; Pessayre *et al.*, 2001).

Cytokine production is increased in nonalcoholic steatohepatitis and is believed to play a role in its pathogenesis. In the liver, TNF-α can contribute to oxidative stress (Tilg and Diehl, 2000), and may contribute to insulin resistance through activation of the inhibitor of Kappa kinase beta (IKK beta) (Yuan *et al.*, 2001). Increased free fatty acid levels, in addition to mediating insulin resistance and causing oxidative stress, can be directly hepatotoxic, leading to cellular injury (Neuschwander-Tetri and Caldwell, 2003).

Leptin: a possible third hit? It may promote insulin resistance, contributes both to oxidative stress and to enhanced secretion of inflammatory cytokines (Loffreda *et al.*, 1998; Chitturi, 2002) and may play a role in causing fibrosis.

Biopsy is the definitive test. Because the clinical and laboratory features of NAFLD are not specific and imaging studies cannot reliably distinguish nonalcoholic steatohepatitis from other forms of NAFLD, liver biopsy from human or samples of histological sectioning from animal models are the only ways to accurately

diagnose NAFLD, particularly steatohepatitis. Histological features include steatosis, lobular inflammation, ballooning degeneration, perisinusoidal fibrosis, (Younossi *et al.*, 1998; Brunt *et al.*, 1999; Neuschwander-Tetri and Caldwell, 2003), and hepatocellular carcinoma (Bugianesi *et al.*, 2002).

Treatment. There currently is no proven effective therapy for nonalcoholic steatohepatitis. Early efforts focused on modifying the associated conditions such as obesity, hypertriglyceridaemia, and diabetes mellitus. More recent efforts have targeted theoretical aspects of the pathogenesis, such as insulin resistance and oxidative stress.

a) Weight loss, particularly of 10% or more, can lead to improvement in hepatic steatosis (Nomura *et al.*, 1987). However, there has been very little histological follow-up in obese subjects who have lost weight. One study reported that two patients with nonalcoholic steatohepatitis who lost weight had significant histological improvement on follow-up liver biopsies (Eriksson *et al.*, 1986; Andersen *et al.*, 1991). A very rapid weight loss, however, may lead to increased portal inflammation and fibrosis (Andersen *et al.*, 1991; Luyckx *et al.*, 1998), while the optimum rate of weight loss is not clear, gradual loss of 10% of baseline weight seems to be a reasonable recommendation (Sanyal, 2002).

b) Lipid-lowering agents, have been evaluated in patients with nonalcoholic steatohepatitis. Patients did not appear to benefit from 12 months clofibrate (Laurin *et al.*, 1996). On the other hand, atorvastatin for 1 year produced significant improvement in ballooning degeneration, and inflammatory scores in NAFLD patients (Horlander and Kwo, 1997; Brunt *et al.*, 1999). While, there is very small number of studies in literature regarding the effect of fenofibrate treatment on the progression of NAFLD, many of these reports indicated an improvement in NAFLD, specifically steatoheptitis as result of fenofibrate administration (Shiri-Sverdlov *et al.*, 2006).

Therefore, in this experiment, the effects of dietary-induced obesity and physiological and pharmacological correction of obesity on liver insulin signalling pathway as well as histological features of the liver were investigated. This study

provides valuable information on the mechanism of obesity-induced hepatic insulin resistance.

8.2 METHODS

8.2.1 Experimental protocol

Animals used in this study were those described in chapter 7. At the end of experimental protocol (as described in chapter 7), rats were killed by CO_2 inhalation after 2 hours of fasting, and the liver was dissected and snap frozen in liquid nitrogen, for later molecular and histological techniques.

8.2.2 Experimental procedures

8.2.2.1 Western-immunoblotting technique

Western-immunoblotting technique was carried out (as described in chapter two) on the four experimental groups (chow-fed, vehicle, diet-to-chow, and fenofibrate treated groups) for measuring the levels of protein expressions for kinases such as: IR-β, IRS-1, IRS-2, PI 3-kinase, AKt, eNOS, Shc, and ERK1/2 in insulin signaling pathways in the liver.

8.2.2.2 Liver triglyceride content

Triglyceride content was assessed in liver samples of all experimental groups: chow-fed, diet-fed, diet-to-chow, and fenofibrate treated groups, using a procedure described in chapter two.

8.2.2.3 Liver histology

The tissue samples of liver were prepared for histological examinations using ordinary tissue processing and stained by Haematoxylin and Eosin staining protocol which all described in chapter two. Ten liver specimens (10 animals) of each experimental group were interpreted by a single pathologist who was blinded to all data of liver samples and the experimental groups under investigation. The Liver samples were analyzed microscopically for the presence or absence of the following histological features: steatosis, steatohepatitis, fibrosis and hepatocellular carcinoma. Histological features were interpreted according to the schema outlined by Brunt et al. (Brunt *et al.*, 1999). Briefly, steatosis was graded on a 3-point scale:

grade 1 = steatosis involving <33% of hepatocytes, grade 2=33-66%, grade 3>66%. Inflammation (steatohepatitis) was graded on a 4 point scale: grade 0=no or negligible inflammation, grade 1 = mild, grade 2 = moderate, grade 3 = severe. Fibrosis was staged on a 5 point scale: stage 0 = no fibrosis, stage 1 = zone 3 perisinusoidal/ perivenular fibrosis, stage 2 = zone 3 and periportal fibrosis, stage 3 = septal/bridging fibrosis, stage 4 = cirrhosis.

8.2.3 Data interpretation and statistical analysis

For Western blotting, the data from chow-fed (control) animals were expressed as 100% response, and the results from other three groups were normalized and subsequently expressed as the percentage of their respective controls. Data are expressed as mean ± S.E.M. Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student 't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p<0.05$. The histological data are presented as number (percentage), each sample represent 10%. There are ten histological samples (10 animals) in each experimental group.

8.3 RESULTS

8.3.1 Body weight

By referring to the results of changes in total body weight in chapter 7, animals given palatable diet progressively gained more weight than their chow-fed counterparts. Untreated diet-fed animals were significantly heavier than chow-fed, diet-to-chow, and fenofibrate treated animals. However, at the end of the experiment, body weight of diet-to-chow and fenofibrate treated animals were significantly ($p<0.01$) lower than untreated diet-fed animals.

8.3.2 Liver weight

Liver weight of untreated obese animals (vehicle) was elevated significantly ($p<0.01$, 1.22 folds) compared with chow-fed group (control). Replacing the highly-palatable diet with standard laboratory chow (diet-to-chow group) completely restored the liver weight to normal range, with no significant difference in comparison to chow-fed group. However, compared with chow-fed and vehicle

groups fenofibrate treatment, produced a marked increase in liver weight up to 2.13-folds (for both, $p<0.01$) (Fig. 8.1).

8.3.3. Triglyceride content of liver

Compared with chow-fed group, untreated obese animals (vehicle) had significantly ($p<0.05$) higher TG levels. Diet-to-chow animals had liver TG similar to that of chow-fed, while TG levels in fenofibrate treated group were significantly reduced ($p<0.01$) compared with chow-fed group (Fig. 8.2).

8.3.4 Expression of protein kinases of insulin signalling pathways in hepatocytes

IR-β. Diet-induced obesity and chronic withdrawal of highly-palatable diet did not affect IR-β levels in hepatocytes with no significant difference compared with chow-fed group. However, fenofibrate treatment induced a significant reduction ($p<0.001$) in the concentration of IR-β (Fig. 8.3).

IRS-1. Similar to IR-β, the levels of IRS-1 in both vehicle and diet-to-chow groups were not affected and there was no significant difference compared with chow-fed group, whereas, fenofibrate treatment produced marked decrease ($p<0.001$) in the concentration of IRS-1 in hepatocytes (Fig. 8.4a).

IRS-2. Concentration of IRS-2 was significantly reduced ($p<0.05$) in vehicle group, while its levels were completely restored to the level of chow-fed group. However, compared with chow-fed group, fenofibrate-treated group had significantly reduced ($p<0.0005$) concentration of IRS-2 (Fig. 8.4b).

PI 3-kinase. Similar to IRS-2, there was a significant decrease ($p<0.05$) in the levels of this protein kinase in vehicle group, while its levels were similar to chow-fed group in diet-to-chow group. fenofibrate treatment induced significant decrease ($p<0.0005$) in PI 3-kinase levels (Fig. 8.5a).

AKt. Although, diet-induced obesity did not affect the levels of AKt, chronic withdrawal of the highly-palatable diet caused marked decrease in AKt protein

(p<0.05). Furthermore, fenofibrate treatment induced a significant reduction (p<0.000005) for the levels of Akt (Fig. 8.5b).

eNOS. The expression of eNOS protein was not affected by the three experimental groups: vehicle, diet-to-chow, and fenofibrate treatment groups. eNOS levels were similar in all groups with no significant difference between the groups (Fig. 8.6).

Shc. The expression of Shc was not affected in both vehicle and diet-to-chow groups, with no significant difference compared with chow-fed group. However, fenofibrate treatment induced significant decrease (p<0.005) in Shc levels compared with chow-fed group (Fig. 8.7a).

ERK1/2. Similar to Shc, the levels of ERK1/2 were not affected by both diet-induced obesity and chronic withdrawal of the highly-palatable diet. However, fenofibrate treatment caused significant reduction (p<0.01) in Akt protein levels (Fig. 8.7b).

8.3.5. The histological features of the liver (non-alcoholic fatty liver disease)

The histological slides of liver tissue of each animal were examined for the presence or absence for the histological features of the different stages of non-alcoholic fatty liver disease (Figs. 8.8a, b, c, and d). Ten liver samples (10 animals) in each experimental group were analysed microscopically, and each sample represent 10% of the total percentage of each group.

Steatosis (accumulation of fat in the liver) was found in 50% (grade 1 of steatosis) of chow-fed control group, while untreated obese animals (vehicle group) having steatosis in 80% of the total samples (40% in grade 1, 30% in grade 2, and 10% in grad 3). Steatosis was present in 50% of the samples of diet-to-chow group (40% in grade 1, and 10% in grade 2). Fenofibrate-treated group having steatosis in only 10% of the total samples of this group (which was classified as grade 2) (Table 8.1).

Steatohepatitis was present in 40% of the samples in chow-fed control, whereas in vehicle group, 70% of the samples showed steatohepatitis. Diet-to-chow group

had 30% of the samples steatohepatitis, while in fenofibrate-treated group only 10% of the samples were positive for steatohepatitis. The positive samples in all groups were of grade one steatohepatitis (Table 8.2).

Fibrosis and hepatocellular carcinoma. The histological examination of samples in all experimental groups did not show signs for the presence of advanced features of NAFLD: fibrosis and hepatocellular carcinoma (Table 8.3). In comparison with chow-fed group, obese animals had 30% greater steatosis, while the percentage of samples with steatosis in diet-to-chow group was similar to that of diet-fed "control". Furthermore, steatosis in fenofibrate treated group was 40% less than that of chow-fed group.

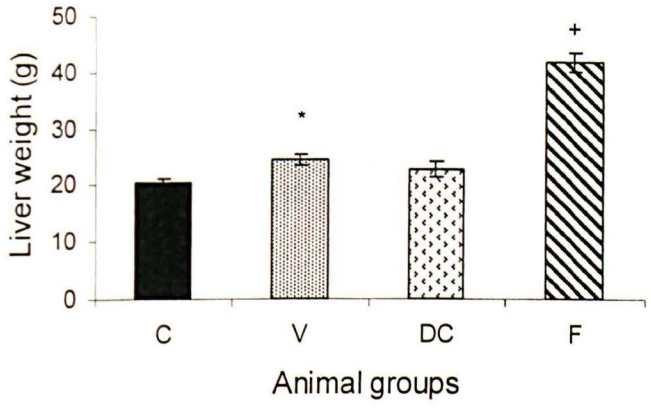


Fig. 8.1. Liver weight of animals at the end of the experiment in the four experimental groups: C) Chow-fed, V) Vehicle, DC) Diet-to-Chow, and F) Fenofibrate-treated groups. Data were represented as mean ± S.E.M. There were significant increase in both vehicle and fenofibrate –treated groups compared with chow-fed group. * =p<0.01, +=p<0.0001 vs chow-fed group.

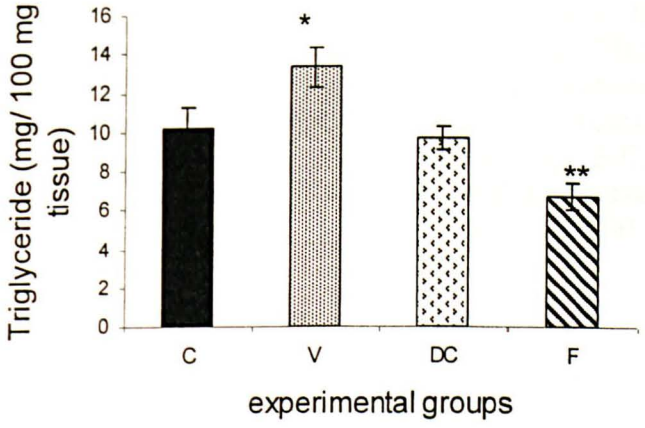


Fig. 8.2. Content of hepatic triglycerides in four experimental groups: C) Chow-fed, V) Vehicle, DC) Diet-to-Chow, and F) Fenofibrate-treated groups. Data are represented as mean ± S.E.M. *=p<0.05, **=p<0.01 both vs. chow-fed group.

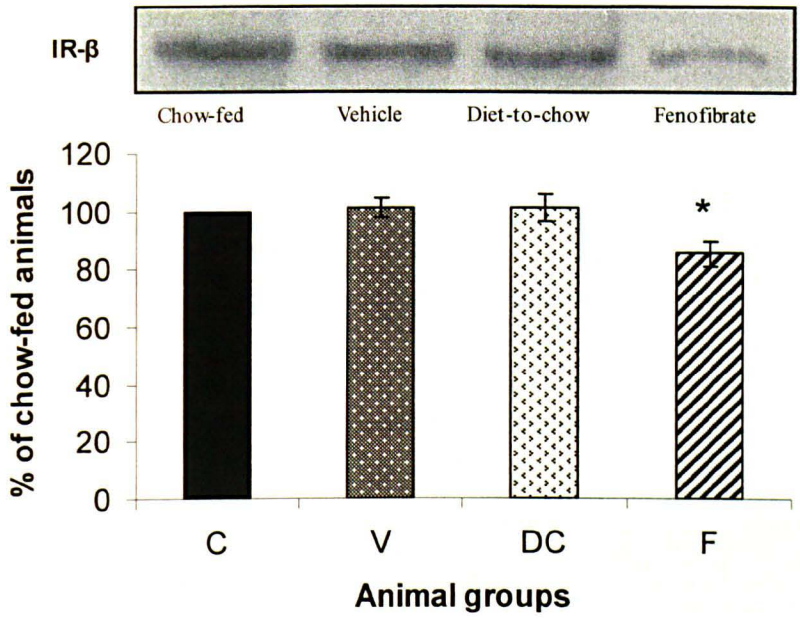


Fig. 8.3 Protein expression of IR-β in the rat liver. Equal amounts (50µg/well) of protein were separated by SDS-PAGE and immunoblotted with IR-β antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * =p<0.00 vs chow fed.

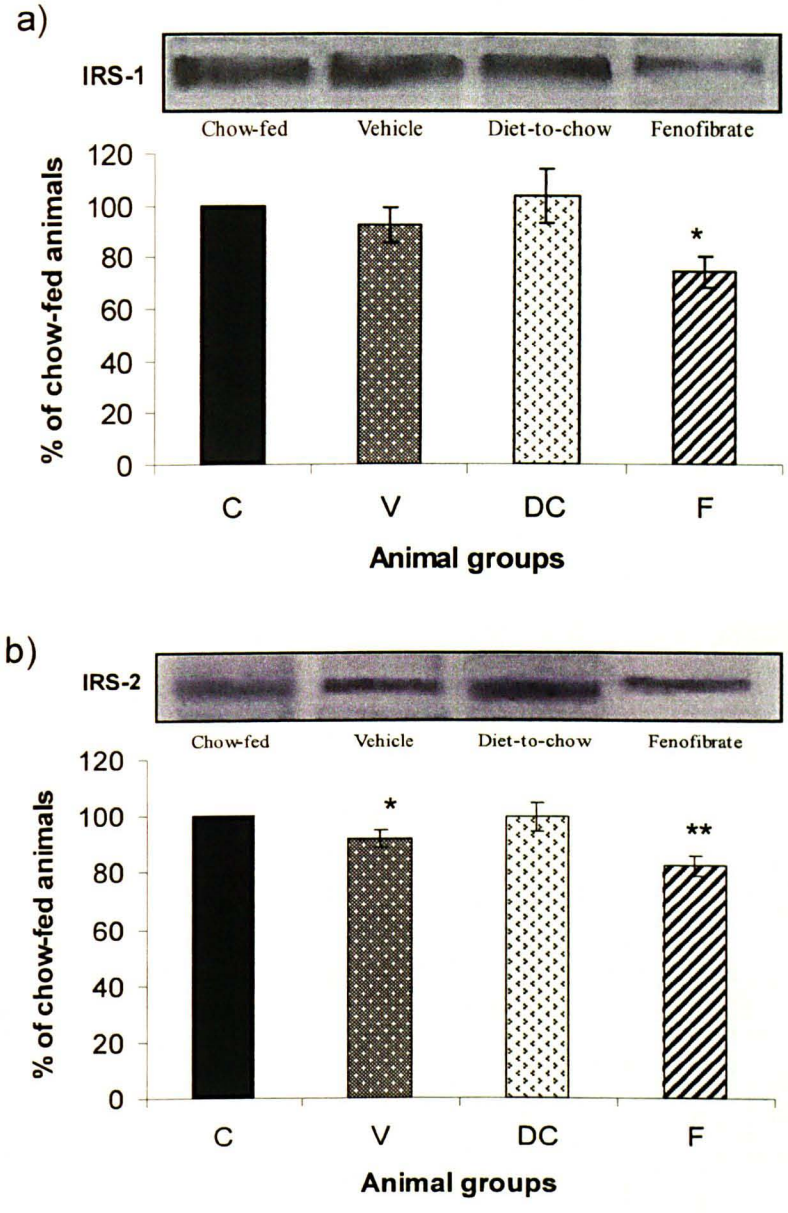


Fig. 8.4. Protein expression of a) IRS-1, and b) IRS-2 in the rat liver. Equal amounts (50µg/well) of protein were separated by SDS-PAGE and immunoblotted with IRS-1 and IRS-2 antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * =p<0.05, ** =p<0.0005 vs. chow-fed.

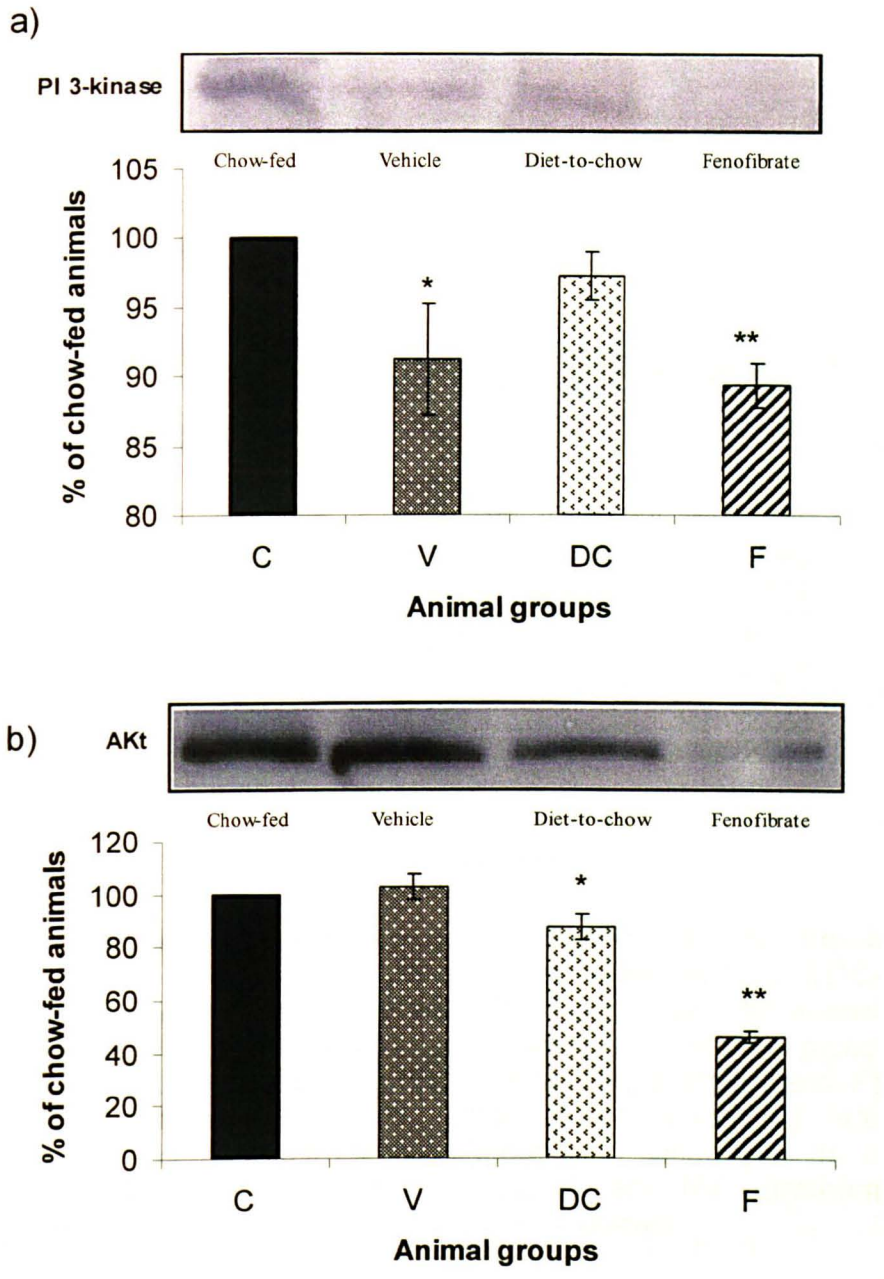


Fig. 8.5. Protein expression of a) PI 3-kinase, and b) AKt in the rat liver. Equal amounts (50µg/well) of protein were separated by SDS-PAGE and immunoblotted with AKt and PI 3-kinase antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M; n=10) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * = $p<0.05$, ** = $p<0.000005$ vs. chow-fed.

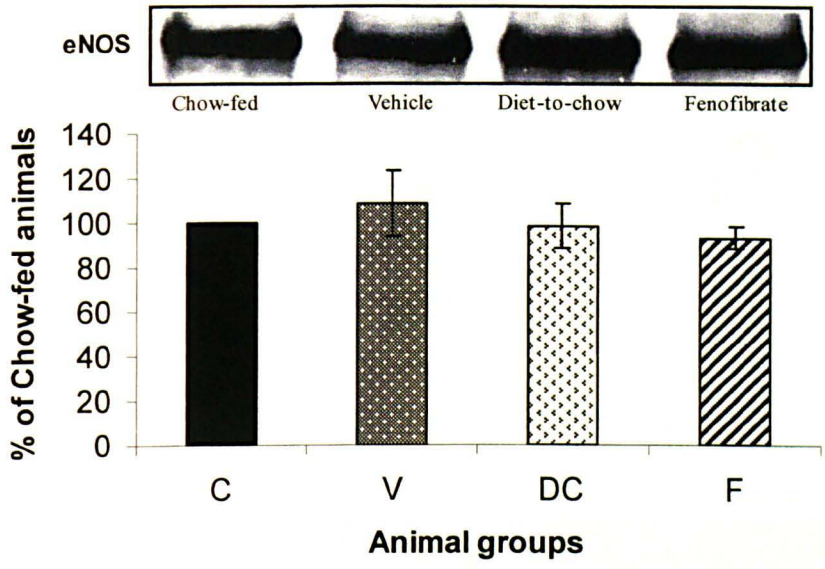


Fig. 8.6. Protein expression of eNOS in the rat liver. Equal amounts (50µg/well) of protein were separated by SDS-PAGE and immunoblotted with eNOS antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. No significant differences were seen between the four groups.

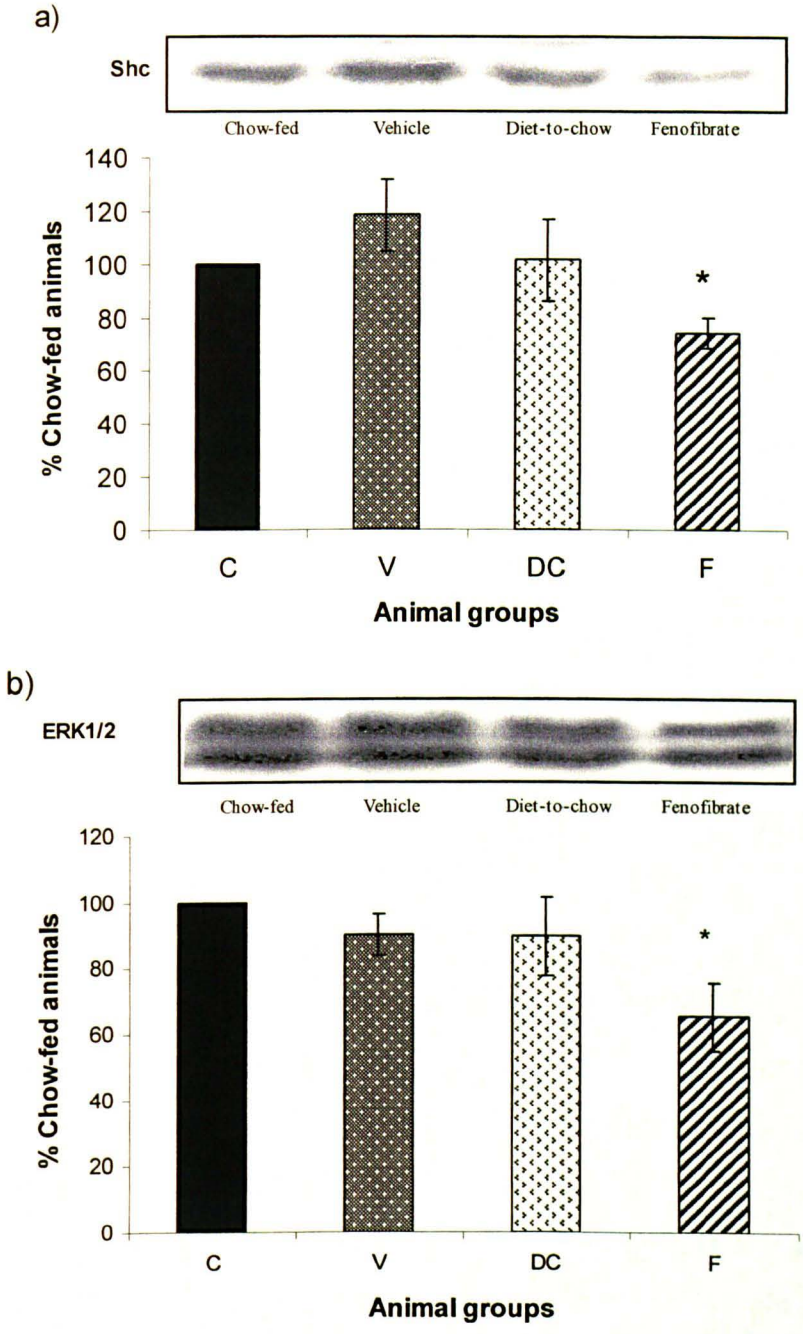


Fig. 8.7. Protein expression of a) Shc, and b) ERK1/2 in the rat liver. Equal amounts (50µg/well) of protein were separated by SDS-PAGE and immunoblotted with Shc and ERK1/2 antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * =$p<0.01$ *vs* chow-fed.

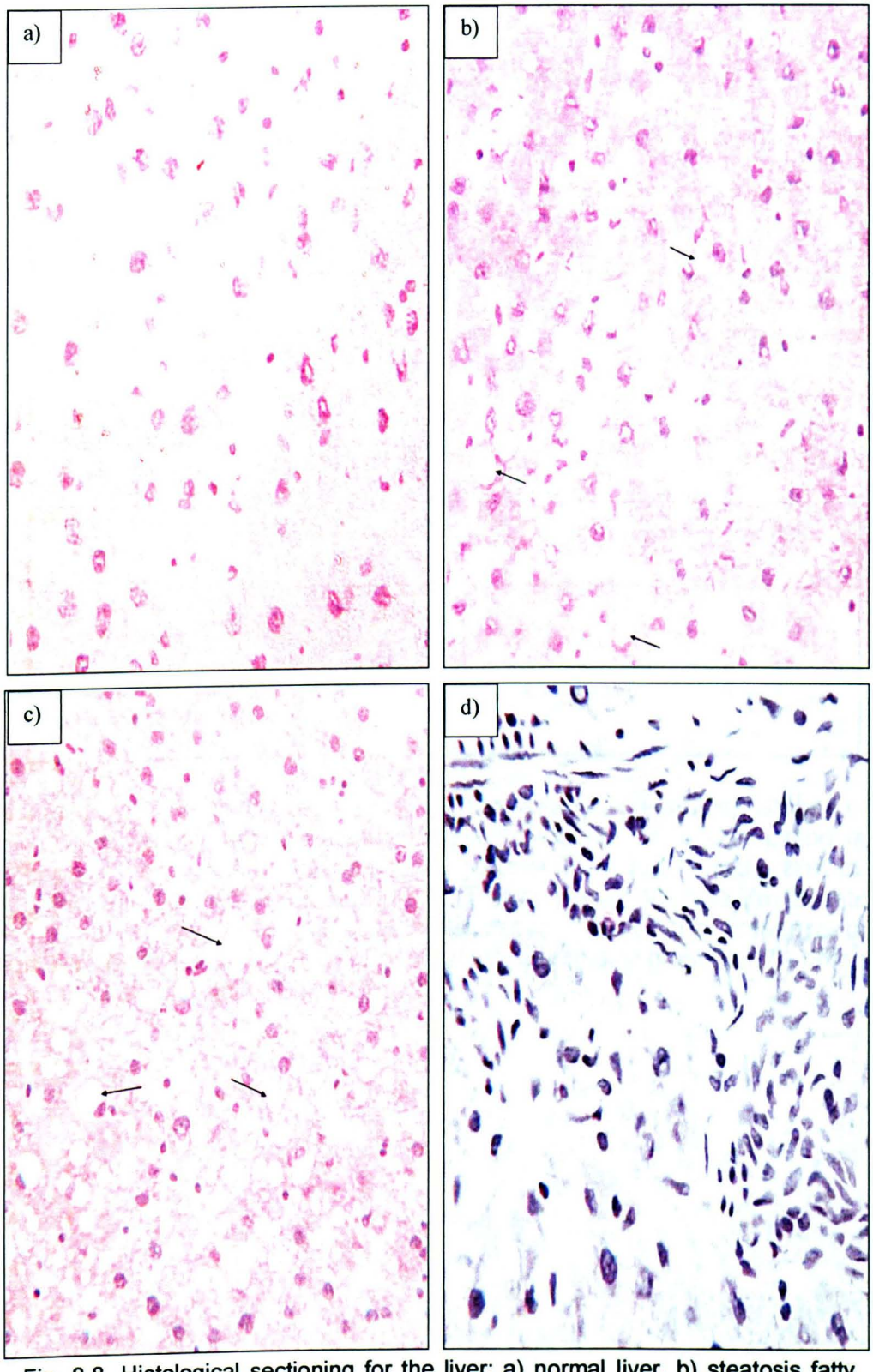


Fig. 8.8. Histological sectioning for the liver: a) normal liver, b) steatosis fatty liver grade one, c) steatosis fatty liver grade two, and d) steatohepatitis grade one.

Animal groups	STEATOSIS (FATTY CHANGE IN THE LIVER)				Total % of fatty change in each group
	Grade				
	0 Negative samples	1	2	3	
Chow-fed (control) (10 animals=100%)	**50%**	50%			50%
Vehicle (obese animals) (10 animals=100%)	**20%**	40%	30%	10%	80%
Diet-to-chow (10 animals=100%)	**50%**	40%	10%		50%
Fenofibrate treatment (10 animals=100%)	**90%**		10%		10%

Table 8.1 Total percentage of hepatic fatty change (steatosis). Stage I of non-alcoholic fatty liver disease: simple steatosis (fat accumulation in hepatocytes) in the four experimental groups: Chow-fed, Vehicle, Diet-to-chow, and Fenofibrate treated group. Tissue liver of 10 male Wister rats from each group were analyzed microscopically for the presence of different grades of Steatosis. Each analyzed animal represent 10% of the total number of animals in each group.

Animal groups	INFLAMMATION				Total % of inflammation in each group
	Grade				
	0 Negative samples	1	2	3	
Chow-fed Control (10 animals=100%)	60%	40 %			**40 %**
Vehicle (obese animals) (10 animals=100%)	30%	70 %			**70 %**
Diet-to-chow (10 animals=100%)	70%	30 %			**30 %**
Fenofibrate teatment (10 animals=100%)	90%	10 %			**10 %**

Table 8.2 Total percentage of hepatic inflammation. Stage II of non-alcoholic fatty liver disease: inflammation (steatohepatitis) in the four experimental groups: Chow-fed, Vehicle, Diet-to-chow, and Fenofibrate treated group. Tissue liver of 10 male Wister rats from each group were analyzed microscopically for the presence of different grades of inflammation. Each analyzed animal represent 10% of the total number of animals in each group.

Animal groups	Fibrosis		Hepatocellular carcinoma	
	% of negative samples	% of positive samples	% of negative samples	% of positive samples
Chow-fed	100%	-	100%	-
Vehicle	100%	-	100%	-
Diet-to-chow	100%	-	100%	-
Fenofibrate-treated	100%	-	100%	-

Table. 8.3 Advanced histological features of non-alcoholic fatty liver disease. Ten liver samples (100%) of each group were analysed microscopically for the presence of: fibrosis and hepatocellular carcinoma. All of the samples were negative for both histological features of non-alcoholic fatty liver disease.

8.4. DISCUSSION

In liver, Insulin has a crucial role in mediating carbohydrate and lipid homeostasis. Therefore, the integrity of hepatic insulin signalling is important for the actions of insulin in hepatocytes which includes stimulation of glycogen synthesis, lipogenesis, and lipoprotein synthesis and suppression of gluconeogenesis / glycogenolysis and VLDL secretion in fed state (Sparks and Sparks, 1994; Zammit, 2000). Insulin also plays a role in hepatic growth and regeneration (Starzl *et al.*, 1976). Deficiency in protein expression of any components of insulin transduction may induce hepatic insulin resistance and subsequently participate induction of systemic insulin resistance and type II diabetes, while local hepatic insulin resistance may cause nonalcoholic fatty liver disease.

Insulin has a major role in glucose homeostasis by uploading circulating glucose into skeletal muscle cells. Therefore, it is expected that malfunctioning of insulin-receptors in skeletal muscle cells may cause imbalance of whole-body glucose homeostasis. However, mice with severe insulin resistance in skeletal muscle due to a muscle-specific insulin receptor knockout have surprisingly normal whole-body glucose homeostasis (Bruning *et al.*, 1998).

In general, protein kinase phosphorylation and activation levels depend on the concentration of the expressed protein. Several studies demonstrated direct relationship between concentration of expressed protein kinase and levels of its activation (Anai *et al.*, 1998). Therefore, in hepatocytes, defective protein kinase expression may cause insulin resistance, with subsequent induction of hepatic insulin resistance-related clinical disorders, namely nonalcoholic fatty liver disease.

In the present study diet-induced obesity did not affect expression of insulin receptor- β (IR-β) and insulin receptor substrate-1 (IRS-1), indicating that the detrimental effects of diet-induced obesity on hepatic insulin signalling is at postreceptor levels. Furthermore, previous studies have demonstrated that levels of both proteins IR-β and IRS-1 in the different types of genetic obesity could be modified that may contribute to inducing hepatic insulin resistance. Friedman and colleagues (Friedman *et al.*, 1997) detected that the genetically obese

hypertensive strain (SHROB) is characterised by reduced IR-β auto-phosphorylation, and this reduction of protein activation was due to inhibited expression of IR-β itself in hepatocytes. Another study revealed that in the genetic obesity *ob/ob* mice, levels and activation of IRS-1 is reduced significantly (Saad *et al.*, 1992). Taken together, these reports have indicated that the molecular mechanism of hepatic insulin resistance in genetic obesity may involve modification in the early steps (IR-β and IRS-1 proteins) of insulin signalling, and that the amount of protein expression may correlate directly with the levels of phosphorylation and activation of the protein (Kim *et al.*, 2000). However in the present study of diet-induced obesity, unlike genetic obesity study, IR-β and IRS-1 did not involve in genesis of hepatic insulin resistance.

Insulin receptor substrate-2 (IRS-2) appears to have a major role in liver by regulating hepatic insulin action, whereas IRS-1 appears to have important functions in skeletal muscle (Sesti *et al.*, 2001). Cell-based experimentation with hepatocytes suggests that IRS-2 is the major effector of both the metabolic and the growth-promoting actions of insulin (Rother *et al.*, 1998). Moreover IRS-2 signalling is essential for the suppression of gluconeogenesis and apoptosis in hepatocytes. IRS-2 is an important regulatory point for the PI 3-kinase-AKt cascade in hepatocytes. PI 3-kinase activity associated with IRS-2 depends mainly on the level of IRS-2 expression and degree of phosphorylation (Rother *et al.*, 1998; Ide *et al.*, 2004). In fact, total insulin-induced PI 3-kinase activity decreased by 50% in IRS-2 (-/-) hepatocytes (Valverde *et al.*, 2003), while other reports have concluded that reduced PI 3-kinase activity may play a role in the pathophysiology of insulin resistant diabetic states, such as that seen in the ob/ob mouse (Folli *et al.*, 1993; Anai *et al.*, 1998). In the present study, levels of expression of both proteins (IRS-2 and PI 3-kinase) were significantly decreased in diet-induced obese animals, suggesting that diet-induced obesity have similar detrimental effects as genetic obesity on IRS-2 and PI 3-kinase proteins in hepatocytes.

The normal concentration of Akt protein in hepatocytes of diet-induced obese animals in the current study is consistent with other reports (Kim *et al.*, 2000) where they found unchanged levels of protein expression and slightly elevated

phosphorylation of Akt in genetic obesity. Therefore, both types of obesity might have similar effects on Akt protein expression.

Much less is known about the expression of eNOS in normal, diseased, and treated livers. eNOS in hepatocytes may regulate hepatic vasculature, regulating blood flow and blood cell interactions (Li and Billiar, 1999). In the present study, expression of eNOS in liver was not affected by the different dietary manipulations or by disease states (normal, steatosis, or steatohepatitis). This finding is in agreement with others demonstrating that the expression of eNOS protein and its activity in hepatocytes was not changed in chronic liver disorders (such as alcoholic cirrhosis, viral hepatitis, cholestasis, and fulminant liver failure) (McNaughton *et al.*, 2002). On the other hand, normal expression of MAP-kinase pathway is a crucial factor in mediating the mitogenic response of insulin. A defect in PI 3-kinase may overactivate MAP-kinase pathway. In the present study, diet-induced obesity did not alter the expression of two important protein kinases (Shc and ERK1/2) in MAP-kinase pathway, despite significant protein deficiencies in PI 3-kinase pathway in hepatocytes.

Interestingly, in the present study the effect of palatable-diet removal on expression of AKt protein in hepatocytes was similar to its affect on Akt in aorta (chapter 7). Moreover, this reduction in Akt expression in hepatocytes might be related to the sharp decrease in calorie intake. It is possible to correlate the reduced AKt levels to improvement in liver histology seen in this study. Therefore, it is possible that, the presence of other isoform(s) of Akt might have greater effect on insulin transduction and glucose metabolism than the studied isoform of AKt in the present study (AKt1). However, other studies have detected the importance of AKt2 protein in mediating the effect of calorie restriction on insulin signalling and glucose homeostasis (McCurdy *et al.*, 2003; McCurdy, 2005). McCurdy and colleagues demonstrated that moderate calorie restriction for 20 days induced a decrease in AKt1 levels. However, in skeletal muscle of rat, there was no change in AKt2 protein abundance but its activation was increased significantly, indicating the importance of (AKt2) in insulin sensitivity (McCurdy *et al.*, 2003). Taking together, the results of the present study and the above reports would indicate that

the levels of AKt1 protein can be affected by the amount of ingested calorie (calorie intake), and might have no major effect on insulin sensitivity in liver and vasculature.

Upregulation of TNF-α is associated with reduced activation of AKt (Gupta and Khandelwal, 2004; Grzelkowska-Kowalczyk and Wieteska-Skrzeczynska, 2006; Tazi *et al.*, 2006). Hence, this study and the other reports together may indicate that overexpression of TNF-α might have inhibitory effects on both protein expression and activity of AKt.

Fatty liver (steatosis), the first stage of NAFLD was apparent in this study. Histological sectioning of liver samples of diet-induced obese animals showed a considerable amount of fat droplets, which was further confirmed by a significant elevation of triglyceride content of the liver samples. Steatohepatitis (inflammation of the liver), the second stage of NAFLD, in untreated obese animals was also confirmed by the histological sectioning of the liver, indicating that steatosis was induced as result of increasing influx of lipid into the liver (Gauthier *et al.*, 2006) in obese animals. Simple steatosis might have also progressed into steatohepatitis due to the increased secretion of inflammatory markers from adipose tissue, which include TNF-α, (Crespo *et al.*, 2001; Li *et al.*, 2003) and CRP (Koruk *et al.*, 2003; Uchihara and Izumi, 2006). In the presence of hepatic insulin resistance, where PI 3-kinase pathway is inhibited and MAP-kinase (ERK1/2) is overactivated liver disease may progress further. Previous studies, have shown that regenerative response to liver injury is impaired in *ob/ob* obese mice with fatty livers (Yang *et al.*, 2001), providing further support for the concept that inhibited regeneration contributes to disease progression in NAFLD. Their results indicate that fatty hepatocytes in *ob/ob* mice become trapped in G_1, the prereplicative phase of the cell cycle. They identified a mechanism that is likely to contribute to this cell cycle arrest. The coordinated activation of the oxidant-sensitive, stress and mitogen-activated protein kinase cascades that normally occurs early in G_1 (Lee *et al.*, 1999) does not occur in fatty hepatocytes, which have adapted to a state of chronic oxidant stress (Yang *et al.*, 2000). Rather, in fatty liver, activation of the mitogen-activated kinase, ERK-1 and ERK-2, is enhanced, whereas induction of the stress-

activated kinase, Jnk, is abolished. ERK and Jnk have multiple cellular targets and interact to orchestrate the complex hepatic response to injury, inducing genes that regulate cell survival, proliferation, and certain-tissues-specific functions (Yan *et al.*, 1994; Fan and Derynck, 1999; Talarmin *et al.*, 1999). Therefore, fatty *ob/ob* hepatocytes fail to proliferate despite robust ERK activation when Jnk induction is abolished (Bost *et al.*, 1997; Lee *et al.*, 1999). Thus, these experiments identify a mechanism (i.e. activation of ERK and Jnk inhibition) that is likely to inhibit proliferation in fatty hepatocytes. Furthermore, TNF-α has been shown to enhance apoptosis, an action which can be inhibited by Jnk (Ventura *et al.*, 2004). Hence, Inhibition of JnK in obesity (Yang *et al.*, 2001) and increased TNF-α in this study may suggest an enhanced apoptosis in obese livers which may contribute to the progression of NAFLD.

A key feature in the molecular pathogenesis of liver fibrosis requires maintenance of the activated hepatic stellate cells phenotype by both proliferation and inhibition of apoptosis. Another study has demonstrated that leptin is a potent hepatic stellate cell mitogen and dramatically inhibits stellate cell apoptosis *via* ERk1/2 and AKt kinases (Saxena *et al.*, 2004). In this study, plasma leptin concentration is elevated in diet-induced obese animals, a finding which may promote the implication of leptin and ERK1/2 in NAFLD progression. Similarly, obese animals had elevated plasma CRP, a protein which has been shown to be an independent risk factor for NAFLD (Park *et al.*, 2004).

The above results of the current study, indicated that the different detrimental factors associated with diet-induced obesity such as increased plasma and liver lipid profile, increased secretion of the different inflammatory markers and adipocytokines (Table 8.4), in addition to hepatic insulin resistance (inactivation of PI 3-kinase and overactivation of MAP-kinase (ERK1/2) pathway) may involve "directly or indirectly" in inducing the different stages of NAFLD.

Lifestyle modification of diet-induced obesity, which represented in the present study by chronic withdrawal of highly-palatable diet (diet-to-chow group), retarded the progression of NAFLD and improved the histological features of this disease to

similar levels of chow-fed animals (Table 8.4). On the other hand, the pharmacological treatment (fenofibrate administration) further improved histological features to levels better than that of chow-fed group. Interestingly, improvements in histological features were paralleled with similar improvement in three detrimental factors associated with diet-induced obesity; namely, TG content of the liver, plasma concentration of TNF-α, and total body weight, suggesting their role in initiating and inducing the different histological features of NAFLD. Moreover, triglyceride content of plasma and liver appears to have a major role in normalising TG content in diet-to-chow and fenofibrate-treated groups, and this may participate in restoring the histological features. Plasma CRP and leptin may also have affected progression or retardation of the histological features of NAFLD, but to a lesser extent. In fenofibrate-treated group, levels of both CRP and leptin were highly elevated, while the histological features were reduced to the minimum level, indicating absence of any considerable correlation between leptin and CRP with development of NAFLD.

Overall, this study suggests that diet-induced obesity results in hepatic insulin resistance possibly by affecting expression and activation of insulin signaling proteins. Moreover, dietary obesity affects liver histology leading to development of NAFLD. Development of NAFLD may be associated by accumulation of triglycerides in liver and/or changes in insulin signaling pathway in the liver.

Table 8.4. Summary for biochemical and hepatic features of the three experimental groups

Biochemical & pathological parameters		Experimental groups			
		Chow-fed	Diet-fed	Diet-to-chow	Feno.
Metabolic Parameters	Insulin	Normal	↑↑	Normal	Normal
	Glucose	Normal	↑↑	Normal	Normal
	Homa	Normal	↑↑	Normal	Normal
	TG	Normal	↑↑	Normal	Normal
	NEFA	Normal	Normal	Normal	Normal
	Body Wt.	Normal	↑↑↑	↑	Normal
Hepatic features	Steatosis +ve samples	50%	80%	50%	10%
	Steatohepatitis +ve samples	40%	70%	30%	10%
	Cirrhosis	0%	0%	0%	0%
	Carcinoma	0%	0%	0%	0%
	TG content	Normal	↑↑↑	Normal	↓↓
	Liver weight	Normal	↑	Normal	↑↑↑
Inflammatory markers	Plasma TNF-α	Normal	↑↑	↑↑	Normal
	Plasma CRP	Normal	↑↑	Normal	↑↑
	Plasma leptin	Normal	↑↑	Normal	↑↑

Chapter 9

RESULTS: Effects of diet-induced obesity on protein expressions of insulin signalling pathways in the skeletal muscle of male Wistar rats

9.1 INTRODUCTION

Insulin resistance is a co-morbidity of overweight and obesity and is a risk factor for development of type 2 diabetes and cardiovascular disease. In obese individuals and patients with type 2 diabetes, skeletal muscle insulin resistance is characterised by markedly blunted insulin-stimulated glucose uptake and metabolism (Dohm *et al.*, 1988). Since skeletal muscle is the primary site of glucose disposal in the human body, the inability of this tissue to uptake glucose in response to insulin most probably explains the reduced *in vivo* disposal of glucose observed in insulin-resistant individuals. At the cellular level, metabolic insulin resistance in diet-induced obesity displays a disturbed protein expression and activation (mainly in skeletal muscle and liver) of signalling *via* the IRS-phosphatidylinositol (PI) 3-kinase pathway, accompanied by a reduction in glucose uptake and utilization (Shulman, 1999).

Systemic lipid accumulation (McGarry, 2002) and increase in the intramuscular concentration of lipid intermediates such as fatty acyl-CoA, ceramides, and diacylglycerols (Despres *et al.,* 1996) not only correlate with insulin resistance, but also directly and indirectly alter insulin signalling (Schmitz-Peiffer, 2002). Considerable evidence linking increased skeletal muscle lipid content to insulin resistance has been derived from animal studies employing acute and chronic high-fat diets (Cooney *et al.*, 2002). Moreover, it has been documented that Protein Kinase-C (PKC) causes insulin resistance in skeletal muscle (Bossenmaier *et al.*, 1997). Furthermore, among the eight PKC isoforms, PKC-β is the only isoform increased in insulin-resistant muscle. Basal PKC-β is higher in the *in vitro* incubated muscles from obese individuals, and insulin increases PKC-β in muscle from obese patients but not from lean individuals (Itani *et al.*, 2000).

Various intramuscular lipid intermediates such as fatty acyl-CoA, ceramides, and DAG inhibit the insulin signalling cascade steps (Schmitz-Peiffer, 2002). Ceramide activates a protein phosphatase that dephosphorylates Akt (protein kinase B), resulting in inhibition of GLUT4 translocation and glycogen synthesis (Long and Pekala, 1996; Chavez *et al.*, 2003).

There is a close relationship between insulin resistance and visceral adiposity. It has become apparent that insulin sensitivity is modulated by adiposity, and this has led to a significant interest in adipocytokines such as TNF-α, leptin, and CRP, which may play pivotal role in skeletal muscle insulin signalling and resistance (Bastard *et al.*, 2006). Moreover, insulin sensitivity is restored by treatments that reduce intramuscular lipid accumulation such as lifestyle modification (i.e. low-fat feeding, fasting and exercise) (Oakes *et al.*, 1997; Monzillo *et al.*, 2003), and pharmacological therapy (e.g. fenofibrate, a PPAR-α activator), which improve lipid profile and insulin resistance (Ye *et al.*, 2001; Lee *et al.*, 2002). However, the exact molecular mechanism(s) involved in insulin transduction in skeletal muscle still not well understood. Therefore, the present study aims to investigate the protein expressions of PI 3-kinase and MAP-kinase insulin signalling pathways in skeletal muscle of diet-induced obese rats, and analysing the relationship between lipid profile, protein expression, and induction of skeletal muscle insulin resistance.

9.2 METHODS

9.2.1. Experimental protocol

Animals used in this study were those described in chapter 7. At the end of experimental protocol (as described in chapter 7), the rats were killed by CO_2 inhalation after 2 hours of fasting, skeletal muscle gastrocnemius was dissected and snap frozen in liquid nitrogen, for later molecular techniques.

9.2.2 Experimental procedures

9.2.2.1 Western-immunoblotting technique

Western-immunoblotting technique was carried out (as described in chapter two, section 2.4) on the four experimental groups (chow-fed, vehicle, diet-to-chow, and fenofibrate treated groups) for measuring the expression of proteins involved in insulin signalling pathways in skeletal muscle. The measured protein kinases were: PKC-β, IR-β, IRS-1, PI 3-kinase, AKt, Shc, ERK1/2 and eNOS.

9.2.2.2 Triglyceride content of gastrocnemius skeletal muscle

A small sample of gastrocnemius muscle (160-200 mg) was homogenised in 500 µl distilled water with polytron homogeniser. The homogenate were centrifuged for

5 minutes at 13000 x g, and the supernatants were transferred to eppendurff tubes. The triglycerides assay was performed on the supernatant to detect the concentration of TG in muscle.

9.2.3 Data interpretation and statistical analysis

For Western blotting, the data from chow-fed (control) animals were expressed as 100% response, and the results from other three groups were normalised and subsequently expressed as the percentage of their respective controls. Data are expressed as mean ± S.E.M. Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student 't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p<0.05$.

9.3. RESULTS

9.3.1 Body weight

By referring to the results of changes in total body weight in chapter 7, animals given palatable diet progressively gained more weight than their chow-fed counterparts. Untreated diet-fed animals were significantly heavier than chow-fed, diet-to-chow, and fenofibrate treated animals. However, at the end of the experiment, body weight of diet-to-chow and fenofibrate treated animals were significantly ($p<0.01$) lower than untreated diet-fed animals (for more statistical details see results in chapter 7).

9.3.2 Triglyceride content of gastrocnemius skeletal muscle

Triglyceride concentration was measured in tissue of skeletal muscle (Fig. 9.1) in all three experimental groups: vehicle, diet-to-chow, and fenofibrate-treated, groups. TG levels in muscle of vehicle group were significantly increased (70%, $p< 0.05$) in comparison with chow-fed control group, while TG concentrations of the other two groups (diet-to-chow and fenofibrate-treated) were comparable with chow-fed control group, with no significant changes (Fig. 9.1).

9.3.3 Expression of protein kinases of insulin signalling pathways in skeletal muscle

PKC-β. Compared with chow-fed group, expression of PKC-β levels were significantly increased in both; vehicle (113%, $p<0.01$) and fenofibrate-treated (137%, $p<0.05$) groups, but not in diet-to-chow group (Fig. 9.2).

IR-β. The expression of IR-β protein was not affected by the three experimental groups: vehicle, diet-to-chow, and fenofibrate-treated groups. IR-β levels were similar in all groups with no significant difference between the groups (Fig. 9.3a).

IRS-1. Similar to IR-β, the expression of IRS-1 was comparable in all three experimental groups; vehicle, diet-to-chow, and fenofibrate-treated, with no significant differences in comparison with chow-fed control group (Fig. 9.3b).

PI 3-kinase. Surprisingly, the concentrations of the regulatory subunit p85 of PI 3-kinase were significantly increased in both vehicle (77%, $p<0.005$), and fenofibrate-treated (68%, $p<0.05$) groups, but not diet-to-chow group compared with chow-fed control (Fig. 9.4a).

AKt. The expression of AKt protein in vehicle group (diet-induced obese animals) did not change significantly in comparison with control group (Fig. 9.4b). However, there were marked reduction in AKt levels in both groups: diet-to-chow (38%, $p<0.001$) and fenofibrate-treated (24%, $p<0.05$) groups when compared with chow-fed group. Moreover, AKt levels in diet-to-chow group were significantly lower ($p<0.05$) than that of vehicle group (Fig. 9.4b).

Shc. In MAP-kinase pathway, expression of two important protein kinases "Shc and ERK1/2" were measured. Compared with chow-fed control group protein levels of Shc in vehicle group were significantly increased (65%, $p<0.01$) (Fig. 9.5a), while the expression of Shc in the other two experimental groups: diet-to-chow and fenofibrate-treated groups were not significantly altered (Fig. 9.5a).

ERK1/2. The expression of ERK1/2 protein in vehicle group was significantly increased (50%, $p<0.005$), while diet-to-chow group was decreased markedly

(28%, $p<0.05$) when compared with control group (Fig. 9.5b). Moreover, ERK1/2 levels in skeletal muscle were not significantly altered by fenofibrate-treatment (Fig. 9.5b).

eNOS. Similar to PI 3-kinase, eNOS protein levels were remarkably augmented in both groups; vehicle (98%, $p<0.001$) and fenofibrate-treated (115%, $p<0.001$) groups in comparison with chow-fed control. However, there were no significant changes in eNOS expression in tissues from diet-to-chow group compared with chow-fed control (Fig. 9.6).

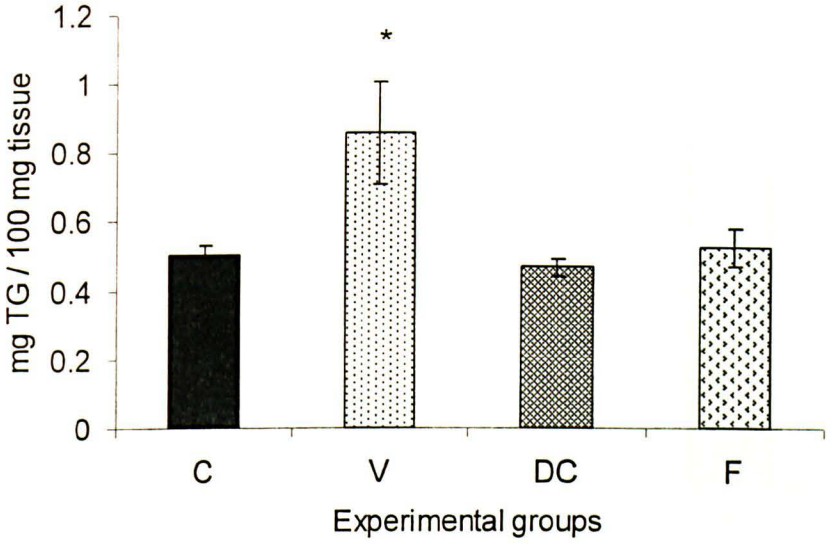


Fig. 9.1. Triglyceride (TG) content of gastrocnemius skeletal muscle in three experimental groups: V) vehicle, DC) diet-to chow, and F) fenofibrate-treated, groups. Data are presented as mean ± S.E.M. * $p<0.05$ vs chow-fed control. TG content of skeletal muscle in vehicle group was significantly higher than chow-fed group.

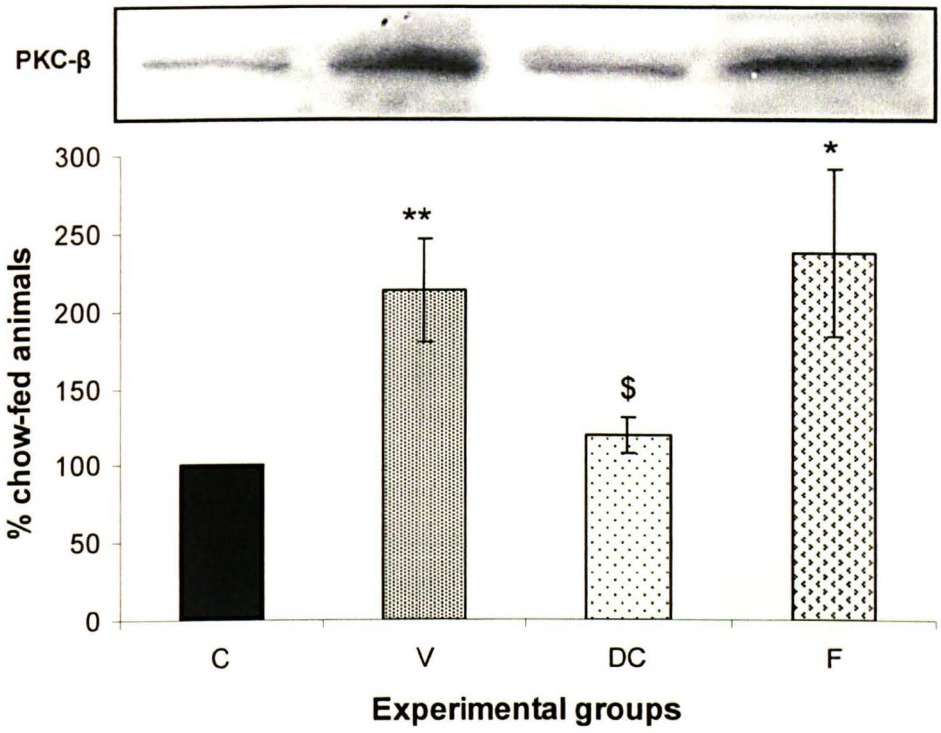


Fig. 9.2. Protein expression of PKC-β in the rat skeletal msucle. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with PKC-β antibody. The animal groups were: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. *=$p<0.05$, ** =$p<0.01$ *vs* chow-fed, $=$p<0.05$ *vs* vehicle.

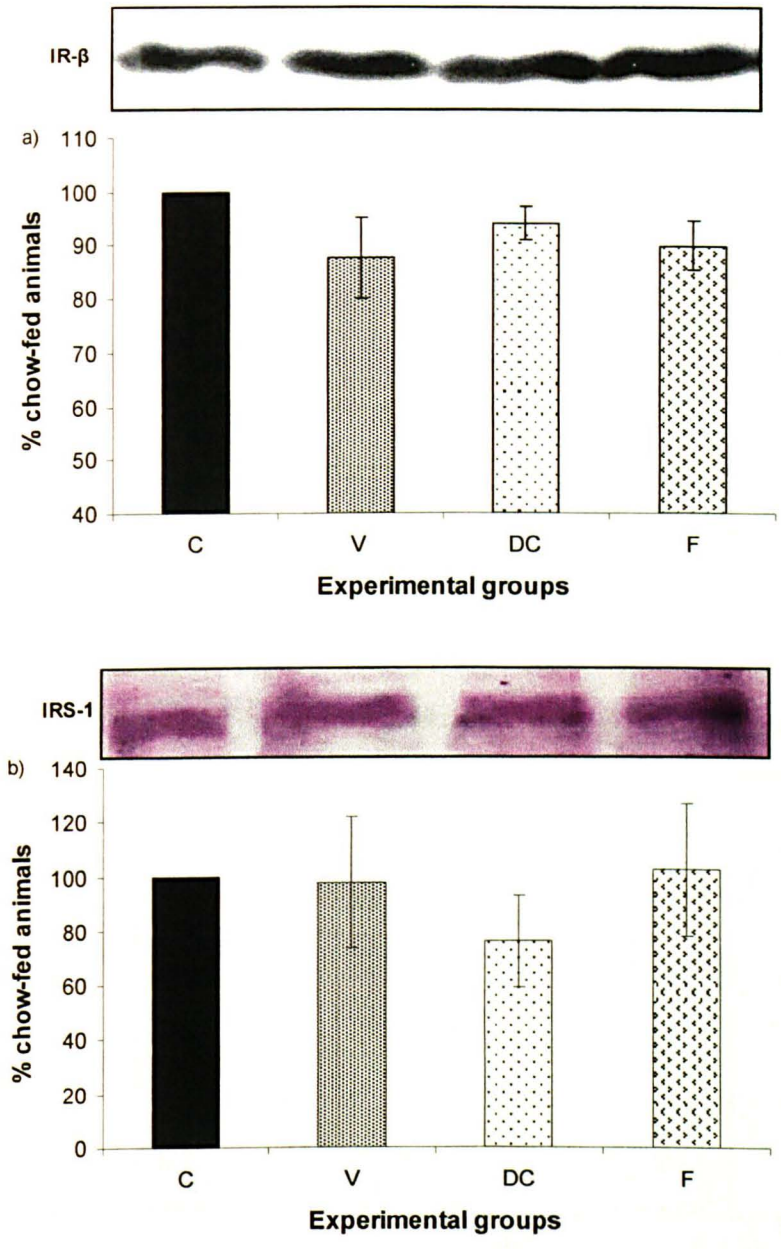


Fig 9.3. Protein expression of a) IR-β, and b) IRS-1 in the rat skeletal muscle. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with IR-β or IRS-1 antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. There was no significant difference in protein expression between any group.

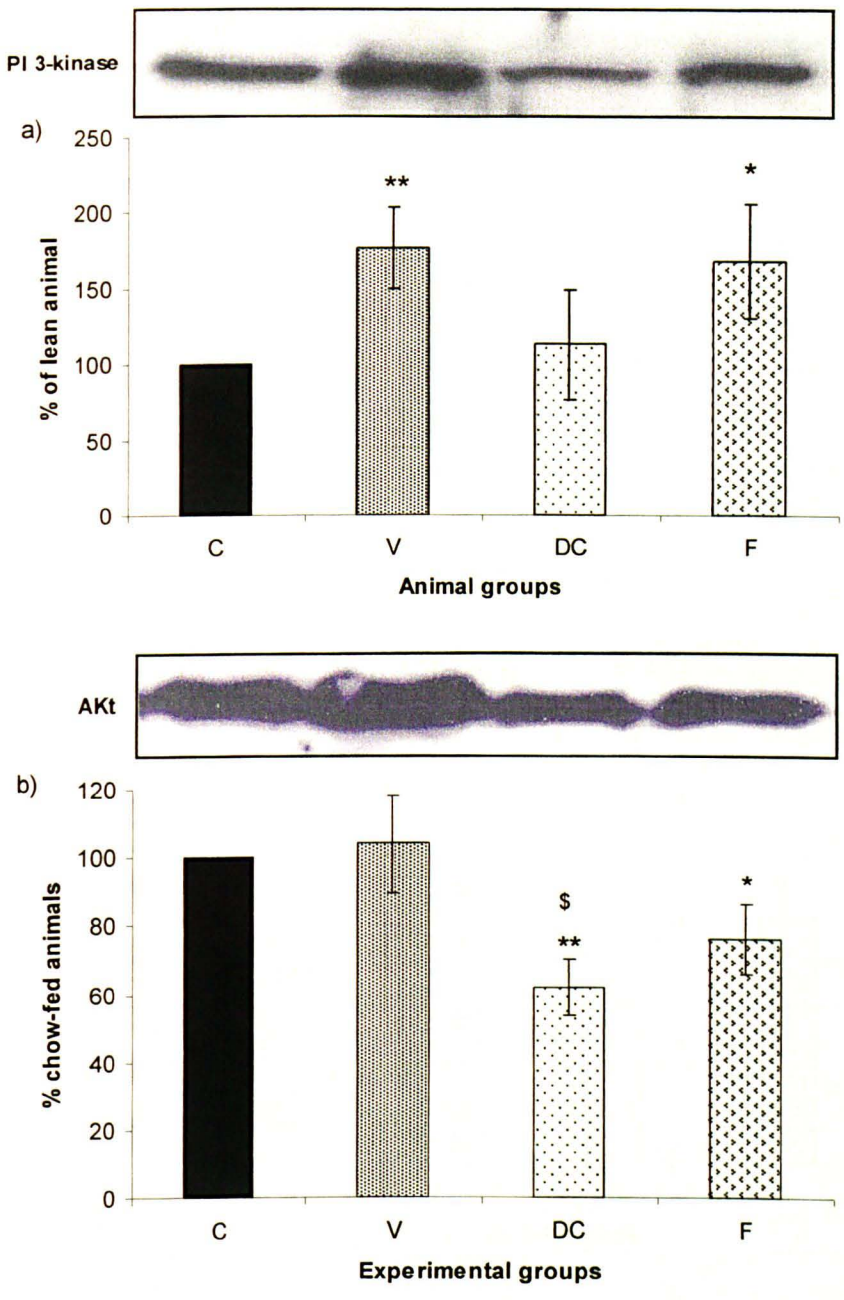


Fig. 9.4. Protein expression of a) PI 3-kinase, and b) AKt in the rat skeletal msucle. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with PI 3-kinase or AKt antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * p=<0.05, ** =p<0.005 *vs* chow-fed. $ =p<0.05 *vs* vehicle.

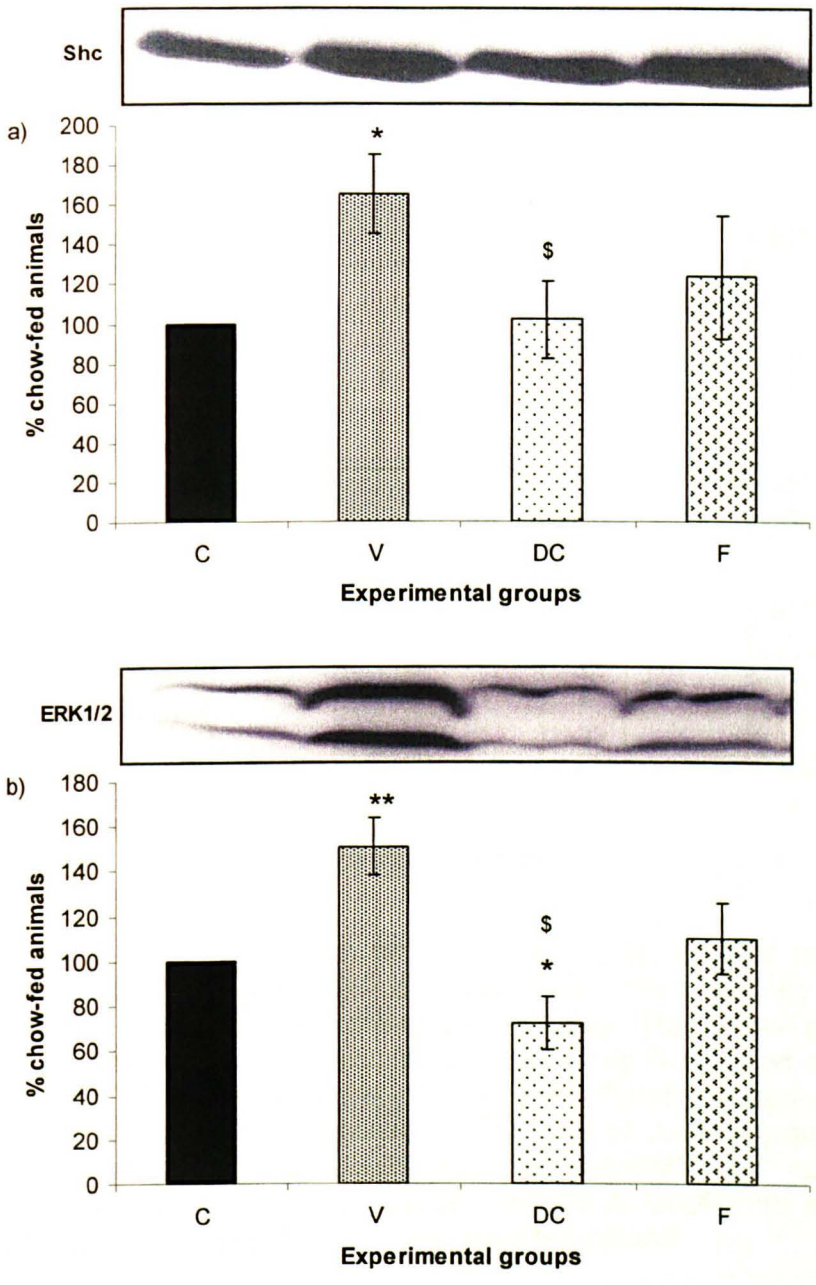


Fig. 9.5. Protein expression of a) Shc, and b) ERK1/2, in the rat skeletal msucle. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with Shc or PERK1/2 antibodies. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. *=p<0.05, ** =p< 0.005 *vs* chow-fed. $ =p<0.05 *vs* vehicle.

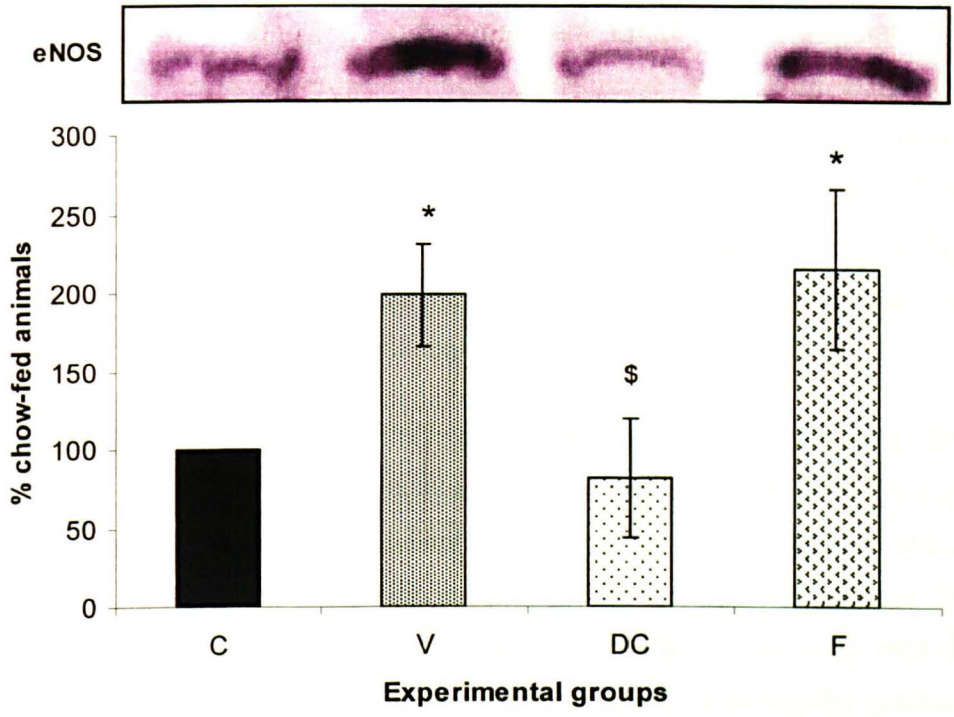


Fig. 9.6. Protein expression of eNOS in the rat skeletal muscle. Equal amounts (40 µg / well) of protein were separated by SDS-PAGE and immunoblotted with eNOS antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± S.E.M) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * =$p<0.05$ *vs* chow-fed. $ =$p< 0.05$ *vs* vehicle. Vehicle & fenofibrate treated groups are significantly higher than chow-fed control.

9.4 DISCUSSION

Normal muscular insulin signalling is an important factor in the regulation of glucose metabolism. In fact, muscle insulin resistance is one of the major contributing factors to the development of diabetes mellitus type 2. In muscle, insulin signalling pathway is composed of cascade of protein kinases. Their expression and activation play crucial roles in maintaining normal glucose homeostasis. Therefore, any reduced strength in this insulin transduction may lead to skeletal muscle and whole body insulin resistance.

In diet-induced obesity, intramuscular TG (and its intermediates: ceramides, fatty acyl-CoA, and diacyl glycerol), hyperinsulinaemia, and the elevated levels of some adipocytokines, in cooperative or separate manner induce muscular insulin resistance by disturbing protein expressions and phosphorylation of kinases that belong to PI 3-kinase and MAP-kinase signalling pathways in skeletal muscle. Disruption of protein phosphorylation of the above cascades in insulin resistant skeletal muscle and obesity has received an adequate attention in literature and scientific research, while the associated disruption in the levels of their protein expressions still not well understood and merits further investigation.

The present study demonstrated that increased intramuscular triglyceride content was detected in diet-induced obese rats, and the greater lipid uptake by the muscle may be a potential mechanism responsible for this accumulation rather than due to a decrease in fatty acid oxidation (Hulver *et al.*, 2003). This hypothesis can be strengthened by a result in chapter five, where it has been detected that plasma levels of triglyceride in untreated diet-fed group (obese rats) were markedly elevated. Furthermore, reduction in fatty acid oxidation is not a necessity for increased intramuscular triglyceride intermediate (fatty acyl-CoA) accumulation (Oakes *et al.*, 1997; Hegarty *et al.*, 2003; Hulver *et al.*, 2003). Therefore, this study suggests that increased influx of lipid into skeletal muscle might play a major role in inducing augmentation of intramuscular triglyceride in diet-induced obesity. Moreover, both types of obesity treatment, chronic removal of highly-palatable diet and fenofibrate administration, caused remarkable reductions in the elevated levels

of intramuscular and plasma triglyceride. These improvements were occurred through two different mechanisms: in removal of highly-palatable diet, reduction in energy (calorie) intake was involved, and thus improving plasma and muscle lipid profile, while in fenofibrate treatment, increasing muscular fatty acid β-oxidation lead to raised levels of fatty acid utilization and clearance.

In the present study, PKC-β expression in gastrocnemius muscle of diet-induced obese rats were remarkably increased; and was associated with insulin resistance (as shown in chapter five), suggesting a role for PKC-β protein expression in glucose uptake in skeletal muscle (Bossenmaier *et al.*, 1997; Itani *et al.*, 2000). Triglyceride intermediates "fatty acyl-CoA, and diacylglycerol" might directly activate PKC-β (Shulman, 2000; Cooney *et al.*, 2002; Itani *et al.*, 2002; Schmitz-Peiffer, 2002). PKC-β activation in turn, may lead to negative effects on insulin transduction cascade in muscle. In this study, treating obesity by lifestyle modification "chronic withdrawal of highly-palatable diet" induced complete normalisation of PKC-β protein expression as well as an improvement in the intramuscular lipid accumulation and subsequent decrease in muscular and whole body insulin resistance. However, pharmacological treatment of obesity in this study (fenofibrate administration) failed to reduce or normalise the muscular expression of PKC-β, despite a remarkable improvement in intramuscular and plasma lipid concentrations and insulin resistance. Elevation of PKC-β levels in the muscle of fenofibrate-treated group could be due to excessive production of reactive oxygen species (ROS) *via* increased fatty acid-β oxidation. The Principal mechanism of lipid reduction by fenofibrate is via simulating fatty acid-β oxidation in skeletal muscle and other tissues (Furuhashi *et al.*, 2002). ROS is one of the end products of fatty acid-β oxidation (Kudin *et al.*, 2005) which may have a major role in increasing expression and the phosphorylation of PKC-β (DelCarlo and Loeser, 2006). This hypothesis could be strengthened further by reports that fenofibrate-treatment is associated with increased ROS production and availability (Arnaiz *et al.*, 1995; Lores Arnaiz *et al.*, 1997; Jiao and Zhao, 2002; Jiao *et al.*, 2003). Hence, these results may indicate that diet-induced obesity can cause an increase in expression of protein kinase PKC-β in skeletal muscle, and this augmented level may participate in inducing muscular and subsequent whole body

insulin resistance. Furthermore, in the present study, the increased level of PKC-β was reversed completely by "chronic withdrawal of highly-palatable diet" but not pharmacologically (fenofibrate treatment), indicating a key role for the diet-content in the elevation of PKC- β.

The protein expression of the early steps of PI 3-kinase insulin signalling pathway such as IR-β and IRS-1 were not affected by diet-induced obesity. However, others have detected that tyrosine phosphorylation of IRS-1 and IR-β in insulin resistant skeletal muscle in obesity is reduced signifgicantly (Goodyear *et al.*, 1995). These deficiencies in phosphorylation could be mediated by increased protein expression and activation of PKC-β, which in turn phosphorylate IR-β and IRS-1 in serine residue, consequently reducing their ability for tyrosine phosphorylation and development of subsequent insulin resistance (Caro *et al.*, 1987; Bossenmaier *et al.*, 1997; Zhou *et al.*, 1999; Kruszynska *et al.*, 2002).

Although, in the current study we didn't measure the protein expression of IRS-2 in skeletal muscle, several studies have indicated that IRS-1 and IRS-2 are not functionally interchangeable in tissues that are responsible for glucose production (Lehmann *et al.*, 1995), and glucose up take (skeletal muscle). Thus, IRS-1 appears to have its major role in skeletal muscle, whereas IRS-2 appears to regulate hepatic insulin action (Yamauchi *et al.*, 1996; Kido *et al.*, 2000; Previs *et al.*, 2000; Sesti *et al.*, 2001).

Interestingly, in this study, diet-induced obesity and/or overfeeding of highly-palatable diet induced a significant increase in protein expression of the regulatory subunit p85 of PI 3-kinase in skeletal muscle. Moreover, this increase in protein expression was synchronised with muscular and whole body insulin resistance. Therefore, it could be postulated that increased level of the p85 regulatory subunit might inhibit PI 3-kinase activity by competing with phosphotyrosine targets. Hence, disruption of the balance between the amounts of PI 3-kinase subunits and excess levels of p85 regulatory subunit could contribute to insulin resistance. The possible mechanism(s) of insulin resistance could be explained as follow: PI 3-kinase consists of a regulatory subunit, p85, and a catalytic subunit, p110

(Shepherd *et al.*, 1998). Normally, the regulatory subunit exists in excess to the catalytic one, resulting in a pool of free p85 monomers not associated with the p110 catalytic subunit. Thus, there is a balance between the free p85 monomer and the complex p85-p110 heterodimer, the latter being responsible for the PI 3-kinase activity. Increases or decreases in expression of p85 shift this balance in favour of either free p85 or p85-p110 complexes (Terauchi *et al.*, 1999; Ueki *et al.*, 2003). Because the monomer and the heterodimer compete for the same binding sites on the IRS proteins, an imbalance could cause either increased or decreased PI 3-kinase activity. This possibility has recently been supported by studies of the insulin-resistant states induced by human placental growth hormone (Barbour *et al.*, 2004), obesity and type 2 diabetes (Bandyopadhyay *et al.*, 2005). If the production of p85 can be enhanced nutritionally, the resulting changes in the ratio of p85 to p110 may be the earliest manifestation of the ensuing insulin resistance. This could also explain how overfeeding and weight gain trigger insulin resistance.

The levels of the protein expression of AKt in skeletal muscle did not change by diet-induced obesity and/or overfeeding of highly-palatable diet for fifteen week. A similar result has been also reported where the phosphorylation of AKt in skeletal muscle of insulin-resistant obese-diabetic subjects was not changed significantly (Kim *et al.*, 1999). Taken together, it can be argued that AKt in skeletal muscle has no role in inducing muscular insulin resistance. However, chronic withdrawal of the highly-palatable diet caused significant reduction in the protein levels of AKt compared to that of control group, despite the complete normalisation in the muscular and whole body insulin resistance, further indicating lack of a significant role for AKt in skeletal muscle insulin resistance. This in agreement with other studies, where Akt had no major role in resistance to insulin's action in muscle of obese type 2 diabetic subjects (Kim *et al.*, 1999). On the other hand, fenofibrate – treated animals, had lower AKt levels in skeletal muscle, despite an improved insulin signalling and muscular lipid concentration. Once again, this is in agreement with others showing that fenofibrate remarkably reduces AKt activity (Kubota *et al.*, 2005; Grabacka *et al.*, 2006). Therefore, it could be postulated that, the triglyceride-induced muscular insulin resistance, appears to be the consequence of a defect in insulin signalling pathway leading to impaired PI 3-

kinase activation. This in turn, may lead to impaired glucose transport through an AKt-independent pathway.

Lifestyle (withdrawal of highly-palatable diet), but not pharmacological modification of obesity improves protein expression of PI 3-kinase cascade of insulin signalling transduction in skeletal muscle. There is little or no information about mechanism of insulin-induced expression and activation of ERK1/2 MAP-kinase in insulin-resistant obese or diabetic animals. PI 3-kinase mediates most of the metabolic effects of insulin and its downstream signalling events (Kanai *et al.*, 1993; Shepherd *et al.*, 1995), while Shc and Grb2/Sos link insulin receptor signalling to activation of the mitogen-activated protein (MAP) kinase (MAPK) isoform p42MAPK/p44MAPK (ERK1ERK2) cascade. The later is not required for insulin's metabolic effects, but mediates mitogenic signalling (Lazar *et al.*, 1995). The increased expression of both Shc and ERK1/2 in diet-induced obese animals in the present study, suggest a role for this pathway in insulin resistance. However, several studies have demonstrated that activation of MAP-kinase pathway by insulin is not reduced in insulin resistance (Paez-Espinosa *et al.*, 1999 ; Krook *et al.*, 2000; Cusi *et al.*, 2000 ; Paez-Espinosa *et al.*, 2001 ; Zecchin *et al.*, 2003). Furthermore, PKC-β may activate MAP-kinase pathway (Formisano *et al.*, 2000). Counteracting obesity by withdrawing highly-palatable diet or by fenofibrate administration, caused reduction in the expression of Shc and ERK1/2 in skeletal muscle, indicating that the augmented levels of these protein kinases in diet-induced obesity could be reversed by losing weight and subsequent improvement in lipid profile and insulin signalling. In fact, previous reports have indicated that lowering intramuscular lipid accumulation by removing fat-enriched diet or fenofibrate treatment improve muscular insulin resistance (Oakes *et al.*, 1997; Hegarty *et al.*, 2003; Ide *et al.*, 2004; Jazet *et al.*, 2005), perhaps by effecting Shc and ERK1/2 expression.

The protein kinase eNOS is present in endothelial cells of capillaries and larger blood vessels (Segal *et al.*, 1999) of skeletal muscle and in the fibre of skeletal muscle itself. Furthermore, insulin signalling shown to induce eNOS expression and activation (Fisslthaler *et al.*, 2003; Toba *et al.*, 2006), an effect which may not

be related to its metabolic functions (Lau *et al.*, 2000; Baum *et al.*, 2004; Williams *et al.*, 2006). The angiogenic factors such as VEGF and the process of angiogenesis (Silha *et al.*, 2005) are elevated in overweight and obese individuals. In the present study, hyperinsulinaemia (results in chapter 5) might be involved in inducing augmented levels of eNOS expression in skeletal muscle of diet-induced obese rats. Therefore, taken together these results, it is possible that elevated levels of eNOS in skeletal muscle from obese rats could have a major role in inducing the angiogenesis in this tissue. Treating obesity by removing the highly-palatable diet had reversed eNOS levels, while fenofibrate treatment was unable to reduce or improve the raised levels of eNOS.

In conclusion, this study indicate that diet-induced obesity results in insulin resistance in skeletal muscle mainly by increasing the protein expression of the regulatory subunit p85 of PI 3-kinase. Moreover, this augmented level was reversible by physiological but not pharmacological manipulation of obesity. This study also indicates that increased intramuscular triglyceride accumulation may play a principal role in triggering protein expression modification and possible subsequent muscular insulin resistance. Furthermore, protein expressions of early steps (IR-β and IRS-1) of PI 3-kinase pathway were not affected by muscular insulin resistance.

Chapter 10

General discussion

The current obesity epidemic is partly due to the consumption of a high energy diet, which is relatively abundant in atherogenic components such as carbohydrates and fat, leading to dyslipidaemia and vascular abnormalities. The atherogenic dietary components have different detrimental effects on various vascular functions. In fact, the present study indicates that all high energy diets, regardless of their varying compositions, were able to induce varieties of metabolic abnormalities and vascular dysfunctions. Moreover, combination of high amounts of atherogenic components (fat and carbohydrates) in one diet would exacerbate vascular dysfunction. For example: diets of combined atherogenic components such as chocolate, and biscuit had the most diverse detrimental effects on the vascular system (Table 10.1), causing increased KCl- and NA-induced vasoconstriction, and attenuation of vasodilatation in response to carbamylcholine (endothelium-dependent vasorelaxation), indicating endothelial dysfunction. On the other hand, other high energy diets such as highly-palatable and high-fat diets, only attenuated endothelium-dependent vasorelaxation (Table 10.1) with no effect on vascular contractility. These general results further argue that, all types of high energy diets (high-fat, high-carbohydrate, or combination of both macronutrients), at least in the present work are directly or summativly involved in inducing vascular abnormalities. Interestingly, by analysing the results of studies in this project, potential role of obesity and atherogenic diets on the induction of vascular insulin resistance and subsequent endothelial dysfunction, leading to cardiovascular diseases and atherosclerosis becomes more evident. However, the degree of vascular abnormalities in response to long term consumption of a certain high energy diet may depend on the combination and/or amounts of the two atherogenic components (fat and carbohydrate) in any given diet. For example, in the present work, the greatest diversity in vascular defects was present in biscuit, and chocolate supplemented diets, where both diets contained high amounts of atherogenic macronutrients (Table 10.1).

Induction of vascular dysfunction by atherogenic macronutrient components of high energy diet is independent of BMI, and occurs well before appearance of any visible sign of obesity. For example, in chapter four, vascular abnormalities induced by high energy diet (chocolate supplementation) were occurred in the

absence of both hyperphagia and/or obesity. Similarly, in chapter three, long term consumption of biscuit diet induced the greatest variation in vascular functions without any remarkable obesity. These indicate that the induced vascular defects could be more related to the types of the macronutrient components in each diet rather than obesity *per se* or the amount of calorie intake (i.e hyperphagia). Therefore, relying on the amount of calorie intake as indicator of vascular defect would not be a plausible way to predict vascular abnormality. Moreover, these studies reveal that obesity *per se* might not have a major role in inducing vascular dysfunction. The presence of attenuated endothelium-dependent vasorelaxation as one of the vascular detrimental effects of the all four types of high energy diets in the present study may indicate that these types of the diets might have induced their vascular dysfunctions, at least partly, *via* involving the disruption and the inhibition of insulin action in vascular system by disturbing the levels of protein expression and/or activation of insulin signalling pathways in vasculature.

The highly-palatable diet was the only diet among the others, associated with a significant obesity, elevated plasma insulin level and subsequent systemic insulin resistance, in addition to attenuated endothelium-dependent vasorelaxation (Table 10.1 & 10.2), indicating a major role for palatable diet in inducing systemic insulin resistance and metabolic changes. Vascular insulin resistance might also be induced by atherogenic components of the diet itself. However, elevated levels of plasma triglyceride in these animals are the principal link to vascular dysfunction. Once again, these studies reveal that combined macronutrients of high fat and carbohydrate are mostly associated with a significant obesity. Nonetheless, long term consumption of any type of high energy diets resulted in synchronisation of both; elevated levels of plasma triglyceride and attenuated endothelial-dependent vasorelaxation. These parallel events may indicate that triglyceride have a major role in disturbing insulin action in vasculature (vascular insulin resistance) with subsequent vascular defect (attenuated endothelium-dependent vasorelaxation) (Table 10.1 & 10.2).

At the molecular level, protein expressions of PI 3-kinase and MAP kinase pathways were demonstrated in three different insulin-sensitive organs namely,

vascular system, liver, and skeletal muscle. The presence of local insulin resistance in each of these organs might lead to induction of a particular systemic disease. For example vascular insulin resistance can cause endothelial dysfunction and subsequent cardiovascular disease and atherosclerosis, liver insulin resistance can induce fatty liver problems such as non-alcoholic fatty liver disease, while insulin resistance in skeletal muscle may cause systemic insulin resistance and subsequently type II diabetes. Unfortunately, exact molecular mechanisms linking local insulin resistance with the clinical disease still not well understood.

In the aorta, obesity-inducing palatable diet caused selective changes in protein levels of PI 3-kinase-dependent signalling pathway. The reduced levels of PI 3-kinase may have a role in inducing insulin resistance in vascular system, contributing to increased incidence of cardiovascular events seen in obese subjects. Chronic withdrawal of obesity-inducing diet caused a complete normalisation of PI 3-kinase, while fenofibrate treatment failed to improve PI 3-kinase concentrations in dietary-obese animals, indicating that lifestyle modification may have a major role in combating diet-induced obesity-related cardiovascular dysfunction. In the skeletal muscle, protein expression of the regulatory subunit p85 of PI 3-kinase was augmented and were reversed by physiological but not pharmacological manipulation of obesity. The protein expressions of Shc and ERK1/2 in MAP-kinase pathway did not change in vascular and hepatic tissues, but were increased in skeletal muscle. Similarly, in the liver, reduction in protein levels of IRS-2 and PI 3-kinase were the only detrimental effects induced by dietary obesity, and these defects are suggested to be the principal causatives in induction of hepatic insulin resistance. Furthermore, disturbed levels of circulating and hepatic content of triglyceride may have crucial role in insulin resistance. Nonetheless, these molecular abnormalities are reversible by physiological but not pharmacological modification. Moreover, the elevated levels of plasma and liver triglyceride in addition to hepatic insulin resistance in diet-induced obesity might have a major role in inducing the detected histological features of non-alcoholic fatty liver disease, which were normalised by both physiological and pharmacological reversals of the obesity.

Chronic removal of highly-palatable diet for seven weeks was relatively the best therapeutical approach for obesity, as it completely reversed all diet-induced metabolic changes, increased lipid profile, insulin resistance, and vascular defects. Pharmacological treatment (fenofibrate administration), despite improving metabolic and insulin resistance, had very limited beneficial effects on vasculature. In general, it is well known that the application of non-pharmacological approaches such as "lifestyle modification" for treating obesity is both easy and financially more acceptable. The present work provides evidence that pharmacological intervention is much less preferable than non-pharmacological approaches in treating obesity, because of the undesirable effects that produced by the pharmacological agents. Non-pharmacological treatment "withdrawal of highly-palatable diet " reduced obesity by removing the source of obesity, and thus reducing the ingestion of high calorie intake, leading to near perfect reversal and normalisation of all altered physiological functions and mechanisms. Pharmacological intervention involves inhibition or acceleration of certain biochemical steps or functions that lead to increased accumulation of harmful end-products. It has been reported that fenofibrate completely improves the vascular dysfunction (Liang *et al.*, 2000; Steiner *et al.*, 2001). However, in the present work, fenofibrate administration was associated with molecular defects, and limited vascular improvement. It is possible that fenofibrate-induced side-effects are result of ROS production *via* accelerating the oxidation of lipid profile. Hence, it is plausible to recommend application of lifestyle modification such as reducing calorie intake for more convenience and satisfactory result, for treating obesity and its associated complications.

Conclusion. Long term consumption of a high calorie diet could be the major causative agent in inducing vascular defects leading to cardiovascular disease and atherosclerosis, regardless of obesity induction, or the amount of calorie intake. However, the consumption of any type of high calorie diets effects vascular functions. Moreover, the diversity of vascular dysfunction-induced by any high energy diet might not depend on the amount of ingested calorie or diet-induced obesity *per se*, rather it may depend on the combination of atherogenic macronutrients such as carbohydrate and fat in a given diet. The major effects of

the diet were on endothelium-dependent vasorelaxation, probably involving triglyceride-induced vascular insulin resistance.

Chocolate bars (Galaxy) supplementation for long period in addition to the chow-diet may induce wide range of vascular defects (increased vasoconstrictions in response to both KCl and NA, and attenuated endothelium-dependent vasorelaxation), while positive effects of fenofibrate treatment on vascular function may be due to its ability to reduce TG levels.

At the molecular level, it is plausible to conclude that: diet-induced obesity in combination with a highly-palatable diet results in significant alterations in the levels of protein expressions of both PI 3-kinase and MAP kinase pathways in the three different insulin-sensitive organs (vascular system, liver, and skeletal muscle). Furthermore, some of these alterations in protein levels are principally participated in triggering the induction of local and systemic insulin resistance. PI 3-kinase, in particular appears to be the key controlling step of insulin transduction in these organs. Moreover, abnormalities in expression of PI 3-kinase could be the main trigger for the induction of insulin resistance in these organs.

In diet-induced obese animals, MAP-kinase pathway does not appear to be deficient in insulin-sensitive tissues, thus allowing more mitogenic response, ultimately promoting the pathological progression. Moreover, the protein expressions of the early steps of insulin signalling were intact, indicating that the molecular defects in insulin transduction are post receptor. Moreover, differences in the molecular effects of diet-induced obesity on insulin-sensitive tissues may depend on the nature of the cellular function(s) in each organ. For example, in vascular tissue the protein expression of eNOS plays a major role in producing NO and subsequent facilitation of the vascular functions (endothelium-dependent vasorelaxation). Therefore, deficient PI 3-kinase pathway leads to abnormal eNOS expression and activation causing reduced NO production and subsequent attenuation of vasorelaxation.

Long term consumption of any type of high energy diet may not induce obesity but it generally associated with a wide range of vascular abnormalities, which is

depended on the type of the macronutrient present in the diet. In the present work, two approaches of obesity treatment were included for investigation. However, the sum of these studies have shown clearly that lifestyle modification, which represented in this work by removal of highly-palatable obesity-inducing diet for treating obesity, has no pronounced side effects. Moreover, it is a safe method, does not induce detrimental effects on the various physiological functions of the body such as: metabolic parameters, vascular functions, and molecular mechanisms (protein expressions), and has convenient and satisfactory results.

Final comment. Obesity is a neglected epidemic disease that is frequently thought of it as a self-inflected condition. Unfortunately, it can represent as a network or a group of pathological illnesses and symptoms that grow up, spread, and detrimentally effects every organ of the body. Therefore, I would like to have this opportunity to express my recommendations and advices for over weight, obese, and non-obese high calorie diet loving individuals, informing them that obesity is a complicated and harmful than it appears, especially at vascular, and metabolic levels, advising them to rethink carefully and have a more healthy lifestyle. It is plausible to change lifestyle by modifying eating behaviour such as by decreasing calorie intake, avoiding overfeeding, and applying more healthy, convenient, and natural therapeutical mechanisms to reduce body weight. I would also like to advise those apparently healthy non-obese consumers of high calorie diets such as biscuit, chocolate, and cakes to reconsider the pathological "vascular" hazards of such diets, regardless of whether they are obese or not. These diets are capable of exposing individuals to incidence of cardiovascular diseases and atherosclerosis even in the absence of high BMI or a significant obesity. Last advice will be for both groups of individuals, to perform a regular medical checkup, especially of the cardiovascular system, liver function tests, and metabolic functions. By doing this medical checkup as early as possible, they could save their vital organs from a permanent pathological damage. Finally, it is definitely the time for all categories of people all over the world, to unite, combat and defeat such silent devastating monster, namely the obesity.

Table 10.1 Relationship between the type of high-energy diet and the type of vascular dysfunction

Type of high energy diet	Type of vascular dysfunction			
	increase vasocontriction in response to:		Attenuation of vasorelaxation In response to:	
	KCl	NA	CCh	SNP
Biscuits	Sig.	Sig.	Sig.	Sig.
High-fat	Not sig.	Not sig.	Sig.	Not sig.
Chocolate	Sig.	Sig.	Sig.	Not sig.
High-palatable	Not sig.	Not sig.	Sig.	Not sig.

KCl = potassium chloride, NA = noradrenaline, CCh = carbamylcholine,
SNP = sodium nitroprosside, Sig. = significant increase or attenuation, Not sig. = not significant

Table 10.2 Relationship between metabolic changes and the different types of high energy diets

Type of high energy diet	Metabolic parameters "Plasma levels"					
	Glucose	**Insulin**	**leptin**	**TG**	**NEFA**	**HOMA INDEX**
Biscuits	normal	normal	normal	⇑	normal	normal
High-fat	normal	normal	normal	⇑	⇧	normal
Chocolate	normal	normal	⇧	⇑	⇧	normal
High-palatable	normal	⇧	⇧	⇑	normal	⇧

Bibliography

Abram, j. (1997). Role of endothelial dysfunction in coronary artery disease. Am. J. **79**: 2-9.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. (2002). Molecular biology of the cell. Molecular biology of the cell. **4th edition**.

Alessi ,D. R. and Cohen, P. (1998). Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. **8(1)**: 55-62.

Aljada, A. and Dandona, P. (2000). Effect of insulin on human aortic endothelial nitric oxide synthase. . Metabolism **49(2)**: 147-150.

Aljada, A., Saadeh, R., Assian, E., Ghanim, H. and Dandona, P. (2000). Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide. J. Clin. Endocrinol. Metab. **85(7)**: 2572-2575.

Allison, D. B., Fontaine, K. R., Manson, J. E., Stevens, J. and VanItallie, T. B. (1999). Annual deaths attributable to obesity in the United States. JAMA. **282(16)**: 1530-1538.

Anai, M., Funaki, M., Ogihara, T., Terasaki, J., Inukai, K., Katagiri, H ,.Fukushima, Y., Yazaki, Y., Kikuchi, M., Oka, Y. and Asano, T. (1998). Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. Diabetes. **47(1)**: 13-23.

Andersen, R. E., Wadden, T. A. and Bartlett, S. J. (1999). Effects of lifestyle activity vs structured aerobic exercise in obese women: a randomized trial. *JAMA* **281**: 335-340.

Andersen, T., Gluud, C., Franzmann, M.B. and Christoffersen, P. (1991). Hepatic effects of dietary weight loss in morbidly obese subjects. J. Hepatol. **12(2)**: 224-229.

Andres, R. (1980). Effects of obesity on total mortality. Int. J. Obes. **4**: 381-86.

Antl, M., V-Bruhl, M.L., Eiglsperger, C., Werner, M., Konrad, I., Kocher, T., Wilm, M., Hofmann, F., Massberg, S. and Schlossmann, J. (2006). IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. Blood **21**: [Epub ahead of print].

Anty, R., Bekri, S., Luciani, N., Saint-Paul, M.C., Dahman, M., Iannelli, A., Amor, I.B., Staccini-Myx, A., Huet, P.M., Gugenheim, J., Sadoul, J.L., Le-Marchand-Brustel, Y. and Tran, A., Gual, P. (2006). The Inflammatory C-Reactive Protein Is Increased in Both Liver and Adipose Tissue in Severely Obese Patients Independently from Metabolic Syndrome, Type 2 Diabetes, and NASH. Am. J. Gastroenterol. **101(8)**: 1824-1833.

Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T., Furihata, K. and Hashimoto ,T. (1998). Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). J. Biol. Chem. **273**: 5678-5684.

Appel, L. J., Sacks, F. M., Carey, V.J., Obarzanek, E., Swain, J. F., Miller, E. R., Conlin, P. R., Erlinger, T. P., Rosner, B. A., Laranjo, N. M., Charleston, J., McCarron, P. and Bishop, L.M. (2005). Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids. Results of the OmniHeart Randomized Trial. . JAMA **294**: 2455-2464.

Arcaro, G., Cretti, A., Balzano, S., Lechi, A., Muggeo, M., Bonora, E. and Bonadonna, R. C. (2002). Insulin causes endothelial dysfunction in humans: sites and mechanisms. Circulation **105(5)**: 576-582.

Arcaro, G., Zamboni, M., Rossi, L., Turcato, E., Covi, G., Armellini, F., Bosello, O. and Lechi, A. (1999). Body fat distribution predicts the degree of endothelial dysfunction in uncomplicated obesity. A. Int. J. Obes. **23**: 936-942.

Archer, Z. A., Rayner, D. V., Duncan, J. S., Bell, L. M. and Mercer, J. G. (2005). Introduction of a high-energy diet acutely up-regulates hypothalamic cocaine and amphetamine-regulated transcript, Mc4R and brown adipose tissue uncoupling protein-1 gene expression in male Sprague-Dawley rats. J. Neuroendocrinol. **17(1)**: 10-17.

Arnaiz, S. L., Travacio, M., Llesuy, S. and Boveris, A. (1995). Hydrogen peroxide metabolism during peroxisome proliferation by fenofibrate. Biochim. Biophys. Acta. **1272(3)**: 175-180.

Aronne, L. J. and Segal, K. R. (2002). Adiposity and fat distribution outcome measures: assessment and clinical implications. Obes. Res. **10(1)**: 14S-21S.

Auwerx, J., Schoonjans, K., Fruchart, J. C. and Staels, B. (1996). Regulation of triglyceride metabolism by PPARs: fibrates and thiazolidinediones have distinct effects. J. Atheroscler. Thromb. **3**: 81-89.

Azula, F. J., Alzola, E.S., Conde, M., Trueba, M., Macarulla, J. M. and Marino, A. (1996). Thrombin-stimulated phospholipase C activity is inhibited without visible delay by a rapid increase in the cyclic GMP levels induced by sodium nitroprusside. Mol. Pharmacol. **50(2)**: 67-79.

Badiou, S., Merle-De Boever, C., Dupuy, A. M., Baillat, V., Cristol, J. P. and Reynes, J. (2004). Fenofibrate improves the atherogenic lipid profile and enhances LDL resistance to oxidation in HIV-positive adults. Atherosclerosis **172(2)**: 273-279.

Bae, J. H., Bassenge, E., Lee, H. J., Park, K. I., Park, C. G., Park, K. Y., Lee, M.K. and Schwemmer, M. (2001). Impact of postprandial hypertriglyceridaemia on vascular responses in patients with coronary artery disease: effects of ACE inhibitors and fibrates. Atherosclerosis **158**: 165-171.

Bandyopadhyay, G. K., Yu, J.G., Ofrecio, J. and Olefsky, J. M. (2005). Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. Diabetes **54(8)**: 2351-2359.

Banks, W. A. and Farrell, C. L. (2003). Impaired transport of leptin across the blood-brain barrier in obesity is acquired and reversible. Am. J. Physiol. Endocrinol. Metab. Clin. North. Am. **285**: E10-E15.

Barbagallo, C. M., Rizzo, M., Noto, D., Frasheri, A., Pernice, V., Rubino, A., Pieri, D., Pinto, V., Cefalù, A. B., Giordano, C., Notarbartolo, A. and Averna, M. R. (2006). Accumulation of apoE-enriched triglyceride-rich lipoproteins in patients with coronary artery disease. . Metab. Clin. Exp. **55**: 662-668.

Barbour, L. A., Shao, J., Qiao, L., Leitner, W., Anderson, M., Friedman, J. E. and Draznin, B. (2004). Human placental growth hormone increases expression of the p85 regulatory unit of phosphatidylinositol 3-kinase and triggers severe insulin resistance in skeletal muscle. Endocrinology **145(3)**: 1144-1150.

Barnard, R. J., Roberts, C. K., Varon, S. M. and Berger, J. J. (1998). Diet-induced insulin resistance precedes other aspects of the metabolic syndrome. J. Appl. Physiol. **84**: 1311-1315.

Baron, A. D. (1994). Hemodynamic actions of insulin. Am. J. Physiol. **267**: E187-E202.

Baron, A. D. and Brechtel, G. (1993). Insulin differentially regulates systemic and skeletal muscle vascular resistance. Am. J. Physiol. **265**: E61-E67.

Barouch, L. A., Berkowitz, D. E., Harrison, R. W., O'Donnell, C. P. and Hare, J.M. (2003). Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. Circulation.**108(6)**:754-759.

Barret-Conner, E. L. (1985). Obesity, atherosclerosis and coronary heart disease. . Ann. Int. Med. **103**: 1010-1019.

Bastard, J. P., Maachi, M., Lagathu, C., Kim, M. J., Caron, M., Vidal, H., Capeau, J. and Feve, B. (2006). Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur. Cytokine Netw. **17(1)**: 4-12.

Baum, O., Da Silva-Azevedo, L., Willerding, G., Wockel, A., Planitzer, G., Gossrau, R., Pries, A. R. and Zakrzewicz, A. (2004). Endothelial NOS is main mediator for shear stress-dependent angiogenesis in skeletal muscle after prazosin administration. Am. J. Physiol. Heart. Circ. Physiol. **287(5)**: H2300-2308.

Bays, H. and Dujovne, C. A. (2006). Adiposopathy is a more rational treatment target for metabolic disease than obesity alone. Curr. Atheroscler. Rep. **8**: 144-156.

Belanger, B., Cupples, L. and D'Agostino, R. (1988). The Framingham Study: An Epidemiologic Study Investigation of Cardiovascular Disease, Section 36: Measure at Each Examination and Interexamination Consistency of Specified Characteristics. Washington, DC: Government Printing Office. 88-2970.

Bergman, R. N. and Ader, M. (2000). Free fatty acids and pathogenesis of type 2 diabetes mellitus. Trends Endocrinol. Metab. **11(9)**: 351-356.

Bhagat, K. and Valance, P. (1997). Inflammatory cytokines impair endothelium-dependant dilatation in human veins in vivo. Circulation **96**: 3042–3047.

Bhakdi, S., Torzewski, M., Klouche, M. and Hemmes, M. (1999). Complement and atherogenesis: binding of CRP to degraded, nonoxidised LDL enhances complement activation. Arterioscler., Thromb., Vasc. Biol. **19**: 109-117.

Biasucci, L. M., Liuzzo, G., Grillo, R. L., Caligiuri, G., Rebuzzi, A. G., Buffon, A., Summaria, F., Ginnetti, F., Fadda, G. and Maseri, A. (1999). Elevated levels of C-reactive protein at discharge in patients with unstable angina predict recurrent instability. Circulation. **99(7)**: 855-860.

Bjorbaek, C., El-Haschimi, K., Frantz, J. D. and Flier, J. S. (1999). The role of SOCS-3 in leptin signaling and leptin resistance. J. Biol. Chem. **274**: 30059-30065.

Bjorntorp, P. (1990). "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. Arteriosclerosis. **10(4)**: 493-496.

Bjorntorp, P. (1997). Obesity. Lancet **350**: 423-426.

Blair, S. N., Cheng, Y. and Holder, J. S. (2001). Is physical activity or physical fitness more important in defining health benefits? Med. Sci. Sports Exerc. **33(6)**: S379-S399.

Bliss, M. (1993). The history of insulin. Diabetes Care **16**: 4-7.

Blundell, J. E. and Macdiarmid, J. I. (1997). Passive overconsumption. Fat intake and short-term energy balance. Ann. N. Y. Acad. Sci. **827**: 392-407.

Boden, G. (1997). Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes. **46(1)**: 3-10.

Boden, G. (1999). Free fatty acids, insulin resistance, and type 2 diabetes mellitus. Proc. Assoc. Am. Physicians. **111(3)**: 241-248.

Bossenmaier, B., Mosthaf, L., Mischak, H., Ullrich, A. and Haring, H.U. (1997). Protein kinase C isoforms beta 1 and beta 2 inhibit the tyrosine kinase activity of the insulin receptor. . Diabetologia **40**: 863–866.

Bost, F., McKay, R., Dean, N. and Mercola, D. (1997). The JUN kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells. J. Biol. Chem. **272(52)**: 33422-33429.

Bouchard, C. and Tremblay, A. (1990). Genetic effects in human energy expenditure components. Int. J. Obes. **14**: 49-55.

Bouchard, C., Tremblay, A., Despres, J., P., Nadeau, A., Lupien, P., J., , Theriault, G., Dussault, J., Moorjani, S., Pinault, S. and Fournier, G. (1990). The response to long-term overfeeding in identical twins. N. Engl. J. Med. **322(21)**: 1477-1482. .

Boulanger, C. M. (1999). Secondary endothelial dysfunction: hypertension and heart failure. J. Mol. Cell. Cardiol. **31**: 39-49.

Bouloumie, A., Drexler, H. C., Lafontan, M. and Busse, R. (1998). Leptin, the product of Ob gene, promotes angiogenesis. Circ. Res. **83(10)**: 1059-1066.

Bouloumie, A ,.Marumo, T., Lafontan, M. and Busse, R. (1999). Leptin induces oxidative stress in human endothelial cells. FASEB. J. **13**: 1231-1238.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos ,N., Cobb, M. H. and Yancopoulos, G. D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell **65(4)**: 663-675.

Bratanova-Tochkova, T. K., Cheng, H., Daniel, S., Gunawardana, S., Liu, Y. J., Mulvaney-Musa, J., Schermerhorn, T., Straub, S. G., Yajima, H. and Sharp, G. W. (2002). Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion. Diabetes **51(1)**: S83-90.

Bray, G. A. and Tartaglia, L. A. (2000). Medicinal strategies in the treatment of obesity. Nature. **404(6778)**: 672-677.

Bray, G.A. (2000). A concise review on the therapeutics of obesity. *Nutrition* **16**: 953-960.

Brown, B. G., Zhao, X. Q., Sacco, D. E. and Albers, J. J. (1993). Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical events in coronary disease. Circulation. **87(6)**: 1781-1791.

Bruning, J. C., Michael, M. D., Winnay, J. N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J. and Kahn, C.R. (1998) .A muscle-specific insulin receptor

knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol. Cell. **2(5)**: 559-569.

Brunt, E. M., Janney, C. G., Di Bisceglie, A. M., Neuschwander-Tetri, B. A. and Bacon, B.R. (1999) .Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am. J. Gastroenterol. **94(9)**: 2467-2474.

Bucher, H.C., Griffith, L.E. and Guyatt, G.H. (1999). Systemic review on the risk and benefits of different cholesterol-lowering interventions. . Arterioscler. Thromb. Vas. Biol. **19**: 187-95.

Buffon, A., Liuzzo, G., Biasucci, L. M., Pasqualetti, P., Ramazzotti, V., Rebuzzi, A. G., Crea, F. and Maseri, A. (1999). Preprocedural serum levels of C-reactive protein predict early complications and late restenosis after coronary angioplasty. J. Am. Coll. Cardiol. **34(5)**: 1512-1521.

Bugianesi, E., Leone, N., Vanni, E., Marchesini, G., Brunello, F., Carucci, P., Musso, A., De Paolis, P., Capussotti, L., Salizzoni, M. and Rizzetto, M. (2002). Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology. **123(1)**: 134-140.

Bullo, M., Garcia-Lorda, P., Megias, I. and Salas- Salvado, J. (2003). Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression . Obesity. Res. **11**: 525-531.

Burks, D. J. and White, M. F. (2001). IRS proteins and beta-cell function. Diabetes **50(1)**: S140-145.

Cai, H. and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ. Res. **87(10)**: 840-844.

Calle, E. E., Thun, M. J., Petrelli, J. M., Rodriguez, C. and Heath, C. W. (1999). Body-mass index and mortality in a prospective cohort of U.S. adults. N. Engl. J. Med. **341(15)**: 1097-1105.

Capell, W. H., DeSouza, C. A., Poirier, P., Bell, M. L., Stauffer, B. L., Weil, K. M., Hernandez, T. L. and Eckel, R. H. (2003). Short-term triglyceride lowering with fenofibrate improves vasodilator function in subjects with hypertriglycerideamia. Arterioscler. Thromb. Vasc. Biol. **23**: 307-313.

Carey, V. J., Walters, E. E., Colditz, G. A., Solomon, C. G., Willett, W. C., Rosner, B. A., Speizer, F. E. and Manson, J. E. (1997) .Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The nurses' health study. Am. J. Epidemiol. **145**: 614-619.

Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., Meelheim, D. and Dohm ,G.L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. Journal of Clinical Investigation **79**: 1330–1337.

Cefalu, W. T. (2001). Insulin resistance: cellular and clinical concepts .Exp. Biol. Med.(Maywood) **226**: 13-26.

Chang, S., Graham, B., Yakubu, F., Lin, D., Peters, J. C. and Hill, J. O. (1990). Metabolic differences between obesity-prone and obesity-resistant rats. Am. J. Physiol. **259**: R1103-1110.

Chavez, J. A., Knotts, T. A ,.Wang, L. P., Li, G., Dobrowsky, R. T., Florant, G. L. and Summers, S. A. (2003). A role for ceramide, but not diacylglycerol, in

the antagonism of insulin signal transduction by saturated fatty acids. Journal of Biological Chemistry **278**: 10297–10303.

Chen , K., Li, F., Li, J., Cai, H., Strom, S., Bisello, A., Kelley, D.E., Friedman-Einat, M., Skibinski, G.A., McCrory, M. A., Szalai, A. J. and Zhao, A. Z. (2006). Induction of leptin resistance through direct interaction of C-reactive protein with leptin. Nat .Med. **12**(4): 425-432.

Cherrington, A. D., Chiasson, J. L., Liljenquist, J. E., Lacy, W. W. and Park, C. R. (1978). Control of hepatic glucose output by glucagon and insulin in the intact dog. Biochem. Soc. Symp. **43**: 31-45.

Chitturi, S., Abeygunasekera, S., Farrell, G. C., Holmes-Walker, J., Hui, J. M., Fung, C., Karim, R., Lin, R., Samarasinghe, D., Liddle, C., Weltman, M. and George, J. (2002). NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. Hepatology. **35(2)**: 373-379.

Chitturi, S., Farrell, G., Frost, L., Kriketos, A., Lin, R., Fung, C., Liddle, C., Samarasinghe, D., George, J. (2002). Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity? Hepatology. **36(2)**: 403-409.

Chua, S. C. Jr., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Liu, S. M., Tartaglia, L. and Leibel, R.L. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science **271(5251)**: 994-996.

Clarke, S. D. (2000). Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. Br. J. Nutr. **83(1)**: S59-66.

Coakley, E. H., Kawachi, I., Manson, J. E., Speizer, F. E., Willet, W. C. and Colditz, G. A. (1998). Lower levels of physical functioning are associated with higher body weight among middle-aged and older women. Int. J. Obes. Relat. Metab. Disord. **22(10)**: 958-965.

Coleman, D. L. (1978). Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia **14(3)**: 141-148.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J. and Bauer, T. L. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese human. New Engl. J. Med. **334**: 292-295.

Cooney, G. J., Thompson, A. L., Furler, S. M., Ye, J. and Kraegen, E. W. (2002). Muscle long-chain acyl CoA esters and insulin resistance. Annals of the New York Academy of Sciences **967**: 196–207.

Corrado, E., Muratori, I., Tantillo, R., Contorno, F., Coppola, G., Strano, A. and Novo, S. (2005). Relationship between endothelial dysfunction, intima media thickness and cardiovascular risk factors in asymptomatic subjects. Int. Angiol. **24**: 52-58.

Correia, M. L., Morgan, D. A., Sivitz, W. I., Mark, A. L. and Haynes, W. G. (2001). Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. Hypertension **37(3)**: 936-942.

Cortez-Pinto, H., Camilo, M. E., Baptista, A., De Oliveira, A. G. and De Moura, M.C. (1999). Non-alcoholic fatty liver: another feature of the metabolic syndrome? Clin. Nutr. **18(6)**: 353-358.

Cowan, G. S., Defibaugh, N., White, T., Hiler, M. L. and Somes, G. (1991). Aorta and iliac arterial size in pre-operative morbidity obese patients: a preliminary report. Obese. Surg. **1**: 155-159.

Crespo, J., Cayon, A., Fernandez-Gil, P., Hernandez-Guerra, M., Mayorga, M., Dominguez-Diez, A., Fernandez-Escalante, J.C. and Pons-Romero, F. (2001). Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. Hepatology **34(6)**: 1158-1163.

Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M. E., Pratipanawatr, T., DeFronzo, R. A., Kahn, C. R. and Mandarino, L. J. (2000). Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J. Clin. Invest. **105(3)**: 311-320.

Damci, T., Tatliagac, S., Osar, Z. and Ilkova, H. (2003). Fenofibrate treatment is associated with better glycemic control and lower serum leptin and insulin levels in type 2 diabetic patients with hypertriglyceridemia. Eur. J Intern. Med. **14(6)**: 357-360.

Dandona, P., Weinstock, R., Thusu, K., Abdel-Rahman, E., Aljada, A. and Wadden, T. (1998). Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. J. Clin. Endocrinol Metab. **83(8)**: 2907-2910.

Day, C. P. and James, O. F. (1998). Steatohepatitis: a tale of two "hits"? Gastroenterology **114(4)**: 842-845.

De Keulenaer, G. W., Alexander, R. W., Ushio-Fukai, M., Ishizaka, N. and Griendling, K. K. (1998). Tumour necrosis factor a activates a p22phox based NADH oxidase in vascular smooth muscle. . Biochem. J. **329**: 653–657.

De Lonlay and Saudubray, J-M. (2000). Persistent hyperinsulinaemic hypoglycaemia. Inborn Metabolic Diseases: Diagnosis and treatment.(3rd ed):Springer, Heidelberg Germany **3**: 117-26.

DeFronzo, R. A. and Ferrannini, E. (1991). Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care. **14(3)**: 173-194.

Deitel, M. and Shikora, S. A. (2002). The development of the surgical treatment of morbid obesity. J. Am. Coll. Nutr. **21(5)**: 365-371.

DelCarlo, M. and Loeser, R. F. (2006). Chondrocyte cell death mediated by reactive oxygen species-dependent activation of PKC-betal. Am. J. Physiol. Cell. Physiol. **290(3)**: C802-811.

Denton, R. M. and Tavare, J. M. (1997). Molecular basis of insulin action on intracellular metabolism. International Textbook of Diabetes Mellitus (2nd ed) John Wiley & Sons, New York **2**: 469-488.

Despres, J. P., Lamarche, B., Mauriege, P., Cantin, B., Dagenais, G. R., Moorjani, S. and Lupien, P. J. (1996). Hyperinsulinemia as an independent risk factor for ischemic heart disease. N. Engl. J. Med. **334(15)**: 952-957.

Desprēs, J. P., Lemieux, I., Salomon, H. and Delaval, D. (2002). Effects of micronized fenofibrate versus atrovastatin in the treatment of dyslipidaemic patients with low plasma HDL-cholestrol levels: a 12-week randomised trial. J. Intern. Med. **251**: 490-499.

Devaraj, S., Rosenson R. S. and Jialal, I. (2004). Metabolic syndrome: an appraisal of the pro-inflammatory and procoagulant status. Endocrinol. Metab. Clin. North. Am. **33**: 431-453.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature **399**: 601-605.

Ding, Y., Vaziri, N. D., Coulson, R., Kamanna, V., S., and Roh, D. D. (2000). Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression. Am. J. Physiol. Endocrinol Metab. **279(1)**: E11-E17.

DiPietro, L., Kohl, H. W., Barlow, C. E. and Blair, S. N. (1998). Improvements in cardiorespiratory fitness attenuate age-related weight gain in healthy men and women: the Aerobics Center Longitudinal Study. Int. J. Obes. Relat. Metab. Disord. **22(1)**: 55-62.

Dobrian, A. D., Davies, M. J., Prewitt, R. L. and Lauterio, T. J. (2000). Development of hypertension in a rat model of diet-induced obesity. Hypertension **35**: 1009-1015.

Dodson, G. and Steiner, D. (1998). The role of assembly in insulin's biosynthesis. Curr. Opin. Struct. Biol. **8**: 189-194.

Dohm, G. L., Tapscott, E. B., Pories, W. J., Dabbs, D. J., Flickinger, E. G., Meelheim, D., Fushiki, T., Atkinson, S. M., Elton, C. W. and Caro, J. F. (1988). An in vitro human muscle preparation suitable for metabolic studies. Decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. Journal of Clinical Investigation **82**: 486–494.

Duflou, J., Vrmani, R., Rabin, I., Farb, A. and Smialek. (1995). Sudden death as a result of heart disease in morbid obesity. Am. Heart J. **130**: 306-313.

Dunaif, A. and Thomas, A. (2001). Current concepts in the polycystic ovary syndrome. Annu. Rev. Med. **52**: 401-419.

Eckel, R. H. and Krauss, R. M.(1998). American Heart Association call to action: obesity as a major risk factor for coronary heart disease. AHA Nutrition Committee. Circulation **97(21)**: 2099-2100.

Empen, K., Frost, R. J., Geiss, H., C.Otto, C. and Parhofer, K. G. (2003). Differential effects of fenofibrate versus atorvastatin on the concentrations of E-selectin and vascular cellular adhesion molecule-1 in patients with type 2 diabetes mellitus and mixed hyperlipoproteinemia: a randomized cross-over trial. Cardiovasc. Diabetol. **2**: 17.

Erdos, B., Snipes, J. A., Miller, A. W. and Busija, D. W. (2004). Cerebrovascular dysfunction in Zucker obese rats is mediated by oxidative stress and protein kinase C. Diabetes **53**: 1352-1359.

Eriksson, K. F. and Lindgarde, F. (1991). Prevention of type 2 (non-insulin-dependent) diabetes mellitus by diet and physical exercise. The 6-year Malmo feasibility study. Diabetologia **34(12)**: 891-898.

Eriksson, S., Eriksson, K.F. and Bondesson, L. (1986). Nonalcoholic steatohepatitis in obesity: a reversible condition .Acta. Med. Scand. **220(1)**: 83-88.

Eringa, E. C., Stehouwer, C. D., Merlijn, T., Westerhof, N. and Sipkema, P. (2002). Physiological concentrations of insulin induce endothelin-mediated

vasoconstriction during inhibition of NOS or PI3-kinase in skeletal muscle arterioles. . Cardiovasc. Res. **56**: 464-471.

Eringa, E. C., Stehouwer, C. D., van, Nieuw. , Amerongen, G. P., Ouwehand, L., Westerhof, N. and Sipkema, P. (2004). Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium. Am. J. Physiol. Heart Circ. Physiol. **287(5)**: H2043-2048.

Evans, D. J., Hoffmann, R. G., Kalkhoff, R. K. and Kissebah, A. H. (1984). Relationship of body fat topography to insulin sensitivity and metabolic profiles in premenopausal women. Metabolism **33(1)**: 68-75.

Fan, H. and Derynck, R. (1999). Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. EMBO J. **18(24)**: 6962-6972.

Ferri, C., Carlomagno, A., Coassin, S., Baldoncini, R., Cassone, Faldetta, M. R., Laurenti, O., Properzi, G., Santucci, A. and De Mattia, G. (1995). Circulating endothelin-1 levels increase during euglycemic hyperinsulinemic clamp in lean NIDDM men .Diabetes Care. **18(2)**: 226-233.

Filippatos, T. D., Kiortsis, D. N., Liberopoulos, E. N., Georgoula, M., Mikhailidis, D. P. and Elisaf, M. S. (2005). Effect of orlistat, micronised fenofibrate and their combination on metabolic parameters in overweight and obese patients with the metabolic syndrome: the FenOrli study. Curr. Med. Res. Opin. **21**(12): 1997-2006.

Fine, J. T., Colditz, G. A., Coakley, E. H., Moseley, G., Manson, J. E., Willett, W. C. and Kawachi, I. (1999). A prospective study of weight change and health-related quality of life in women. JAMA **282(22)**: 2136-2142.

Finer, N., James, W. P., Kopelman, P. G., Lean, M. E. and Williams, G. (2000). One-year treatment of obesity: a randomized, double-blind, placebo-controlled, multicentre study of orlistat, a gastrointestinal lipase inhibitor. Int. J. Obes. Relat. Metab. Disord. **24(3)**: 306-313.

Fisslthaler, B., Benzing, T., Busse, R. and Fleming, I. (2003). Insulin enhances the expression of the endothelial nitric oxide synthase in native endothelial cells: a dual role for Akt and AP-1. Nitric Oxide **8(4)**: 253-261.

Flegal, K. M., Carroll, M. D., Kuczmarski, R. J. and Johnson, C. L. (1998). Overweight and obesity in the United States: prevalence and trends, 1960-1994. Int. J. Obes. Relat. Metab. Disord. **22(1)**: 39-47.

Flegal, K. M., Carroll, M. D., Ogden, C. L., Johnson, C. L. (2002). Prevalence and trends in obesity among US adults, 1999-2000. JAMA **288(14)**: 1723-1727.

Fleming, I. and Busse, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am. J. Physiol. Regul. Integr. Comp. Physiol. **284(1)**: R1-12.

Folli, F., Saad, M. J., Velloso, L., Hansen, H., Carandente, O., Feener, E. P. and Kahn, C. R. (1999). Crosstalk between insulin and angiotensin II signalling systems. Exp. Clin. Endocrinol Diabetes. **107(2)**: 133-139.

Folli, F., Saad, M. J., Backer, J. M. and Kahn, C.R. (1993). Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. J. Clin. Invest. **92(4)**: 1787-1794.

Fontes, R. C. A., Almeida, L., Paiva, I., Tavares, P., Cabrita, S., Wulfroth, P., Matos, B. M. L. and Teixeira, F. (1998). Influence of 0.1 or 0.2% cholesterol-enriched diets on the induction of atherosclerosis and aorta reactivity in vitro. J. Cardiovasc. Pharmacol. **3**: 690-699.

Formisano, P., Oriente, F., Fiory, F., Caruso, M., Miele, C., Maitan, M. A., Andreozzi, F., Vigliotta, G., Condorelli, G. and Beguinot, F. (2000). Insulin-activated protein kinase Cbeta bypasses Ras and stimulates mitogen-activated protein kinase activity and cell proliferation in muscle cells. Mol. Cell. Biol. **104(6)**: 733-741.

Franks, S. (1995). Polycestic ovary syndrome. N. Engl. J. Med. **333**: 853-861.

Friedman, J. E., Ishizuka, T., Liu, S., Farrell, C. J., Bedol, D., Koletsky, R. J., Kaung, H. L. and Ernsberger, P. (1997). Reduced insulin receptor signaling in the obese spontaneously hypertensive Koletsky rat. Am. J. Physiol. **273**: E1014-1023.

Friedman, M. I. (1998). Fuel partitioning and food intake .Am. J. Clin. Nutr. **67(3)**: 513S-518S.

Fruchart, J. C., Duriez, P. and Staels, B. (1999). Molecular mechanism of action of the fibrates. . J. Soc. Biol. **193**: 67-75.

Furuhashi, M., Ura, N., Murakami, H., Hyakukoku, M., Yamaguchi, K., Higashiura, K. and Shimamoto, K. (2002). Fenofibrate improves insulin sensitivity in connection with intramuscular lipid content, muscle fatty acid-binding protein, and beta-oxidation in skeletal muscle. J. Endocrinol. **174(2)**: 321-9.

Gallagher, D., Heymsfield, S. B., Heo, M., Jebb, S. A., Murgatroyd, P. R. and Sakamoto, Y. (2000). Healthy percentage body fat ranges: an approach for developing guidelines based on body mass index. Am. J. Clin. Nutr. **72(3)**: 694-701.

Garanty-Bogacka, B ,.Syrenicz, M., Syrenicz, A., Gebala, A. and Walczak, M. (2005). Relation of acute-phase reaction and endothelial activation to insulin resistance and adiposity in obese children and adolescents. Neuro. Endocrinol. Lett. **26(5)**: 473-479.

Gauthier, M. S., Favier, R. and Lavoie, J. M. (2006). Time course of the development of non-alcoholic hepatic steatosis in response to high-fat diet-induced obesity in rats. Br. J Nutr. **95(2)**: 273-281.

Gavrilova, O. (2000). Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. J. Clin. Invest. **105**: 271-278.

Geliebter, A., Maher, M. M., Gerace, L., Gutin, B., Heymsfield, S. B. and Hashim, S. A. (1997). Effects of strength or aerobic training on body composition, resting metabolic rate, and peak oxygen consumption in obese dieting subjects. Am. J. Clin. Nutr. **66(3)**: 557-563.

George, D. K., Goldwurm, S., MacDonald, G. A., Cowley, L. L., Walker, N. I., Ward, P. J., Jazwinska, E. C. and Powell, L. W. (1998). Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology. **114(2)**: 311-318.

Gerber, R. T., Anwar, M. A. and Poston, L. (1998). Enhanced acetylcholine induced relaxation in small mesenteric arteries from pregnant rats: an important role for endothelium-derived hyperpolarizing factor (EDHF). Br. J. Pharmacol. **125**: 455-460

German, J. B. and Dillard, C.J. (2004). Saturated fats: what dietary intake? Am. J. Clin. Nutr. **80**: 550-559.

Ginsberg, H. N. (2000). Insulin resistance and cardiovascular disease. J. Clin. Invest. **106(4)**: 453-458.

Giorgino, F., Laviola, L. and Eriksson, J. W. (2005). Regional differences of insulin action in adipose tissue:insights from in vivo and in vitro studies. Acta. Physiol. Scand. **183**: 13-30.

Giovannucci, E., Rimm, E. B ,.Chute, C. G., Kawachi, I., Colditz, G. A., Stampfer, M. J. and Willett, W. C. (1994). Obesity and benign prostatic hyperplasia. Am. J. Epidemiol. **140(11)**: 989-1002.

Glueck, C. J., Lang, J. E., Tracy, T., Sieve-Smith, L. and Wang, P. (1999). Contribution of fasting hyperinsulinemia to prediction of atherosclerotic cardiovascular disease status in 293 hyperlipidemic patients. Metabolism **48**: 1437-1444.

Goetze, S., Bungenstock, A. and Czupalla, C. (2002). Leptin induces endothelial cell migration through Akt, which is inhibited by PPAR-γ ligands. Hypertension **40**: 748-754

Golay, A., Chen, N., Chen, Y. D., Hollenbeck, C. and Reaven, G. M. (1990). Effect of central obesity on regulation of carbohydrate metabolism in obese patients with varying degrees of glucose tolerance. J. Clin. Endocrinol. Metab. **71(5)**: 1299-304.

Gonzalez, F. J., Peters, J. M. and Cattley, R. C. (1998). Mechanism of action of the nongenotoxic peroxisome proliferators: Role of the peroxisome proliferator-activated receptor alpha. Journal of the National Cancer Institute **90(22)**: 1702-1709.

Goodyear, L. J., Giorgino, F., S, herman, L. A., Carey, J., Smith, R. J. and Dohm, G. L. (1995). Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. J. Clin. Invest. **95(5)**: 2195-2204.

Goya, K., Sumitani, S., Xu, X., Kitamura, T., Yamamoto, H., Kurebayashi, S., Saito, H., Kouhara, H., Kasayama, S. and Kawase, I. (2004). Peroxisome proliferator-activated receptor alpha agonists increase nitric oxide synthase expression in vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. **24(4)**: 658-663.

Grabacka, M., Plonka, P.M., Urbanska, K. and Reiss, K. (2006). Peroxisome proliferator-activated receptor alpha activation decreases metastatic potential of melanoma cells in vitro via down-regulation of Akt. Clin. Cancer Res. **12(10)**: 3028-3036.

Green, S. and Wahli, W. (1994). Peroxisome proliferator-activated receptors: finding the orphan a home. Mol. Cell. Endocrinol. **100**: 149-153.

Griendling, K. K. and FitzGerald, G. A. (2003). Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation **108(16)**: 1912-1916.

Griffin, M. E., Marcucci ,M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F. and Shulman, G. I. (1999). Free fatty acid-induced insulin resistance is associated with activation of

protein kinase C theta and alterations in the insulin signaling cascade. Diabetes **48(6)**: 1270-1274.

Grigorescu, F., Flier, J. S. and Kahn, C. R. (1986). Characterization of binding and phosphorylation defects of erythrocyte insulin receptors in the type A syndrome of insulin resistance. Diabetes. **35(2)**: 127-138.

Grunberger, G., Zick, Y. and Gorden, P. (1984). Defect in phosphorylation of insulin receptors in cells from an insulin-resistant patient with normal insulin binding. Science **223(4639)**: 932-934.

Grzelkowska-Kowalczyk, K. and Wieteska-Skrzeczynska, W .(2006) .Exposure to TNF-alpha but not IL-1beta impairs insulin-dependent phosphorylation of protein kinase B and p70S6k in mouse C2C12 myogenic cells. Pol. J. Vet. Sci. **9(1)**: 1-10.

Guerre-Millo, M., Gervois, P., Raspe, E., Madsen, L., Poulain, P., Derudas, B., Herbert, J.M., Winegar, D.A., Willson, T.M., Fruchart, J.C., Berge, R.K. and Staels, B. (2000). Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. . J. Biol. Chem. **275**: 16638-16642.

Gupta , D. and Khandelwal, R. L. (2004). Modulation of insulin effects on phosphorylation of protein kinase B and glycogen synthesis by tumor necrosis factor-alpha in HepG2 cells. Biochim. Biophys. Acta. **1671**: 51-58.

Gutin, B., Barbeau, P., Owens, S., Lemmon ,C.R., Bauman, M., Allison, J., Kang, H.S. and Litaker, M.S. (2002). Effects of exercise intensity on cardiovascular fitness, total body composition, and visceral adiposity of obese adolescents. . Am. J. Clin. Nutr. **75**: 818-826.

Haak, T., Haak, E., Kusterer, K., Weber, A., Kohleisen, M. and Usadel, K. H. (1998). Fenofibrate improves microcirculation in patients with hyperlipidemia. . Eur. J. Med. Res. **3**: 50-54.

Haffner, S. M. (1999). Epidemiology of insulin resistance and its relation to coronary artery disease. Am. J. Cardiol. **84(1A)**: 11J-14J.

Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K. and Friedman, J. M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science **269(5223)**: 543-546.

Hall, J. E., Hildebrandt, D. A. and Kuo, J. (2001). Obesity hypertension: role of leptin and sympathetic nervous system. Am. J. Hypertens. **14**: 103S-115S.

Hannum, S. M. and Erdman, J. W. (2000). Emerging health benefits from cocoa and chocolate. J. Med. Food. **3**: 73-75.

Harrold, J. A., Williams, G. and Widdowson, P. S. (2000). Early leptin response to a palatable diet predicts dietary obesity in rats: key role of melanocortin-4 receptors in the ventromedial hypothalamic nucleus .J. Neurochem. **74(3)**: 1224-1228.

Harrold, J. A., Widdowson, P. S., Clapham, J. C. and Williams, G. (2000). Individual severity of dietary obesity in unselected Wistar rats: relationship with hyperphagia. Am. J. Physiol. Endocrinol. Metab. **279(2)**: E340-347.

Hasdai, D., Rizza, R. A., Holmes, D. R., Richardson, D. M., Cohen, P. and Lerman, A. (1998). Insulin and insulin-like growth factor-I cause coronary vasorelaxation in vitro. Hypertension **32(2)**: 228-234.

Hegarty, B. D., Furler, S. M., Ye, J., Cooney, G. J. and Kraegen, E. W .(2003) .The role of intramuscular lipid in insulin resistance. Acta. Physiol. Scand. **178(4)**: 373-383.

Heinrich, P. C., Castell, J. V. and Andus, T. (1990). Interleukin-6 and the acute phase response. Biochem. J. **265**: 621-636.

Helmrich, S. P., Ragland, D.R ,.Leung, R. W. and Paffenbarger, R. S. Jr. (1991). Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus. *N. Engl. J Med.* **325**: 147-152.

Hernandez-Rodriguez, J., Seggara, M. and Vilardell, C. (2003). Elevated production of interleukin-6 is associated with a lower incidence of disease-related ischemic events in patients with giant-cell arteritis: angiogenic activity of interleukin-6 as a potential protective mechanism. Circulation **107**: 2428–2434.

Hill, J. O., Hauptman, J., Anderson, J. W., Fujioka, K., O'Neil, P. M., Smith, D. K., Zavoral, J. H. and Aronne, L. J. (1999). Orlistat, a lipase inhibitor, for weight maintenance after conventional dieting: a 1-y study. Am. J. Clin. Nutr. **69(6)**: 1108-1116.

Hodge, A., M., Dowse, G., K., Zimmet, P., Z., and Collins, V., R. (1995). Prevalence and secular trends in obesity in Pacific and Indian Ocean island populations. Obes. Res. **3(2)**: 77s-87s.

Holzinger, C., Weissinger, E. and Zuckermann, A. (1993). Effects of interleukin-1, -2, -4 ,6- ,interferon-g and granulocyte/macrophage colony stimulating factor on human vascular endothelial cells. Immunol. Lett. **35**: 109–117.

Home, P. D. (1997). Insulin therapy. IInternational Textbook of Diabetes Mellitus. Johan Wiley & Sons, New York **2**:899-928.

Hop le, T. and Xuan Ngoc, T. T. (2004). Overweight/obesity situation and the relation to lipid disorders and hypertension in women aged 20 to 59 years old in Ba Dinh district, Hanoi City. . Asia. Pac. J. Clin. Nutr. **13**: S139.

Horlander, J. and Kwo, P. (1997). Atorvastatin for the treatment of NASH. Hepatology **26**: 544A.

Hotamisligil, G. S. (1999). The role of TNFalpha and TNF receptors in obesity and insulin resistance. J. Intern. Med. **245(6)**: 621-625.

Hotamisligil, G. S. (1999). Mechanisms of TNF-alpha-induced insulin resistance. Exp. Clin. Endocrinol. Diabetes. **107(2)**: 119-125.

Hsueh, W. A. and Law, R. E. (1999). Insulin signaling in the arterial wall. Am. J. Cardiol. **84(1A)**: 21J-24J.

Hughes, J. R. (1984). Psychological effects of habitual aerobic exercise: a critical review. *Prev. Med.* **13**: 66-78.

Hulver, M. W., Berggren, J. R., Cortright, R. N., Dudek, R. W., Thompson, R. P., Pories, W. J., MacDonald, K. G., Cline, G. W., Shulman, G. I., Dohm, G. L. and Houmard, J. A. (2003). Skeletal muscle lipid metabolism with obesity. American Journal of Physiology **284**: E741–E747.

Hunter, S. J. and Garvey, W. T. (1998). Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. Am. J. Med. **105**: 331-45.

Ide, T., Tsunoda, M., Mochizuki, T. and Murakami, K. (2004). Enhancement of insulin signaling through inhibition of tissue lipid accumulation by activation

of peroxisome proliferator-activated receptor (PPAR) alpha in obese mice. Med Sci Monit. **10(10)**: BR388-395.

Idzior-Walus, B., Sieradzki, J., Rostworowski, W., Zdzienicka, A., Kawalec, E., Wojcik, J., Zarnecki, A. and Blane, G. Eur J (2000). Effects of comicronised fenofibrate on lipid and insulin sensitivity in patients with polymetabolic syndrome X. Clin. Invest. **30**: 871-878.

Inoue, I., Goto, S. and Matsunaga, T. (2001). The ligands/activators for peroxisome proliferator-activated receptor alpha (PPARalpha) and PPARgamma increase Cu2+,Zn2+-superoxide dismutase and decrease p22phox message expressions in primary endothelial cells. Metabolism **50**: 3-11.

Itani, S.I., Ruderman, N.B., Schmieder, F. and Boden, G. (2002). Lipidinduced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C ,and IkappaBalpha. Diabetes **51**: 2005–2011.

Itani, S. I., Zhou, Q., Pories, W. J., MacDonald, K. G. and Dohm, G. L. (2000). Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. Diabetes **49(8)**: 1353-1358.

Jakicic, J. M., Winters, C., Lang, W. and Wing, R. R. (1999). Effects of intermittent exercise and use of home exercise equipment on adherence, weight loss, and fitness in overweight women: a randomized trial. JAMA. **282(16)**: 1554-1560.

James, P. T., Leach, R., Kalamara, E. and Shayeghi, M. (2001). The worldwide obesity epidemic. *Obes. Res.* **9(5)**: S228-S233.

Janssen, I., Katzmarzyk, P. T. and Ross, R. (2002). Body mass index, waist circumference, and health risk: evidence in support of current National Institutes of Health guidelines. Arch. Intern. Med. **162(18)**: 2074-2079.

Jazet, I. M., Ouwens, D. M., Schaart, G., Pijl, H., Keizer, H., Maassen, J. A. and Meinders, A. E.(2005). Effect of a 2-day very low-energy diet on skeletal muscle insulin sensitivity in obese type 2 diabetic patients on insulin therapy. Metabolism **54(12)**: 1669-1678.

Jen, C. J., Chan, H. P. and Chen, H. I. (2002). Chronic exercise improves endothelial calcium signalling and vasodilatation in hypercholesterolemic rabbit femoral artery. . Arterioscler. Thromb. Vasc. Biol. **22**: 1219-1224.

Ji, H., Outterbridge, L. V. and Friedman, M. I. (2005). Phenotype-based treatment of dietary obesity: differential effects of fenofibrate in obesity-prone and obesity-resistant rats. Metabolism **54**(4): 421-429.

Jiang, Z. Y., Lin, Y. W., Clemont, A., Feener, E. P., Hein, K. D., Igarashi, M., Yamauchi, T., White, M. F. and King, G. L. (1999). Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats. J. Clin. Invest. **104(4)**: 447-457.

Jiao, H. L., Ye, P. and Zhao, B. L. (2003). Protective effects of green tea polyphenols on human HepG2 cells against oxidative damage of fenofibrate. Free Radic. Biol. Med. **35(9)**: 1121-1128.

Jiao, H. L. and Zhao, B. L. (2002). Cytotoxic effect of peroxisome proliferator fenofibrate on human HepG2 hepatoma cell line and relevant mechanisms. Toxicol. Appl. Pharmacol. **185(3)**: 172-179.

Kahn, B. B. and Flier, J. S. (2000). Obesity and insulin resistance. J. Clin. Invest. **106(4)**: 473-481.

Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993). Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. Biochem. Biophys. Res. Commun. **195(2)**: 762-768.

Karam, J. H. (1997). Pancreatic Hormones and Diabetes Mellitus. Basic and Clinical Endocrinology. Appleton & Lange, Stamford CT. U.S.A. 601-2.

Kasuga, M. F., Karlsson, A. and Kahn, C. R. (1982). insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science **215**: 185-186.

Katakam, P. V., Tulbert, C. D., Snipes, J. A., Erdos, B., Miller, A. W. and Busija, D. W. (2005). Impaired insulin-induced vasodilation in small coronary arteries of Zucker obese rats is mediated by reactive oxygen species. Am. J. Physiol. Heart Circ. Physiol. **288(2)**: H854-H860. .

Kennedy, E. T., Bowman, S. A., Spence, J. T., Freedman, M. and King, J. (2001). Popular diets: correlation to health, nutrition, and obesity. J. Am. Diet. Assoc. **101(4)**: 411-420.

Keogh, J. B., Grieger, J. A., Noakes, M. and Clifton, P. M. (2005). Flow-mediated dilatation is impaired by a high-saturated fat diet but not by a high-carbohydrate diet. . Arterioscler. Thromb. Vasc. Biol. **25**: 1274-1279.

Kern, P. A., Saghizadeh, M., Ong, J. M., Bosch, R. J., Deem, R. and Simsolo, R. B. (1995). The expression of tumour necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. . J. Clin. Invest. **95**: 2111–2119.

Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F. and Accili, D. (2000). Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. J. Clin. Invest. **105**: 199-205.

Kido, Y., Nakae, J. and Accili, D. (2001). The insulin receptor and its cellular targets. J. Clin Endocrinol. Metab. **86**: 972-979.

Kim, F., Gallis, B. and Corson, M. A. (2001). TNF-α inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. Am. J. Physiol. Cell Physiol. **280**: C1057-C1065.

Kim, J., Gavrilova, O., Chen, Y., Reitman, M. and Shulman, G.I. (2000). Mechanisms of insulin resistance in A-ZIP/F-1 fatless mice. J. Biol. Chem. **275**: 8456-8460.

Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R. and Kahn, B. B. (1999). Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. J. Clin. Invest. **104(6)**: 733-741.

Kim, Y. B., Peroni, O. D., Franke, T. F. and Kahn, B. B. (2000). Divergent regulation of Akt1 and Akt2 isoforms in insulin target tissues of obese Zucker rats. Diabetes. **49(5)**: 847-856.

Klein, S. (1999). The war against obesity: attacking a new front. Am. J. Clin. Nutr. **69(6)**: 1061-1063.

Kobayashi, S., Inoue, N., Ohashi, Y., Terashima, M., Matsui, K., Mori, T., Fujita, H., Awano, K., Kobayashi, K., Azumi, H., Ejiri, J., Hirata, K., Kawashima, S.,

Hayashi, Y., Yokozaki, H., toh, H. and Yokoyama, M. (2003). Interaction of oxidative stress and inflammatory response in coronary plaque instability: important role of C-reactive protein. Arterioscler. Thromb. Vasc. Biol. **23(8)**: 1398-1404.

Koh, K. K., Han, S. H., Quon, M. J., Yeal Ahn, J. and Shin, E. K. (2005). Beneficial effects of fenofibrate to improve endothelial dysfunction and raise adiponectin levels in patients with primary hypertriglyceridemia. Diabetes Care **28(6)**: 1419-1424.

Koruk, M., Taysi, S., Savas, M. C., Yilmaz, O., Akcay, F. and Karakok, M. (2003). Serum levels of acute phase proteins in patients with nonalcoholic steatohepatitis. Turk. J. Gastroenterol. **14(1)**: 12-17.

Koumentaki, A., Anthony, F., Poston, L. and Wheeler ,T. (2002). Low-protein diet impairs vascular relaxation in virgin and pregnant rats. Clin Sci (Lond) **102(5)**: 553-560.

Kraegen, E. W., Clark, P. W., Jenkins, A. B., Daley, E. A., Chisholm, D. J. and Storlien, L. H. (1991). Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. Diabetes **40(11)**: 1397-403.

Krauss, R. M. (1998). Triglycerides and atherogenic lipoproteins: rationale for lipid mangement. Am. J. Med. **105**: 805-812.

Krauss, R. M., Eckel, R. H. and Howard, B. (2000). AHA Dietary Guidelines, revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* **102**: 2284-2299.

Krauss, R. M., Siri, P.W. (2004). Metabolic abnormalities: triglyceride and low-density lipoprotein. Endocrinol. Metab. Clin. North Am. **33(2)**: 405-415.

Krauss, R. M., Winston, M., Fletcher, B. J. and Grundy, S. M. (1998). Obesity : impact on cardiovascular disease. Circulation **98(14)**: 1472-1476.

Krook, A., Bjornholm, M., Galuska, D., Jiang, X.J., Fahlman, R., Myers, M.G. Jr, Wallberg-Henriksson, H. and Zierath, J.R. (2000). Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes **49(2)**: 284-292.

Kruszynska, Y. T., Worrall, D. S., Ofrecio, J., Frias, J. P., Macaraeg, G. and Olefsky, J. M. (2002). Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation. J. Clin. Endocrinol. Metab. **87(1)**: 226-234.

Kuboki, K. (1998). Mechanism of insulin's effect on endothelial nitric oxide synthase (eNOS) in vivo and in vitro. Diabetes. **47**: A42.(Abstr.).

Kuboki, K., Jiang, Z. Y., Takahara, N., Ha, S. W., Igarashi, M., Yamauchi, T., Feener, E. P., Herbert, T. P., Rhodes, C. J. and King, G. L. (2000). Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo : a specific vascular action of insulin. Circulation **101(6)**: 676-681.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S. and Kadowaki, T. (1999). PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol. Cell. **4**: 597-609.

Kubota, T., Yano, T., Fujisaki, K., Itoh, Y. and Oishi, R. (2005). Fenofibrate induces apoptotic injury in cultured human hepatocytes by inhibiting phosphorylation of Akt. Apoptosis **10(2)**: 349-358.

Kuczmarski, R. J., Carroll, M. D., Flegal, K. M. and Troiano, R. P. (1997). Varying body mass index cutoff points to describe overweight prevalence among U.S. adults: NHANES III (1988 to 1994). Obes. Res. **5(6)**: 542-548.

Kuczmarski, R. J., Flegal, K. M., Campbell, S. M. and Johnson, C. L. (1994). Increasing prevalence of overweight among US adults. The National Health and Nutrition Examination Surveys, 1960 to 1991. JAMA. **272(3)**: 205-211.

Kudin, A. P., Debska-Vielhaber ,G. and Kunz, W. S. (2005). Characterization of superoxide production sites in isolated rat brain and skeletal muscle mitochondria. Biomed. Pharmacother. **59(4)** 163-168.

Kuno, T., Hozumi, M., Morinobu, T., Murata, T., Mingci, Z. and Tamai, H. (1998). Antioxidant vitamin levels in plasma and low- density lipoprotein of obese girls. . Free Radic. Res. **28**: 81-86.

Kunsch, C. and Medford, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. Circ. Res. **85**: 753-766.

Kushner, R.F. (1993). Body weight and mortality. *Nutr. Rev.* **51**: 1-10.

Laight, D. W., Kaw, A. V., Carrier, M.J. and Anggard, E. E. (1998). Pharmacological modulation of endothelial function by insulin in the rat aorta. J. Pharm. Pharmacol. **50(10)**: 1117-1120.

Lakka, H. M., Lakka, T. A., Tuomilehto, J. and Salonen, J. T. (2002). Abdominal obesity is associated with increased risk of acute coronary events in men. Eur. Heart. J. **23**: 706-713.

Lartey, J., Gampel, A., Pawade, J., Mellor, H., and Bernal, A. L. (2006). Expression of RND proteins in human myometrium. Biol. Reprod. **75**:452-61.

Lau, K. S., Grange, R. W., Isotani, E., Sarelius, I. H., Kamm, K. E., Huang, P. L. and Stull, J. T. (2000). nNOS and eNOS modulate cGMP formation and vascular response in contracting fast-twitch skeletal muscle. Physiol. Genomics. **24;2(1)**: 21-27.

Laurin, J., Lindor, K. D., Crippin, J. S., Gossard, A., Gores, G. J., Ludwig, J., Rakela, J. and McGill, D. B. (1996). Ursodeoxycholic acid or clofibrate in the treatment of non-alcohol-induced steatohepatitis: a pilot study. Hepatology. **23(6)**: 1464-1467.

Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P. and Saltiel, A. R. (1995). Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J. Biol. Chem. **270(35)**: 20801-20807.

Lee, F.Y., Li, Y., Zhu, H., Yang, S., Lin, H.Z., Trush, M. and Diehl, A. M. (1999). Tumor necrosis factor increases mitochondrial oxidant production and induces expression of uncoupling protein-2 in the regenerating mice [correction of rat] liver. Hepatology. **29(3)**: 677-687.

Lee, H. J., Choi, S. S., Park, M. K., An, Y. J., Seo, S. Y., Kim, M. C., Hong, S. H., Hwang, T. H., Kang, D. Y., Garber, A. J. and Kim, D. K. (2002). Fenofibrate lowers abdominal and skeletal adiposity and improves insulin sensitivity in OLETF rats. Biochem. Biophys. Res. Commun. **296(2)**: 293-299.

Lee, I. M. and Paffenbarger, R. S. Jr. (2000). Associations of light, moderate, and vigorous intensity physical activity with longevity: the Harvard Alumni Health Study. *Am. J. Epidemiol.* **151**: 293-299.

Lee, R. J., Albanese, C., Stenger, R.J., Watanabe, G., Inghirami, G., Haines, G.K. 3rd., Webster, M., Muller, W.J., Brugge, J.S., Davis, R.J. and Pestell, R. G. (1999). pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells. J. Biol. Chem. **12;274(11)**: 7341-7350.

Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W. and Kroetz, D. L. (1995). Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. **15**: 3012-3022.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M. and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J. Biol.Chem. **270**: 12953-12956.

Leinonen, E., Hurt-Camejo, E., Wiklund, O., Hulten, L.M., Hiukka, A. and Taskinen, M.R. (2003). Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. Atherosclerosis **166(2)**: 387-394.

Lemberger, T., Braissant, O., Juge-Aubry, C., Keller, H., Saladin, R. and Staels, B. (1996). PPAR tissue distribution and interactions with other hormone-signaling pathways. Ann. N. Y. Acad. Sci. **804**: 231-251.

Lembo, G., Vecchione, C. and Fratta, L. (2000). Leptin induces direct vasodilation through distinct endothelial mechanisms. Diabetes **49**: 293-297.

Leon, A. S., Myers, M. J. and Connett, J .(1997) .Leisure time physical activity and the 16-year risks of mortality from coronary heart disease and all-causes in the Multiple Risk Factor Intervention Trial (MRFIT). Int. J. Sports Med. **18(3)**: S208-S215.

Lewis, T. V., Dart, A. M. and Chin-Dusting, J.P. (1999). Endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridemic humans with normal levels of plasma LDL cholesterol. J. Am. Coll. Cardiol. **33**: 805-812.

Li, J. and Billiar, T. R. (1999). Nitric Oxide. IV. Determinants of nitric oxide protection and toxicity in liver. Am. J. Physiol. **276**: G1069-1073.

Li, Z., Yang, S., Lin, H., Huang, J., Watkins, P. A., Moser, A. B., Desimone, C., Song, X. Y. and Diehl, A. M. (2003). Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. Hepatology **37(2)**: 343-350.

Liang, B., McMaster, J. C., Kroeger, E. A., Hatch, G. M., Mymin, D., Dembinski, T., Arthur, G., Shen, G., Man, R.Y. and Choy, P.C. (2000). The effect of fenofibrate treatment on endothelium-dependent relaxation induced by oxidative modified low density lipoprotein from hyperlipidemic patients. Mol. Cell. Biochem. **207**: 123-129.

Liao, J. K., Shin, W. S., Lee, W. Y. and Clark, S. L. (1995). Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. J. Biol. Chem. **270**: 319-324.

Libby, P., Ridker, P. M. and Maseri, A. (2002). Inflammation and atherosclerosis. Circulation **105(9)**: 1135-1143.

Lin, R., Liu, J., Gan, W. and Yang, G. (2004). C-reactive protein-induced expression of CD40-CD40L and the effect of lovastatin and fenofibrate on it in human vascular endothelial cells. Biol. Pharm. Bull. **27**(10): 1537-1543.

Lind, L., Berne, C. and Lithell, H. (1995). Prevalence of insulin resistance in essential hypertension. J. Hypertens. **12**: 1456-1462.

Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell Death Differ. **104**: 487–501.

Loffreda, S., Yang, S.Q., Lin, H.Z., Karp, C.L., Brengman, M. L., Wang, D.J., Klein, A.S., Bulkley, G.B., Bao, C., Noble, P.W., Lane, M.D. and Diehl, A. M. (1998). Leptin regulates proinflammatory immune responses. FASEB J. **12(1)**: 57-65.

Long, S. D. and Pekala, P. H. (1996). Lipid mediators of insulin resistance : ceramide signalling down-regulates GLUT4 gene transcription in 3T3-L1 adipocytes. . Biochemical Journal **319**: 179–184.

Lores Arnaiz, S., Travacio, M., Monserrat, A. J., Cutrin, J. C., Llesuy, S. and Boveris, A. (1997). Chemiluminescence and antioxidant levels during peroxisome proliferation by fenofibrate. Biochim. Biophys. Acta. **1360(3)**: 222-228.

Lovejoy, J. C., de la Bretonne, J. A., Klemperer, M. and Tulley, R. (1996). Abdominal fat distribution and metabolic risk factors: effects of race. . *Metabolism* . **45**: 1119-1124.

Ludwig, J., Viggiano, T. R., McGill, D. B. and Oh, B. J. (1980). Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo. Clin. Proc. **55(7)**: 434-438.

Luque, C. A. and Rey, J. A. (1999). Sibutramine: a serotonin-norepinephrine reuptake-inhibitor for the treatment of obesity. Ann. Pharmacother. **33(9)**: 968-978.

Luyckx, F. H., Desaive, C., Thiry, A., Dewe, W., Scheen, A. J., Gielen, J.E. and Lefebvre, P.J. (1998). Liver abnormalities in severely obese subjects: effect of drastic weight loss after gastroplasty. Int. J. Obes. Relat. Metab. Disord. **22(3)**: 222-226.

Luyckx, F. H., Lefebvre, P. J., Scheen, A. J. (2000). Non-alcoholic steatohepatitis: association with obesity and insulin resistance, and influence of weight loss. Diabetes Metab. **26(2)**: 98-106.

Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y. and Matsubara, K. (1996). cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). Biochem Biophys Res Commun. **221(2)**: 286-289.

Maeda, K., Okubo, K., Shimomura, I., Mizuno, K., Matsuzawa, Y. and Matsubara, K. (1997). Analysis of an expression profile of genes in the human adipose tissue. Gene **190(2)**: 227-235.

Malaisse, W. J. (1997). Insulin biosynthesis and secretion in vitro. International Textbook of Diabetes Mellitus. Johan Wiley & Sons, New York **2**: 315-336.

Malik, J., Melenovsky, V., Wichterle, D., Haas, T., Simek, J., Ceska, R. and Hradec, J. (2001). Both fenofibrate and atrovastatin improve vascular reactivity in combined hyperlipidaemia (fenofibrate versus atrovastatin trial-FAT). Cardiovasc. Res. **52**: 290-298.

Mancini, F. P., Lanni, A. and Sabatino, L. (2001). Fenofibrate prevents and reduces body weight gain and adiposity in diet-fed obese rats. FEBS **491**: 905-908.

Mancini, F. P., Lanni, A., Sabatino, L., Moreno, A., Giannino, F., Contaldo, V. and Gogila, F. (2001). Fenofibrate prevents and reduces body weight gain and adiposity in diet-fed obese rats. FEBS Letters **491**: 154-158.

Mannaerts, G. P. and Van Veldhoven, P. P. (1993). Metabolic pathways in mammalian. peroxisomes. Biochimie. **75**: 147-58.

Mano, T., Masuyama, T., Yamamoto, K., Naito, J., Kondo, H., Nagano, R., Tanouchi, J., Hori, M., Inoue, M. and Kamada, T. (1996). Endothelial dysfunction in the early stage of atherosclerosis precedes appearance of intimal lesions assessable with intravascular ultrasound. Am. Heart J. **131(2)**: 231-238.

Mantha, L., Russell, J.C., Brindley, D.N. and Deshaies, Y. (2002). Developmental changes in adipose and muscle lipoprotein lipase activity in the atherosclerosis-prone JCR:LA-corpulent rat. Int. J. Obes. Relat. Metab. Disord. **26**: 308-317.

Marchesini, G. and Babini, M. (2006). Nonalcoholic fatty liver disease and the metabolic syndrome. Minerva. Cardioangiol. **54(2)**: 229-239.

Marchesini, G., Brizi, M., Morselli-Labate, A.M., Bianchi, G., Bugianesi, E., McCullough, A.J., Forlani, G. and Melchionda, N. (1999). Association of nonalcoholic fatty liver disease with insulin resistance. Am. J. Med. **107(5)**: 450-455.

Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A. J., Natale, S., Forlani, G., Melchionda, N. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. **50(8)**: 1844-1850.

Mark, A. L., Correia, M., Morgan, D. A., Shaffer, R. A. and Haynes, W. G. (1999). State-of-the-art-lecture: Obesity-induced hypertension: new concepts from the emerging biology of obesity. Hypertension **33**: 537-541.

Marques-Lopes, I., Forga, L. and Martinez, J. A. (2003). Thermogenesis induced by a high-carbohydrate meal in fasted lean and overweight young men: insulin, body fat, and sympathetic nervous system involvement. . J.A. Nutrition. **19**: 25-29.

Marx, N., Mach, F. and Sauty, A. (2000). Peroxisome proliferator-activated receptor-gamma activators inhibit IFN- gamma-induced expression of the T cell-active CXC chemokines IP-10, Mig, and I-TAC in human endothelial cells. J. Immunol. **164**: 6503-6508.

Marx, N., Sukhova, G. K. and Collins, T. (1999). PPARalpha activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. Circulation. **99**: 3125-3131.

Mather, k., Anderson, T. J. and Verma, S. (2001). Insulin action in the vasculature: physiology and pathophysiology. J. Vasc. Res. **38**: 415-422.

Matsuda, K., Teragawa, H., Fukuda, Y., Nakagawa, K., Higashi, Y. and Chayama, K. (2003). Leptin causes nitric-oxide independent coronary artery vasodilation in humans. Hypertens. Res. **26**: 147-152

Matsuda, M. and Shimomura, I. (2004). [Adipocytokines and metabolic syndrome--molecular mechanism and clinicalimplication]. Nippon. Rinsho. **62(6)**: 1085-1090.

Matsui, N., Ito, R., Nishimura, E., Yoshikawa, M., Kato, M., Kamei, M., Shibata, H., Matsumoto, I., Abe, K. and Hashizume, S. (2005). Ingested cocoa can prevent high-fat diet-induced obesity by regulating the expression of genes for fatty acid metabolism. . Nutrition **21(5)**: 594-601.

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F. and Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. **28(7)**: 412-419.

McCurdy, C. E., Cartee, G. D. (2005). Akt2 is essential for the full effect of calorie restriction on insulin-stimulated glucose uptake in skeletal muscle. Diabetes **54(5)**: 1349-1356.

McCurdy, C. E., Davidson, R. T. and Cartee, G. D. (2003). Brief calorie restriction increases Akt2 phosphorylation in insulin-stimulated rat skeletal muscle. Am. J. Physiol. Endocrinol. Metab. **285(4)**: E693-700.

McGarry, J. D. (2002). Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes **51**: 7–18.

McKay, M. K. and Hester, R. L. (1996). Role of nitric oxide, adenosine, and ATP-sensitive potassium channels in insulin-induced vasodilation. Hypertension. **28(2)**: 202-208.

McNaughton, L., Puttagunta, L ,.Martinez-Cuesta, M. A., Kneteman, N., Mayers, I., Moqbel, R., Hamid, Q. and Radomski, M.W. (2002). Distribution of nitric oxide synthase in normal and cirrhotic human liver. Proc. Natl. Acad. Sci. U S A. **99(26)**: 17161-17166.

Melinek, J., Livingston, E., Cortina, G. and Fishbein, M. C. (2002). Autopsy findings following gastric bypass surgery for morbid obesity. Arch Pathol Lab Med. **126(9)**: 1091-1095.

Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. and Kahn, C. R.(2000). Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol. Cell. **6(1)**: 87-97.

Michell, B. J., Griffiths, J. E. and Mitchelhill, K. I. (1999). The Akt kinase signals directly to endothelial nitric oxide synthase. . Curr. Biol. **9**: 845-848.

Mittelman, S. D., Van Citters, G. W., Kim, S. P., Davis, D. A., Dea, M. K., Hamilton-Wessler, M. and Bergman, R.N. (2000). Longitudinal compensation for fat-induced insulin resistance includes reduced insulin clearance and enhanced beta-cell response. Diabetes. **49(12)**: 2116-2125.

Miyazawa- Hoshimoto, S., Takahashi, K., Bujo, H., Hashimoto, N. and Saito, Y. (2003). Elevated serum vascular endothelial growth factor is associated with

visceral fat accumulationin human obese subjects. Diabetologia **46**: 1483-1488.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D. R., Miles, J. M., Yudkin, J. S., Klein, S. and Coppack, S. W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J. Clin. Endocrinol. Metab. **82(12)**: 4196-4200.

Mokdad, A. H., Serdula, M. K., Dietz, W. H., Bowman, B. A., Marks, J. S. and Koplan, J. P. (1999). The spread of the obesity epidemic in the United States, 1991-1998. JAMA **282(16)**: 1519-1522.

Montagnani ,M., Golovchenko, I., Kim, I., Koh, G. Y., Goalstone, M. L., Mundhekar, A. N., Johnsen, M., Kucik, D.F., quoni, M. J. and Draznin, B. (2002). Inhibition of phosphatidylinsitol 3-kinase enhance mitogenic actions of insulin in endothelial cells. J. Biol. Chem. **277**: 1794-1799.

Montagnani, M. and Quon, M. J. (2000). Insulin action in vascular endothelium: potential mechanisms linking insulin resistance with hypertension. Diabetes Obes. Metab. **2(5)**: 285-292.

Monzillo, L. U., Hamdy, O., Horton, E. S., Ledbury, S ,.Mullooly, C., Jarema, C., Porter, S., Ovalle, K., Moussa, A. and Mantzoros, C.S. (2003). Effect of lifestyle modification on adipokine levels in obese subjects with insulin resistance. Obes. Res. **11(9)**: 1048-1054.

Moor-de Burgos, A., Wartanowics, M. and Zeimlanski, S. (1992). Blood vitamins and lipid levels in overweight and obese women. . Eur. J. Clin. Nutr. **46**: 803-808.

Murakami, H., Murakami, R., Kambe, F., Cao, X., Takahashi, R., Asai, T., Hirai, T., Numaguchi, Y., Okumura, K., Seo, H. and Murohara, T .(2006) .Fenofibrate activates AMPK and increases eNOS phosphorylation in HUVEC. Biochem. Biophys. Res. Commun. **341(4)**: 973-978.

Musaiger, A. (1996). Diet-Related Non-communicable Diseases in the Arab Countries of the Gulf. Food and Agriculture Organisation of the United Nations, Cairo. 99-117.

Must, A., Spadano, J., Coakley, E. H., Field, A. E., Colditz, G. and Dietz, W. H. (1999). The disease burden associated with overweight and obesity. JAMA. **282(16)**: 1523-1529.

Myers, M.G. and Jr. (1992). IRS-1 Activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc.Natl. Acad. Sci. USA. **89**: 10350-10354.

Myers, P. R., Parker, J. L., Tanner, M. A. and Adams, H. R. (1994). Effects of cytokines tumour necrosis factor a and interleukin 1b on endotoxin mediated inhibition of endothelium derived relaxing factor bioactivity and nitric oxide production in vascular endothelium. Shock **1**: 73–78.

NAASO, North American Association for the Study of Obesity. (2 .(000The practical guide: Identification, evaluation, and Treatment of Overweight and Obesity in Adults. U. S Department of Health and Human Services, public health service, NIH publication no. 00-4084.

Naderali, E. K., Brown, M. J., Pickavance, L. C., Wilding, J. P., Doyle, P. J. and Williams, G. (2001a). Dietary obesity in the rat induces endothelial

dysfunction without causing insulin resistance: a possible role for triglycerides. Clin. Sci. **101**: 499-506.

Naderali, E. K., Fatani, S. and Wiliams, G. (2004a). Chronic withdrawal of a high-palatable obesity-inducing diet completely reverses metabolic and vascular abnormalities associated with dietary-obesity in the rats. Atherosclerosis **172**: 63-69.

Naderali, E. K., Fatani, S., and Wiliams, G. (2004b). Fenofibrate lowers adiposity and corrects metabolic abnormalities, but only partially restores endothelial function in dietary obese rats. Atherosclerosis **177**: 307-312.

Naderali, E. K., Pickavance, L. C., Widling, J. P. H., Doyle, P. J. and Williams ,G. (2001). Troglitazone corrects metabolic changes but not vascular dysfunction in dietary-obese rats. European Journal of Pharmacology **416**: 133-139.

Naderali, E. K., Pickavance, L. C., Wilding, J. P. H. and Williams, G. (2001c). . Diet-induced endothelial dysfunction in the rat is independent of the degree of increase in total body weight. . Clin. Sci. **100**: 635-41.

Naderali, E. K. and Williams, G. (2001b). Effects of short-term feeding of a highly palatable diet on vascular reactivity in rats. Euro. J. Clin. Invest. **31**: 1024-28.

Naderali, E. K. and Williams, G. (2003). Prolonged endothelial-dependent and -independent arterial dysfunction induced in the rat by short-term feeding with a high-fat, high-sucrose diet. Atherosclerosis **166**: 253-259.

Nagaev, I. and Smith, U. (2001). Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. Biochem. Biophys. Res. Commun. **285(2)**: 561-564.

Nesto, R. (2004). C-reactive protein, its role in inflammation, Type 2 diabetes and cardiovascular disease, and the effects of insulin-sensitizing treatment with thiazolidinediones. Diabet. Med. **21(8)**: 810-817.

Neuschwander-Tetri, B. A. and Caldwell, S. H. (2003). Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology. **37(5)**: 1202-1219.

Nguyen, N. U., Henriet, M. T., Dumoulin, G., Widmer, A. and Regnard, J. (1994). Increase in calciuria and oxaluria after a single chocolate bar load. Horm. Metab. Res. **26**: 383-386.

NHLBI, National Health Lung and Blood Institute. (1998). Clinical Guidelines on the identification evaluation and treatment of overweight and obesity in adults-the evidence report. Obes. Res. **6(2)**: 51S-290S.

NIH, conference. (1991). Gastrointestinal surgery for severe obesity. Consensus Development Panel. Ann Intern Med. **115**: 956-961.

Nomura, F., Ohnishi, K., Ochiai, T. and Okuda, K. (1987). Obesity-related nonalcoholic fatty liver: CT features and follow-up studies after low-calorie diet. Radiology **162(3)**: 845-847.

Oakes, N. D., Bell, K. S., Furler, S. M., Camilleri, S., Saha, A. K., Ruderman, N. B., Chisholm, D. J. and Kraegen, E. W. (1997). Diet-induced muscle insulin resistance in rats is ameliorated by acute dietary lipid withdrawal or a single bout of exercise: parallel relationship between insulin stimulation of glucose

uptake and suppression of long-chain fatty acyl-CoA. . Diabetes **46**: 2022–2028.

Oakes, N. D., Cooney, G. J., Camilleri, S., Chisholm, D. J. and Kraegen, E. W. (1997). Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. Diabetes. **46(11)**: 1768-1774.

Obata, T. (1996). Insulin signaling and its regulation of system A amino acid uptake in cultured rat vascular smooth muscle cells .Circ. Res. **79**: 1167-1176.

Ong, P. J., Dean, T. S., Hayward, C. S., Della Monica, P. L., Sanders, T. A. B. and Collins, P. (1999). Effect of fat and carbohydrate consumption on endothelial function. Lancet **354**: 2134.

Orshal, J. M. and Khalil, R. A. (2004 .(Interleukin-6 impairs endothelium-dependent NO-cGMP-mediated relaxation and enhances contraction in systemic vessels of pregnant rats. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **286**: R1013–R1023.

Ota, T., Takamura, T., Hirai, N. and Kobayashi ,K-I. (2002). Preobesity in World Health Organization classification involves the metabolic syndrome in Japanese. . Diab. Care. **25**: 1252-1253.

Paez-Espinosa, E. V., Rocha, E. M., Velloso, L. A., Boschero, A. C. and Saad, M. J. (1999). Insulin-induced tyrosine phosphorylation of Shc in liver, muscle and adipose tissue of insulin resistant rats. Mol. Cell. Endocrinol. **156(1-2)**: 121-129.

Paez-Espinosa, V., Rocha, E. M., Velloso, L. A. and Saad, M. J. (2001). Regulation of insulin-stimulated tyrosine phosphorylation of Shc and Shc/Grb2 association in liver, muscle, and adipose tissue of epinephrine- and streptozotocin-treated rats. Endocrine. **14(3)**: 295-302.

pagliassotti, M. J. and Horton, T. J. (1994). Hormonal and neural regulation of hepatic glucose uptake. In the role of the liver in maintaining glucose homeostasis. Austin, TX: R. G. Landes: 45-70.

Pannacciulli, N., Cantatore, F. P., Minenna, A., Bellacicco, M., Giorgino, R. and De, Pergola, G. (2001). C-reactive protein is independently associated with total body fat, central fat, and insulin resistance in adult women. Int. J. Obesity. **25**: 1416-1420.

Park, J, S., Hong, G, R., Baek, S. W., Shin, D, G., Kim, Y. J. and Shim, B, S. (2002). expression and regulation of endothelial nitric oxide synthase by vascular endothelial growth factor in ECV 304 cells. J. Korean Med. Sci. **17**: 161-167.

Park, S. H., Kim, B. I., Yun, J. W., Kim, J. W., Park, D. I., Cho, Y. K., Sung, I. K., Park, C. Y., Sohn, C. I., Jeon, W. K., Kim, H., Rhee, E. J., Lee, W. Y. and Kim, S. W. (2004). Insulin resistance and C-reactive protein as independent risk factors for non-alcoholic fatty liver disease in non-obese Asian men. J. Gastroenterol. Hepatol. **19(6)**: 694-698.

Parker, L., Lamont, D. W., Unwin, N., Pearce, M. S., Bennett, S. M., Dickinson, H. O., White, M., Mathers, J. C., Alberti, K. G. and Craft, A. W. (2003). A lifecourse study of risk for hyperinsulin-aemia, dyslipidaemia and obesity (the central metabolic syndrome) at age 49-51 years. . Diabet. Med. **20**: 406-415.

Pelkman, C. L., Fishell, V. K., Maddox ,D. H., Pearson, T. A., Mauger, D. T. and Kris-Etherton, P. M. (2004). Effects of moderate-fat (from monounsaturated fat) and low-fat weight-loss diets on the serum lipid profile in overweight and obese men and women. . Am. J. Clin. Nutr. **79**: 204-212.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. Science **269(5223)**: 540-543.

Peng, H. B., Spiecker, M. and Liao, J. K. (1998). Inducible nitric oxide: an autoregulatory feedback inhibitor of vascular inflammation. J. Immunol. **161(4)**: 1970-1976.

Perseghin, G., Petersen, K. and Shulman, G. I. (2003). Cellular mechanism of insulin resistance: potential link with inflammation. Int. J. Obes .Relat. Metab. Disord. **27(3)**: S6-11.

Perticone, F., Cervolo, R., Mafald, C., Ventura, G., Iacopino, S., Sinopoli, F. and Mattioli, P.L. (2001). Obesity and body fat distribution induce endothelial dysfunction by oxidative stress. Diabetes **50**: 159-1.65

Pessayre, D., Berson, A., Fromenty ,B. and Mansouri, A. (2001). Mitochondria in steatohepatitis. Semin. Liver Dis. **21(1)**: 57-69.

Pessin, J. E. and Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. J. Clin. Invest. **106(2)**: 165-169.

Pi-Sunyer, F. X. (2002). The obesity epidemic: pathophysiology and consequences of obesity. Obes. Res. **10(2)**: 97S-104S.

Piatti, P. M., Monti, L. D., Galli, L., Fragasso, G., Valsecchi, G., Conti, M., Gernone, F. and Pontiroli, A. E. (2000). Relationship between endothelin-1 concentration and metabolic alterations typical of the insulin resistance syndrome. Metabolism **49(6)**: 748-752.

Pickavance, L.C., Tadayyon, M., Widdowson, P.S., Buckingham, R.E. and Wilding, J.P. (1999). Therapeutic index for rosiglitazone in dietary obese rats: separation of efficacy and haemodilution. Br. J. Pharmacol. **128(7)**: 1570-1576.

Playford, D. A., Watts, G. F., Best, J. D. and Burke, V. (2002). Effects of fenofibrate on brachial artery flow-mediated dilatation in type 2 diabetes mellitus. Am. J. Cardiol. **90**: 1254-1257.

Plotnick, G. D., Correti, M. C. and Vogel, R. A. (1997). Effect of antioxidant vitamins on the transient impairment of endothelium-dependent brachial artery vasoactivity following a single high-fat mean. JAMA **278**: 1682-1686.

Pories, W. J., Swanson ,M. S., MacDonald, K. G., Long, S. B., Morris, P. G., Brown, B. M., Barakat, H. A., deRamon, R. A., Israel, G. and Dolezal, J. M. (1995). Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. Ann .Surg. **222(3)**: 339-350.

Prentice, A. M., Black, A. E., Coward, W. A. and Cole, T. J. (1996). Energy expenditure in overweight and obese adults in affluent societies: an analysis of 319 doubly-labelled water measurements. Eur. J. Clin. Nutr. **50(2)**: 93-97.

Prescott-Clarke, P. and Primatesta, P. (1998). Health Survey for England 1996. HMSO , London.

Previs, S. F., Withers, D. J., Ren, J. M., White, M. F. and Shulman, G. I. (2000). Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. J. Biol .Chem. **275(50)**: 38990-38994.

Prochaska, J. O. and Velicer, W. F. (1997). The transtheoretical model of health behavior change. Am. J. Health Promot. **12(1)**: 38-48.

Pyorala, M., Miettinen, H., Laakso, M. and Pyorala, k. (2000). Plasma insulin and all-cause ,cardiovascular, and noncardiovascular mortality: the 22 year follow-up results of Helsinki policemen study. Diabetes Care **23**: 1097-1102.

Quyyumi, A. A. (1998). Endothelial function in health and disease: new insights into the genesis of cardiovascular disease. Am. J. Med. **105(1A)**: 32S-39S.

Racette, S, B., Deusinger, S. S. and Deusinger, R. H. (2003). Obesity: Overview of Prevalence, Etiology, and Treatment. Pysical therapy **83(3)**: 276-288.

Racette, S. B., Schoeller, D. A., Kushner, R. F. and Neil, K. M .(1995) .Exercise enhances dietary compliance during moderate energy restriction in obese women. Am. J. Clin. Nutr. **62(2)**: 345-349.

Racette, S. B., Schoeller, D. A., Kushner, R. F., Neil, K. M. and Herling-laffaldano, K. (1995). Effects of aerobic exercise and dietary carbohydrate on energy expenditure and body composition during weight reduction in obese women. Am. J. Clin. Nutr. **61(3)**: 486-494.

Randle, P. J., Garland, P. B., Hales, C. N. and Newsholme, E. A. (1963). The glucose fatty-acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet **i**: 785–789.

Randle, P. J., Garland, P. B., Newsholme, E. A. and Hales, C. N. (1965). The glucose fatty-acid cycle in obesity and maturity onset diabetes mellitus. Ann. NY Acad .Sci. **131**: 324-333.

Rask-Madsen, C., Dominguez, H., Ihlemann, N., Hermann, T., Kober, L. and Torp-Pedersen, C. (2003). Tumor necrosis factor-alpha inhibits insulin's stimulating effect on glucose uptake and endothelium-dependent vasodilation in humans. Circulation. **108(15)**: 1815-1821.

Rask-Madsen, C. and King, G. L. (2005). Proatherosclerotic mechanisms involving protein kinase C in diabetes and insulin resistance. Arterioscler. Thromb. Vasc. Biol. **25(3)**: 487-496.

Ravussin, E., Lillioja, S., Knowler, W. C ,.Christin, L., Freymond, D., Abbott, W. G., Boyce, V., Howard, B. V. and Bogardus, C. (1988). Reduced rate of energy expenditure as a risk factor for body-weight gain. N. Engl. J. Med. **318(8)**: 467-472.

Reaven, G. M. (1988). Role of insulin resistance in human disease. Diabetes **37** 1595-1607.

Reaven, G. M. (1993). Role of insulin resistance in human disease (syndrome x):an expanded definition. Annu. Rev. Med. **44**: 121-131.

Reaven, G. M., Lithel, H. and Lnadsberg, L. (1996). Mechanisms of disease: Hypertensio and associated metabolic abnormalities. the role of insulin resistance and the sympathoadrenal system. N. Engl. J. Med. **334**: 374-381.

Recasens, M., Ricart, W. and Fernandez-Real, J. M. (2004). [Obesity and inflammation]. Rev. Med. Univ. Navarra. **48(2)**: 49-54.

Reddy, J. K. and Chu, R. (1996). Peroxisome proliferator-induced pleiotropic responses: pursuit of a phenomenon. Ann. N. Y Acad. Sci. **804**: 176-201.

Reddy, J. K. and Rao, M. S. (1992). Peroxisome proliferation and hepatocarcinogenesis. IARC Sci. Publ. 225-235.

Renier, G., Desfaits, A. C. and Serri, O. (2000). Gliclazide decreases low-density lipoprotein oxidation and monocyte adhesion to the endothelium. . Metabolism **49**: 17-22.

Rennie, K. L. and Jebb, S. A. (2005). National Prevalence of Obesity. Prevalence of obesity in Great Britain. Obesity reviews **6**: 11-12.

Rissanen, A. M., Heliovaara, M., Knekt, P., Reunanen, A. and Aromaa, A. (1991). Determinants of weight gain and overweight in adult Finns. Eur. J. Clin. Nutr. **45(9)**: 419-430.

Roach, P., Zick, Y., Formisano, P., Accili, D., Taylor, S. I. and Gorden, P. (1994). A novel human insulin receptor gene mutation uniquely inhibits insulin binding without impairing posttranslational processing. Diabetes. **43(9)**: 1096-1102.

Roberts, C. K., Barnard, R. J., Sindhu, R. K., Jurczak, M., Ehdaie, A. and Vaziri, N. D. (2005). A high-fat, refined-carbohydrate diet induces endothelial dysfunction and oxidant/antioxidant imbalance and depresses NOS protein expression. J. Appl. Physiol. **98(1)**: 203-210.

Roberts, S. B. and Leibel, R.L. (1998). Excess energy intake and low energy expenditure as predictors of obesity. Int. J. Obes. **22**: 385-386.

Rosenbaum, M., Leibel, R. L. and Hirsch, J. (1997). Obesity. N. Engl. J. Med. **337(6)**: 396-407.

Ross, R. (1999). Atherosclerosis--an inflammatory disease. N. Engl. J. Med. **340(2)**: 115-126.

Rossner, S. and Flaten, H. (1997). VLCD versus LCD in long-term treatment of obesity .*Int. J. Obes. Relat. Metab. Disord.* **21**: 22-26.

Rother, K. I., Imai, Y., Caruso, M., Beguinot, F., Formisano, P. and Accili, D. (1998). Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. J. Biol. Chem. **273(28)**: 17491-17497.

Rui ,L., Fisher, T. L., Thomas, J. and White, M. F. (2001). Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J. Biol. Chem. **276(43)**: 40362-40367.

Saad, M. J., Araki, E., Miralpeix , M., Rothenberg, P. L., White, M. F. and Kahn, C. R. (1992). Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. J. Clin. Invest. **90(5)**: 1839-1849.

Saltzman, E. and Roberts, S. B. (1995). The role of energy expenditure in energy regulation: findings from a decade of research. Nutr. Rev. **53(8)**: 209-220.

Sanyal, A. J. (2002). AGA technical review on nonalcoholic fatty liver disease. Gastroenterology. American Gastroenterological Association. **123(5)**: 1705-1725.

Saris, W. H. (2001). Very-low-calorie diets and sustained weight loss. Obes. Res. **9(4)**: 295S-301S.

Sarkar, R., Meinberg, E. G., Stanley, J. C., Gordon, D. and Webb, R. C. (1996). Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. Circ Res. **78(2)**: 225-230.

Sasaki, S., Higashi, Y., Nakagawa , K., Kimura, M., Noma, K., Sasaki, S., Hara, K., Matsuura, H., Goto, C., Oshima, T. and Chayama, K. (2002). A low-caloric

diet improves endothelium-dependent vasodilataion in obese patients with essential hypertension. Am. J Hypertens. **15**: 302-309.

Sato, J., O'Brien, T., Katusic, Z. S., Fu, A., Nygren, J., Singh, R. and Nair, K.S. (2002). Dietary antioxidants preserve endothelium dependent vasorelaxation in overfed rats. Atherosclerosis **161**: 327-333.

Saxena ,N. K., Titus, M. A., Ding, X., Floyd, J., Srinivasan, S., Sitaraman, S. V. and Anania, F.A. (2004). Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk (and Akt phosphorylation. FASEB J. **18(13)**: 1612-1614.

Scherrer, U., Randin, D., Vollenweider, P., Vollenweider, L. and Nicod, P. (1994). Nitric oxide release accounts for insulin's vascular effects in humans. J. Clin. Invest. **94(6)**: 2511-2515.

Schmitz-Peiffer, C. (2002). Protein kinase C and lipid-induced insulin resistance in skeletal muscle. Annals of the New York Academy of Sciences **967**: 146–157.

Schoonjans, K., Martin, G., Staels, B. and Auwerx, J. (1997). Peroxisome proliferatoractivated receptors, orphans with ligands and functions. Curr. Opin. Lipidol. **8**: 159-166.

Schroeder, Jr, C. A., , Chen, Y-.L. and Messina, E. J. (1999). Inhibition of NO synthesis or endothelium removal reveals a vasoconstrictor effect of insulin on isolated arterioles. . Am. J. Physiol. **276**: H815-H820.

Schwartz, M. W., Woods, S.C., Porte D, J. r., Seeley, R. J. and Baskin, D. G . .(2000)Central nervous system control of food intake. . Nature **404(6778)**: 661-671.

Segal, S. S., Brett, S. E. and Sessa, W. C. (1999). Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. Am. J. Phyiol. Heart Circ. Physiol. **277**: H1167-H1177.

Seidell, J., C. (1996). The impact of obesity on health status: some implications for health care costs. Int. J. Obes. Relat. Metab. Disord. **19 (6)**: S13-S16.

Seo, T., Qi, K., Chang, C., Liu, Y., Worgall, T. S., Ramakrishnan, R. and Deckelbaum, R.J. (2005). Saturated fat-rich diet enhances selective uptake of LDL cholesteryl esters in the arterial wall. J. Clin. Invest. **115**: 2214-2222.

Serafini, M., Bugianesi, r., maiani, G., Valtuena, S., De santis, S. and Crozier ,A. (2003). plasma antioxidants from chocolate. Nature **424**: 1013.

Sesti, G., Federici, M., Hribal, M. L., Lauro, D., Sbraccia, P. and Lauro, R. (2001). Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. FASEB J. **15(12)**: 2099-2111.

Shamsuzzaman, A. M., Winnicki, M., Wolk, A., Phillips, B.G., Davison, D.E. and Berger, P. B. (2004). Independent association between plasma leptin and C-reactive protein in healthy humans. Circulation **109**: 2181-2185.

Shek, E. W., Brands, M. W. and Hall, J.E. (1998). Chronic leptin infusion increases arterial pressure. Hypertension **31**: 409-414.

Shepherd, P. R., Nave, B. T. and Siddle, K. (1995). Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of

phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase. Biochem. J. **305 (Pt 1)**: 25-28.

Shepherd, P. R., Withers, D. J. and Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. **333**: 471-490.

Shiri-Sverdlov, R., Wouters, K., van Gorp, P. J., Gijbels, M. J., Noel, B., Buffat, L., Staels, B., Maeda, N., van Bilsen, M. and Hofker, M.H. (2006). Early diet-induced non-alcoholic steatohepatitis in APOE2 knock-in mice and its prevention by fibrates. J. Hepatol. **44(4)**: 732-741.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. J. Clin. Invest. **106(2)**: 171-176.

Shulman, G. I. (1999). Cellular mechanisms of insulin resistance in humans. Am. J. Cardiol. **84(1A)**: 3J-10J.

Shulman, G. I .(2000) .Cellular mechanisms of insulin resistance. Journal of Clinical Investigation. **106**: 171-176

Sierra-Honigmann, M. R., Nath, A. K. and Murakami, C. (1998). Biological action of leptin as an angiogenic factor. Science (Washington, D.C.) **281**: 1683-1686.

Silha, J. V., Krsek, M., Sucharda, P. and Murphy, L. J. (2005). Angiogenic factors are elevated in overweight and obese individuals. Int. J. Obes. (Lond) **29(11)**: 1308-1314.

Simopoulous, A. P. and Van Itallie, T.B. (1984). Body weight, health, and longevity . *Ann Intern Med.* **100**: 285-295.

Singh, R. B., Rastogi, V., Rastogi, S. S., Niaz, M. A. and Beegom, R. (1996). Effects of diet and moderate exercise on central obesity and associated disturbances, myocardial infarction and mortality in patients with and without coronary artery disease. J. Am. Coll. Nutr. **15**: 592-601.

Singhal, A., Farooqi, S. and Cole, T. J. (2002). Influence of leptin on arterial distensibility. . Circulation **106**: 1919-1924.

Skolink, E.Y. (1993). the function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science **260**: 1953-1955.

Skolink, E.Y. (1993). The SH2/SH3 Domain-containing protein GRB2 interacts with tyrosine phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. EMBO J. **12**: 1929-1936.

Skoog ,T., Dichtl, W., Boquist, S., Skoglund-Andersson, C., Karpe, F., Tang, R., Bond, M.G., de Faire, U., Nilsson, J., Eriksson, P. and Hamsten, A. (2002). Plasma tumour necrosis factor-alpha and early carotid atherosclerosis in healthy middle-aged men. Eur .Heart. J. **23(5)**: 376-383 .

Smith, U. (2002). Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intoleranceis insulin resistance initiated in the adipose tissue? Int. J. Obes. Relat. Metab. Disord. **26**: 897-904.

Soderberg, S., Ahren, B., Jansson, J. H., Johnson, O., Hallmans, G., Asplund, K. and Olsson, T. (1999). Leptin is associated with increased risk of myocardial infarction. J. Intern. Med. **246**: 409-418.

Soria, B., Quesada, I., Ropero, A. B., Pertusa, J. A., Martin, F. and Nadal, A. (2004). Novel players in pancreatic islet signaling: from membrane receptors to nuclear channels. Diabetes **53(1)**: S86-91.

Sparks, J. D. and Sparks, C. E. (1994). Insulin regulation of triacylglycerol-rich lipoprotein synthesis and secretion. Biochim. Biophy.s Acta. **1215(1-2)**: 9-32.

Spiegelman, B. M. and Flier, J. S. (1996). Adipogenesis and obesity: rounding out the big picture. Cell **87**: 377-389.

St Jeor, S. T., Howard, B. V. and Prewitt, T. E. (2001). Dietary protein and weight reduction :a statement for healthcare professionals from the Nutrition Committee of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. *Circulation* **104**: 1869-1874.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E. and Fruchart, J. C. (1998). Mechanism of action of fibrates on lipid and lipoprotein metabolism. . Circulation **98**: 2088-2093.

Staels, B., Koenig, W. and Habib, A. (1998). Activation of human aortic smooth muscle cells is inhibited by PPAR[alpha] but not by PPAR[gamma] activators. Nature **393**: 790-793.

Starzl, T. E., Watanabe, K., Porter, K. A. and Putnam, C. W. (1976). Effects of insulin, glucagon, and insuling/glucagon infusions on liver morphology and cell division after complete portacaval shunt in dogs. Lancet. **1(7964)**: 821-825.

Steinberg, H. O. and Baron, A. D. (2002). Vascular function, insulin resistance and fatty acids. Diabetologia **45**: 623-634.

Steinberg, H. O., Brechtel, G., Johnson, A., Fineberg, N. and Baron, A. D. (1994). Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. J. Clin. Invest. **94(3)**: 1172-1179.

Steinberg, H.O., Chacker, H., Leaming, R., Johnson, A., Brechtel, G. and Baron, A.D. (1996). Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance. J. Clin. Invest. **97**: 2601-2610.

Steinberg, H.O., Tarshoby, M., Monestel, R., Hook, G., Cronin, J., Johnson, A., Bayazeed, B. and Baron, A.D. (1997). Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. . J. Clin. Invest. **100**: 1230-1239.

Steiner, G., Hamsten, A., Hoskin, J., Stewart, D., McLaughlin, P., Gladstone, P., Sole, M. and Syvanne, M. (2001). Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes. . Lancet **357**: 905-910.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S. and Lazar, M. A. (2001). The hormone resistin links obesity to diabetes. Nature **409(6818)**: 307-312.

Stunkard, A. J., Foch, T. T. and Hrubec, Z. (1986). A twin study of human obesity. JAMA **256(1)**: 51-54.

Sugerman, H. J. (2000). The epidemic of severe obesity: the value of surgical treatment. Mayo. Clin. Proc. **75(7)**: 669-672.

Sugerman, H. J., Starkey, J. V. and Birkenhauer, R. (1987). A randomized prospective trial of gastric bypass versus vertical banded gastroplasty for morbid obesity and their effects on sweets versus non-sweets eaters. Ann. Surg. **205(6)**: 613-624.

Sun, X.J. (1991). The structure of insulin receptor substarte IRS-1 defines a unique signal transduction protein. Nature **352**: 73-77.

Suter, P.M. (2005). Carbohydrates and dietary fiber. Handb. Exp. Pharmacol. **170**: 231-261.

Suwaidi, J.A., Hamasaki, S., Higano, S.T., Nishimura, R.A., Holmes, D.R. and Lerman, A. (2000). Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation **101**: 948-954.

Talarmin, H., Rescan, C., Cariou, S., Glaise, D., Zanninelli, G., Bilodeau, M., Loyer, P., Guguen-Guillouzo, C. and Baffet, G. (1999). The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of G(1) phase progression in proliferating hepatocytes. Mol. Cell. Biol. **19(9)**: 6003-6011.

Tamaroglio, T, A., and Lo, C, S. (1994). Regulation of fibronectin by insulin-like growth factor-1 in cultured rat thoracic smooth muscle cells and glomerular mesangial cells. Exp. Cell Res. **21**: 338-346.

Taylor, C. B., Sallis, J. F. and Needle, R. (1985). The relationship between physical activity and exercise and mental health. *Public Health Rep.* **100**: 195-202.

Taylor, S. I. and Arioglu, E. (1998). Syndromes associated with insulin resistance and acanthosis nigricans. J. Basic Clin. Physiol. Pharmacol. **9(2-4)**: 419-439.

Taylor, S. I., Cama, A., Accili, D., Barbetti, F., Quon, M. J., de la Luz Sierra, M., Suzuki, Y., Koller, E., Levy-Toledano, R. and Wertheimer, E. (1992). Mutations in the insulin receptor gene. Endocr. Rev. **13(3)**: 566-595.

Tazi, K. A., Quioc, J. J., Saada, V., Bezeaud, A., Lebrec, D. and Moreau, R. (2006). Upregulation of TNF-alpha production signaling pathways in monocytes from patients with advanced cirrhosis: possible role of Akt and IRAK-M. J. Hepatol. **45(2)**: 280-289.

Tchernof, A., Nolan, A., Sites, C. K., Ades, P. A. and Poehlman, E. T. (2002). Weight loss reduces C-reactive protein levels in obese postmenopausal women. Circulation **105**: 564-569.

Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., Inukai, K., Asano, T., Kaburagi, Y., Ueki, K., Nakajima, H., Hanafusa, T., Matsuzawa, Y., Sekihara, H., Yin, Y., Barrett, J.C., Oda, H., Ishikawa, T., Akanuma, Y., Komuro, I., Suzuki, M., Yamamura, K., Kodama, T., Suzuki, H., Yamamura, K., Kodama, T., Suzuki, H., Koyasu, S., Aizawa, S., Tobe, K., Fukui, Y., Yazaki, Y. and Kadowaki, T. (1999). Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. Nat. Genet. **21(2)**: 230-235.

Tilg, H. and Diehl, A. M. (2000). Cytokines in alcoholic and nonalcoholic steatohepatitis. N. Engl. J Med. **343(20)**: 1467-1476.

Tka, I., Kimball, B. P., Lewis, G., Uffelman, K. and Steiner, G. (1997). The severity of coronary atherosclerosis in type 2 diabetes mellitus is related to the number of circulating triglyceride-rich lipoprotein particles. Arterioscler. Thromb. Vasc. Biol. **17**: 3633-3638.

Toba, H., Gomyo, E., Miki, S., Shimizu, T., Yoshimura, A., Inoue, R., Sawai, N., Tsukamoto, R., Asayama, J., Kobara, M. and Nakata, T. (2006).

Hyperinsulinaemia increases the gene expression of endothelial nitric oxide synthase and the phosphatidylinositol 3-kinase/Akt pathway in rat aorta. Clin. Exp. Pharmacol. Physiol. **33(5-6)**: 440-447.

Tooke, J. E. (1995). Microvascular function in human diabetes. A physiological perspective. Diabetes **44(7)**: 721-726.

Tordjman, K., Bernal-Mizrachi, C. and Zemany, L. (2001). PPAR[alpha] deficiency reduces insulin resistance and atherosclerosis in apoE-null mice. J. Clin. Invest. **107**: 1025-1034.

Tremblay, A., Despres, J. P., Leblanc, C., Craig, C. L., Ferris, B., Stephens, T. and Bouchard, C. (1990). Effect of intensity of physical activity on body fatness and fat distribution. Am. J. Clin. Nutr. **51(2)**: 153-157.

Tschop, M. and Heiman, M. L. (2001). Rodent obesity models: an overview. Exp. Clin. Endocrinol. Diabetes. **109(6)**: 307-319.

Uchihara, M. and Izumi, N. (2006). [High-sensitivity C-reactive protein (hs-CRP): a promising biomarker for the screening of non-alcoholic steatohepatitis (NASH)]. Nippon. Rinsho. **64(6)**: 1133-1138.

Ueki, K., Fruman, D. A., Yballe, C. M., Fasshauer, M., Klein, J., Asano, T., Cantley, L. C. and Kahn, C. R. (2003). Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling. J. Biol. Chem. **278(48)**: 48453-48466.

USDHHS, U S Department of Health and Human Services. (2000). *Healthy People 2010.* Conference ed, 2 volumes. Washington, DC: US Dept of Health and Human Services.

USDHHS, U S Department of Health and Human Services. (2001). The surgeon general's call to action to prevent and decrease overweight and obesity. U S Department of Health and Human Services ,public health service, offices of the surgeon general.

USDHHS, US Dept of Health and Human Services. (1998). *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults: The Evidence Report.* . Washington, DC: US Dept of Health and Human Services *The Evidence Report* ***(98-4083).***

Vague, J. (1996). The degree of masculin differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease.1956. Obes. Res. **4**: 204-212.

Valverde, A. M., Burks, D. J., Fabregat, I., Fisher, T. L., Carretero, J., White, M. F. and Benito, M. (2003). Molecular mechanisms of insulin resistance in IRS-2-deficient hepatocytes. Diabetes. **52(9)**: 2239-2248.

Ventura, J. J., Cogswell, P ,.Flavell, R. A., Baldwin, A. S. J.r. and Davis, R. J. (2004). JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. Genes Dev. **18(23)**: 2905-2915.

Verhamme, P., Quarck, R., Hao, H., Knaapen, M., Dymarkowski, S., Bernar, H., Van, Cleemput, J., Janssens, S., Vermylen, J., Gabbiani, G., Kockx, M. and Holvoet, P. (2002). Dietary cholesterol withdrawal reduces vascular inflammation and induces coronary plaque stabilization in miniature pigs. Cardiovasc. Res. **56**: 135-144.

Verma, S., Li, S. H., Badiwala, M. V., Weisel, R. D., Fedak, P. W., Li, R. K., Dhillon, B .and Mickle, D. A. (2002). Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. Circulation **105(16)**: 1890-1896.

Vincent, H. K., Powers, S. K., Stewart, D. J., Shanely, R. A., Demirel, H. and Naito, H .(1999) .Obesity is associated with increased myocardial oxidative stress. Int. J. Obes. Relat. Metab. Disord. **23**: 67-74.

Virkamaki, A., Ueki, K. and Kahn, C. R. (1999). Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J. Clin. Invest. **103(7)**: 931-943.

Visser, M., Bouter, L. M., McQuillan, G. M., Wener, M. H. and Harris, T. B. (1999). Elevated C-reactive protein levels in overweight and obese adults. J. Am. Med. Assoc. **282**: 2131-2135.

Vogel, R. A., Corretti, M. C. and Plotnick, G. D. (1997). Effects of a single high-fat meal on endothelial function in healthy subjects. Am. J. Cardiol. **79**: 350-354.

Volek, J. S. and Feinman, R. D. (2005). Carbohydrate restriction improves the features of Metabolic Syndrome. Metabolic Syndrome may be defined by the response to carbohydrate restriction. Nutr. Metab. **2**: 31-47.

Wadden, T. A. and Foster, G. D. (2000). Behavioral treatment of obesity. Med. Clin. North Am. **84(2)**: 441-461.

Wajant, H., Pfizenmaier, K. and Scheurich, P. (2003). Tumor necrosis factor signaling. Cell Death Differ. **10**: 45-65.

Walker, A. B., Savage M. W., Dores, J. and Williams, G. (1997). Insulin-induced attenuation of noradrenaline-mediated vasoconstriction in resistance arteries from Wistar rats is nitric oxide dependent. Clin. Sci. **93(3)**: 147-152.

Walker, A. B., Dores, J., Buckingham, R. E., Savage, M. W. and Williams, G. (1997). Impaired insulin-induced attenuation of noradrenaline-mediated vasoconstriction in insulin-resistant obese Zucker rats. Clin. Sci. **93**: 235-241.

Wallace, A. M., McMahon, A. D., Packard, C. J., Kelly, A., Shepherd, J., Gaw, A. and Sattar, N. (2001). Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). Circulation **104**: 3052-3056.

Wambach, G. K. and Liu, D. (1992). Insulin attenuates vasoconstriction by noradrenaline, seratonin and potassium chloride in rat mesenteric arterioles. Clin. Exp. Hypertens. A. **14**: 733-740.

Wang, C. C., Gurevich, I. and Draznin, B. (2003). Insulin affects vascular smooth muscle cell phenotype and migration via distinct signaling pathways. Diabetes **52(10)**: 2562-2569.

Wang, P., Ba, Z. F. and Chaudry, I. H. (1994). Administration of TNF-a in vivo depresses endothelium dependant relaxation. Am. J. Physiol. **266**: H2535–H2541.

Wang, P. R., Guo, Q. and Ippolito, M. (2001). High fat fed hamster, a unique animal model for treatment of diabetic dyslipideamia with peroxisome proliferator activated receptor alpha selective agonists. . Eur. J. Pharmacol. **427**: 285-293.

Wang, Q., Hassager, C. and Ravn, P. (1994). Total and regional body composition changes in early postmenopausal women: age-related or menopause-related? . *Am. J. Clin. Nutr.* **60**: 843-848.

Webb, D. J. (1997). Endothelin: from molecule to man. Br. J. Clin. Pharmacol. **44**: 9-20.

weber, M. A. (1999). Clinical cardiovascular effects of sibutramine. . Cardiovasc. Rev. Rep. **20**: 271-276.

Weintraub, J. M., Willett, W. C., Rosner, B., Colditz, G. A., Seddon, J. M. and Hankinson, S. E. (2002). A prospective study of the relationship between body mass index and cataract extraction among US women and men. Int. J. Obes. Relat. Metab. Disord. **26(12)**: 1588-1595.

White, M. F. (2002). IRS proteins and the common path to diabetes. Am. J. Physiol. Endocrinol. Metab. **283(3)**: E413-422.

Whitehead, J. P., Humphreys, P., Krook, A., Jackson, R., Hayward, A., Lewis, H., Siddle, K. and O'Rahilly, S. (1998). Molecular scanning of the insulin receptor substrate 1 gene in subjects with severe insulin resistance: detection and functional analysis of a naturally occurring mutation in a YMXM motif. Diabetes **47(5)**: 837-839.

Whiteman, E. L., Cho, H. and Birnbaum, M. J. (2002). Role of Akt/protein kinase B in metabolism. Trends. Endocrinol. Metab. **13**: 444-451.

WHO, World Health Organization. (1995). Physical status: The use and interpretation of Anthropometry. WHO Tech. Rep. Ser. no. 854 **Ser. no. 854**.

WHO, World Health Organization. (1997). Obesity epidemic puts millions at risk from related diseases. Press release WHO **46**.

WHO, World Health Organization. (1997). Obesity: Preventing and Managing the Global Epidemic. WHO, Geneva.

WHO, World Health Organization. (1998). Obesity: Preventing and Managing the Glopal Epidemic. WHO Geneva.276-1 :1998

WHO, World Health Organization. (2000). Obesity: Preventing and Managing the global Epidemic Report of a WHO Consultation Technical Report Series. World Health Origanization, Geneva.

WHO, World Health Organization. (2002). Controlling the global obesity epidemic. http://www.who.int/nut/obs htm.

Wilding, J. P., Gilbey, S. G., Jones, P. M., Mannan, M. M., Ghatei, M. A. and Bloom, S. R. (1992a). Dexfenfluramine treatment and hypothalamic neuropeptides in diet-induced obesity in rats. Peptides. **13(3)**: 557-563.

Wilks, R. (1996). The Origins and Consequences of Obesity (eds Chadwick, D. & Cardew,G.). Wiley, Chichester: 37-53.

Williams, J. L., Cartland D., Hussain, A. and Egginton, S. (2006). A differential role for nitric oxide in two forms of physiological angiogenesis in mouse. J. Physiol. **570**: 445-454.

Williamson, D. F. (1993). Descriptive epidemiology of body weight and weight change in US adults. *Ann. Int. Med.* **119**: 646-649.

Williamson, J. R. (1966). Mechanism for the stimulation in vivo of hepatic gluconeogenesis by glucagon. Biochem. J. **101(1)**: 11C-14C.

Wilmink, H. W., Twickler, M. B., Banaga, J.D., Dallinga-Thie, G.M., Etltink, H., Erkelens, D. W., Rabelink, T. J. and Stores, E. S. (2001). Effects of statin versus fibrate on post-prandial endothelial dysfunction: role of remnant-like particles. Cardiovasc. Res. **50**: 577-582.

Wing, R. R. and Hill, J. O. (2001). Successful weight loss maintenance. Annu. Rev. Nutr. **21**: 323-341.

Wing, R. R., Jeffery, R. W. (2001). Food provision as a strategy to promote weight loss. . *Obes. Res.* **9(5)**: S271-S275.

Winkler, G., Lakatos, P., Salamon, F., Nagy, Z., Speer, G., Kovacs, M., Harmos, G., Dworak, O. and Cseh, K. (1999). Elevated serum TNF-alpha level as a link between endothelial dysfunction and insulin resistance in normotensive obese patients. Diabet. Med. **16(3)**: 207-211.

Withers, D. J. and White, M. (2000). Perspective: The insulin signaling system--a common link in the pathogenesis of type 2 diabetes. Endocrinology **141(6)**: 1917-1921.

Wolever, T. M. (1990). The glycemic index. World. Rev. Nutr. Diet. **62**: 120-85.

Wolf, A. M. and Colditz, G. A. (1998). Current estimates of the economic cost of obesity in the United States. *Obes. Res.* **6**: 97-106.

Xu, J., Nakamura, M. T., Cho, H. P. and Clarke, S. D. (1999). Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. J. Biol. Chem. **274(33)**: 23577-23583.

Yamaguchi, K., Higashiura, K., Ura, N., Murakami, H., Hyakukoku, M., Furuhashi, M. and Shimamoto, K. (2003). The effect of tumor necrosis factor-alpha on tissue specificity and selectivity to insulin signaling. Hypertens. Res. **26(5)**: 389-396. .

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y ,.Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P. and Kadowaki, T. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat. Med. **7(8)**: 941-946.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi ,M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N. H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T. (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature **423(6941)**: 762-769.

Yamauchi, T., Tobe, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto-Honda, R., Takahashi, Y., Yoshizawa, F., Aizawa, S., Akanuma, Y., Sonenberg, N., Yazaki, Y. and Kadowaki, T. (1996). Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. Mol. Cell. Biol. **16(6)**: 3074-384.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R. and Templeton, D.J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature. **372(6508)**: 798-800.

Yang, S. Q., H., Li, Y., Lin, H. Z., Gabrieson, K., Trush, M. A. and Diehl, A. M. (2000). Mitochondrial adaptations to obesity-related oxidant stress. Arch. Biochem. Biophys. **378**: 259-268.

Yang, S. Q., Lin, H. Z., Mandal, A. K., Huang, J. and Diehl, A. M. (2001). Disrupted signaling and inhibited regeneration in obese mice with fatty livers: implications for nonalcoholic fatty liver disease pathophysiology. Hepatology. **34(4 Pt 1)**: 694-706.

Yanovski, S. Z. and Yanovski, J. A. (2002). Obesity. N. Engl. J. Med. **346(8)**: 591-602.

Ye , J.M., Doyle, P. J., Iglesias, M. A., Watson, D. G., Cooney, G. J. and Kraegen, E. W. (2001). Peroxisome proliferator-activated receptor (PPAR)-alpha activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: comparison with PPAR-gamma activation. Diabetes. **50(2)**: 411-417.

Yeh, E. T., Anderson, H. V., Pasceri, V. and Willerson, J. T. (2001). C-reactive protein: linking inflammation to cardiovascular complications. Circulation **104(9)**: 974-975.

Yki-Jarvinen, H. (2003). Insulin resistance and endothelial dysfunction. Best Pract. Res. . Clin. Endocrinol. Metab. **17**: 411-430.

Yki-Jarvinen, H. and Utriainen, T. (1998). Insulin-induced vasodilatation: physiology or pharmacology? Diabetologia **41**: 369-379.

Yoshizumi, M., Perrella, M. A., Burnett, Jr, J. C., and Lee, M. E. (1993). Tumour necrosis factor down regulates endothelial nitric oxide synthase m-RNA by shortening its half life. Circ. Res. **73**: 205–209.

Younossi, Z. M., Gramlich, T., Bacon, B. R., Matteoni, C. A., Boparai, N., O'Neill, R .and McCullough, A.J. (1999). Hepatic iron and nonalcoholic fatty liver disease. Hepatology. **30(4)**: 847-850.

Younossi, Z. M., Gramlich, T., Liu, Y. C., Matteoni, C., Petrelli, M., Goldblum, J., Rybicki, L. and McCullough, A. J. (1998). Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. Mod. Pathol. Jun. **11(6)**: 560-565.

Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P. and McMahon, G. (1995). Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem. **270**: 23975-23983.

Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M. and Shoelson, S. E. (2001). Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science. **293(5535)**: 1673-1677.

Yudkin, J. S., Kumari, M., Humphries, S. E. and Mohamed-Ali, V. (2002). Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis **148**: 209-214.

yudkin, S. J., Stehouwer, C. D., Emeis, J. J. and Coppack, S. W. (1999). C-reactive protein in healthy subjects: associations with obesity, insulin resistance and endothelial dysfunction: a potential role for cytokines

originating from adipose tissue? Arterioscler Thromb Vasc. Biol. **19**: 972-978.

Zammit, V. A. (2000). Use of in vivo and in vitro techniques for the study of the effects of insulin on hepatic triacylglycerol secretion in different insulinaemic states. Biochem. Soc. Trans. **28(2)**: 103-109.

Zecchin, H. G., Bezerra, R. M., Carvalheira, J. B., Carvalho-Filho, M. A., Metze, K., Franchini, K. G. and Saad, M. J. (2003). Insulin signalling pathways in aorta and muscle from two animal models of insulin resistance--the obese middle-aged and the spontaneously hypertensive rats. Diabetologia **46(4)**: 479-491.

Zeman, M., Zak, A., Vecka, M. and Tvrzicka, E. (2005). [Insulin resistance, metabolic syndrome and cardiovascular diseases] Cas. Lek. Cesk. **144(1)**: 22-26.

Zeng, G., Nystrom, F, H., Ravichandran, L . V., Cong, L, N., Kirby, M., Mostowski, H. and Quon, M. (2000). Roles for insulin receptor, PI3-kinase and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation **101**: 1539-1545.

Zeng, G. and Quon, M. J. (1996). Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. J. Clin. Invest. **98(4)**: 894-898.

Zhao, S. P., Liu, L., Gao, M., Zhou, Q. C., Li, Y. L. and Xia, B. (2001). Impairment of endothelial function after a high-fat meal in patients with coronary artery disease. . Coron. Artery. Dis. **12**: 561-565.

Zhou, Q., Dolan, P. L. and Dohm, G. L. (1999). Dephosphorylation increases insulin-stimulated receptor kinase activity in skeletal muscle of obese Zucker rats. Molecular and Cellular Biochemistry **194**: 209–216.

Ziccardi, P., Nappo, F., Giugliano, G., Esposito, K., Marfella, R., Cioffi, M., D'Andrea, F., Molinari, A. M. and Giugliano, D. (2002). Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. Circulation **105(7)**: 804-809.

Appendix

Nutrition & Metabolism

BioMed Central

Differential vascular dysfunction in response to diets of differing macronutrient composition: a phenomenonological study

Nutrition & Metabolism 2007, **4**:15 doi:10.1186/1743-7075-4-15

Sameer Fatani (sfatani@liv.ac.uk)
Lucy C Pickavance (lucyp@liv.ac.uk)
Claire J Sadler (claire.sadler@syngenta.com)
Joanne A Harrold (harrold@liv.ac.uk)
Roslyn Cassidy (Roslyn_Cassidy@yahoo.com)
John PH Wilding (J.P.H.Wilding@liv.ac.uk)
Ebrahim K Naderali (e.k.naderali@liv.ac.uk)

ISSN 1743-7075

Article type Brief communication

Submission date 21 September 2006

Acceptance date 14 June 2007

Publication date 14 June 2007

Article URL http://www.nutritionandmetabolism.com/content/4/1/15

Brief Communication

Differential vascular dysfunction in response to diets of differing macronutrient composition: a phenomenonological study

Sameer Fatani[1*], Lucy C Pickavance[2*§], Claire J Sadler[1], Joanne A Harrold[3], Roslyn Cassidy[1], John PH Wilding[1], Ebrahim K Naderali[1]

[1]School of Clinical Sciences, University of Liverpool, Liverpool, UK
[2]Department of Veterinary Preclinical Sciences, University of Liverpool, Liverpool, UK
[3]School of Psychology, University of Liverpool, Liverpool, UK

***These authors contributed equally to this work.**
[§]Corresponding author

Email addresses:

SF:	sfatani@liverpool.ac.uk
LCP :	lucyp@liverpool.ac.uk
CJS :	claire.sadler@syngenta.com
JAH :	harrold@liverpool.ac.uk
RC :	Roslyn_Cassidy@yahoo.com
JPHW :	J.P.H.Wilding@liverpool.ac.uk
EKN :	e.k.naderali@liverpool.ac.uk

Abstract

Background: Vascular dysfunction can develop from consumption of an energy-rich diet, even prior to the onset of obesity. However, the roles played by different dietary components remain uncertain. While attempting to develop models of obesity in a separate study, we observed that two high-energy diets of differing macronutrient compositions affected vascular function differently in overweight rats.

Methods: Male Wistar rats (n=6/group) were fed diets providing varying percentages of energy from fat and carbohydrate (CHO). For 10 weeks, they were fed either chow, as control diet (10% of energy from fat; 63% from CHO), chow supplemented with chocolate biscuit (30% fat; 56% CHO) or a high-fat diet (45% fat; 35% CHO). Blood concentrations of biochemical markers of obesity were measured, and epididymal fat pads weighed as a measure of adiposity. Mesenteric arteries were dissected and their contractile and relaxant properties analysed myographically. Data were tested by analysis of variance (ANOVA).

Results: Weight gain and plasma concentrations of glucose, insulin and leptin were similar in all groups. However, biscuit-fed animals showed increased food intake (+27%; p<0.01) and elevated concentrations of TGs and non- NEFAs (+41% and +17%; both p<0.05). High-fat-fed animals showed an increase only in NEFAs (+38%; p<0.01). Arterial vasoconstriction in response to NA and KCl increased only in biscuit-fed rats (both p<0.01), while vasorelaxation in response to CCh and SNP, but not histamine, was attenuated in both groups (both p<0.01). Furthermore, whereas the effect of the high-fat diet was most pronounced in endothelium-dependent vasorelaxation, the biscuit diet had the greater effect on endothelium-independent vasorelaxation.

Conclusions: Vascular dysfunction resulting from consumption of a high-fat or combined relatively high-fat/high-CHO diet occurs through different physiological processes, which may be attributable to their differing macronutrient compositions. Combining potentially atherogenic macronutrients induces more extensive vascular impairment than that of high-fat alone, and may be attributable to the more marked dyslipidaemia observed with such a diet. Thus, these findings help clarify the role of dietary components in vascular impairment, which has implications for clinical approaches to preventing cardiovascular disease.

Background

The link between obesity and vascular dysfunction is well-established [1-8], but its causes remain uncertain. Of the circulating factors increased in obesity – including leptin, insulin, NEFAs and TGs [5, 6, 9-14] – only the latter two are raised by short-term high-energy feeding (prior to obesity onset), suggesting they may play the more critical role [15-18]. Dissecting out the relative importance of dietary macronutrients in atherogenic dyslipidaemia is complex [19], but it is believed that carbohydrate plays the primary role by stimulating insulin secretion, downstream of which fatty acid metabolism is determined [20, 21]. The mechanisms by which these dietary constituents' effects alter vascular function, particularly in animal models, is less clear, although some attempts have been made by different methods in humans [16, 22]. In view of this, we took the opportunity to investigate these mechanisms during an exploratory study in which we compared a number of high-energy diets for their abilities to induce weight gain in rats.

Methods

Male Wistar rats (192 ± 4 g) were fed diets of differing compositions for 10 weeks (n=6/group). Controls received standard chow (CRM Biosure, Cambridge, UK; 'chow' group). One experimental group received chow supplemented with chocolate biscuit (McVitie's 'HobNobs', Ashby de la Zouch, Leics, UK; 'biscuit' group), and the other received a fat-enriched diet (Research Diets, Inc, New Brunswick, NJ, USA; 'high-fat' group; Table 1). Food intake was measured daily and body weight weekly.

At termination, fasting blood samples were collected and plasma glucose, NEFA and TG concentrations determined by diagnostic kit, and insulin and leptin by RIA. A

single epididymal fat pad was dissected from each animal and weighed as a measure of adiposity. Length-tension characteristics of mesenteric arteries (6/rat) were determined, followed by evaluation of arterial contractility and relaxation, as described previously [18].

Before statistical analysis, vascular reactivity data were quantified as AUC. Experimental groups were compared on all parameters to controls by one-way ANOVA followed by *post hoc* analysis.

Results

Biscuit-fed rats showed a higher daily energy intake and increased TG concentrations. The high-fat diet increased NEFAs by twice the proportion of the biscuit diet. There were non-significant rises in body weight and epididymal fat mass on both experimental diets, but neither affected glucose, insulin or leptin concentrations (Table 2).

The experimental diets did not alter vessel diameter (data not shown; both $p>0.05$ *vs* chow). Both KCl and NA, however, increased vasoconstriction in the biscuit group ($p<0.01$ and $p<0.001$), but had no effect in the high-fat group (both $p>0.05$; Fig. 1).

In response to cumulative addition of CCh (10 nM-100 μM), NA-pre-constricted arteries from biscuit- and high-fat-fed rats displayed a significant rightward shift of concentration-response curves, compared with controls (EC_{50} biscuit: 0.28 ± 0.01; high-fat: 0.52 ± 0.04 μM; both $p<0.001$ *vs* chow: 0.11 ± 0.01 μM; Fig. 2a). Maximal vasorelaxation to 100 μM CCh was reduced by 13% in both biscuit- and high-fat-fed

rats (both $p<0.01$ *vs* chow-fed), but was more attenuated in the latter at lower CCh concentrations (3.16-316 nM; $p<0.01$ *vs* biscuit; Fig. 2a). Histamine-induced vasorelaxation was similar in all groups (Fig. 2b).

The endothelium-independent vasodilator, SNP, also induced concentration-dependent vasorelaxation in arteries from all groups, the curves shifted to the right in the biscuit- and high-fat-fed groups (EC_{50} biscuit: 1.58 ± 0.02; high-fat: 0.24 ± 0.01; both $p\leq0.01$ *vs* chow: 0.13±0.02). However, maximum SNP-induced responses were similar in all groups. Compared with the chow- and high-fat-fed groups, SNP-induced relaxation in biscuit-fed rats was attenuated by more than 2-fold (both $p<0.001$). Attenuation of SNP responses was also significant, though less pronounced, in high-fat-fed animals (*vs* control: $p<0.05$; Fig. 2c).

Discussion

This study confirms previous observations that high-energy diets lead to vascular dysfunction [5, 6, 17, 18, 23, 24]. The high-fat and biscuit diets induced similar degrees of modest weight gain and adiposity despite different macronutrient compositions, suggesting these account for the differences observed in vascular reactivity. The biscuit diet had a detrimental effect on both vasoconstriction, and –relaxation, whereas the high-fat diet affected only the latter. The attenuation of vasorelaxation in response to the cGMP-mediated actions of both CCh and SNP shows that this effect of both diets is mediated through both endothelium and vascular smooth muscle. Consumption of other high-energy diets has shown this dual process [5, 17, 18], although some such diets exert their effects only through one mechanism [23], further highlighting the specificity of effects depending on dietary components.

The failure of either diet to alter cAMP-mediated vasorelaxation (i.e., histamine-induced responses) also appears to be a common feature of high-energy diets [17, 18].

Intriguingly, the degree of abnormality seen in both endothelium- and smooth muscle-mediated vasodilation also differed according to diet. CCh-induced vasorelaxation was more severely attenuated by the high-fat diet, and the SNP response more so by the biscuit diet. These findings suggest that different dietary components affect vasorelaxation by different processes, that a diet high in fat has greater deleterious effects on the arterial endothelial lining, whereas a diet relatively rich in carbohydrate *and* fat largely compromises the vascular smooth muscle. There is substantial supporting evidence that dietary components play a role in vascular function. Humans and animals fed diets high in fat, particularly saturated fat [19, 24-26], show endothelial abnormalities. On the other hand, reports of the protective effects of high-carbohydrate (low-saturated fat) diets and diets high in unsaturated fats are contentious [16, 19, 22, 27, 28]. The biscuit diet, although lower in both total and saturated fat than the high-fat diet, contained a significantly greater amount of MUFAs. Although these are associated with improved cardiovascular risk [27, 29], at high levels they can acutely impair endothelial function [16]. Recent reviews of the literature note that stimulation of insulin release by increased carbohydrates promotes adipogenesis, weight gain and atherogenesis, all associated with the metabolic syndrome [19, 28, 30]. The biscuit diet was higher in overall carbohydrate content than the high-fat diet, and it may be this, in combination with relatively high fat content, that accounts for its more detrimental effects. The relatively lower saturated fat and higher carbohydrate content may also be more palatable and account for the increased daily intake in the biscuit-fed animals, and, hence, epididymal adiposity

(though short of significance) and overall greater hyperlipidaemia. This would have to be more carefully analysed in future, however, by matching intake volume and overall energy content between groups. An adipocentric view would suggest that the increased adiposity *per se* of biscuit-fed animals is critically linked with the worsened vascular defects in this group, with excessive fat mass in this depot resulting in fat cell dysfunction, which in turn contributes to the metabolic disorders that increase the risk of atherosclerosis [31].

High-carbohydrate meals may also stimulate sympathetic nervous system activity *in vivo* [32], and counteract insulin-induced vasodilation. Although fasting insulin levels were similar in all groups, it is possible that differences in post-prandial insulin (which would be expected to be higher in rats fed a carbohydrate-enriched diet and resulting adipogenesis) and then raised plasma TGs, resulting in part from *de novo* lipogenesis, may account for the vascular differences. Indeed, hypertriglyceridaemia is a recognized atherogenic risk factor (e.g., [5, 19, 33]). This can be the case even in the absence of insulin resistance [5] or symptoms of atherosclerosis [34]. Although our animal model did not adequately mimic human obesity in terms of weight gain and some of its metabolic disturbances – probably due in part to a lack of statistical power - it remains predictive of vascular dysfunction in the absence of these and therefore further highlights the insidiousness of the disorder.

Finally, although % energy derived from protein was lower in both biscuit and high-fat diets compared to chow, the total average daily protein intake was similar (67 g and 78 g/day, respectively; biscuit *vs* high-fat: $p>0.05$). Hence, it is unlikely that protein deficiency explains the differences in vascular function. Dietary antioxidant

levels may also play a role (e.g., [35, 36]), but it is not possible to comment further on these as they were not measured.

Conclusion

The limitations of this study, therefore, revolve around the fact that the nutrient contents of the diets were not sufficiently controlled to draw very precise conclusions regarding their comparative effects. We cannot know whether their differential effects on vascular function were direct effects of nutrient content or indirect effects of resulting adiposity and/or circulating lipids. However, this study does show that high-energy diets of varying compositions can induce vascular dysfunction to varying degrees in the rat via mechanisms involving different layers of the vascular wall. The combination of high-fat and high-carbohydrate diets may be particularly damaging, possibly through increased hyperlipidaemia.

Abbreviations

ANOVA: analysis of variance

AUC: area under the curve

CCh: carbamylcholine

CHO: carbohydrate

KCl: potassium chloride

MUFA: monounsaturated fatty acid

NA: noradrenaline

NEFA: non-esterified fatty acid

PUFA: polyunsaturated fatty acid

RIA: radioimmunoassay

TG: triglyceride

SNP: sodium nitroprusside

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SF carried out the myography and participated in tissue collection, statistical analysis and drafting of the manuscript.

LCP calculated the diet composition and participated in data collection, tissue sampling, statistical analysis and drafting of the manuscript.

CJS participated in data collection, tissue sampling and statistical analysis, and carried out metabolic assays.

JAH participated in data collection, tissue sampling and statistical analysis, and carried out metabolic assays.

RC participated in data collection and tissue sampling.

JPHW participated in drafting of the manuscript.

EKNA participated in the conception and design of the vascular study, the myography and in drafting of the manuscript.

All authors read and approved the final manuscript.

Acknowledgements

We thank the staff of the Biomedical Services Unit, University of Liverpool, for their conscientious care of the animals and Dr. Joanne Elliott for her assistance with data collection and tissue sampling. This study was supported in part by a grant from the British Heart Foundation (FS/02/002). EKN is a BHF research fellow.

References

1. Lakka HM, Lakka TA, Tuomilehto J, Salonen JT: **Abdominal obesity is associated with increased risk of acute coronary events in men.** *Eur Heart J* 2002, **23:**706-713.
2. Arcaro G, Zamboni M, Rossi L, Turcato E, Covi G, Armellini F, Bosello O, Lechi A: **Body fat distribution predicts the degree of endothelial dysfunction in uncomplicated obesity.** *Int J Obes* 1999, **23:**936-942.
3. Cowan GS, Defibaugh N, White T, Hiler ML, Somes G: **Aorta and iliac arterial sizes in pre-operative morbidly obese patients: a preliminary report.** *Obes Surg* 1991, **1:**155-159.
4. Dobrian AD, Davies MJ, Prewitt RL, Lauterio TJ: **Development of hypertension in a rat model of diet-induced obesity.** *Hypertension* 2000, **35:**1009-1015.
5. Naderali EK, Brown MJ, Pickavance LC, Wilding JPH, Doyle PJ, Williams G: **Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role of triacylglycerols.** *Clin Sci* 2001, **101:**499-506.
6. Naderali EK, Pickavance LC, Wilding JPH, Williams G: **Diet-induced endothelial dysfunction in the rat is independent of the degree of increase in total body weight.** *Clin Sci* 2001, **100:**635-641.
7. Perticone F, Ceravolo R, Candigliota M, Ventura G, Iacopino S, Sinopoli F, Mattioli PL: **Obesity and body fat distribution induce endothelial dysfunction by oxidative stress: protective effect of vitamin C.** *Diabetes* 2001, **50:**159-165.
8. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD: **Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance.** *J Clin Invest* 1996, **97:**2601-2610.
9. Despres J-P, Lamarche B, Mauriege P, Cantin B, Dagenais GR, Moorjani S, Lupien P-J: **Hyperinsulinemia as an independent risk factor for ischemic heart disease.** *N Engl J Med* 1996, **334:**952-958.
10. Glueck CJ, Lang JE, Tracy T, Sieve-Smith L, Wang P: **Contribution of fasting hyperinsulinemia to prediction of atherosclerotic cardiovascular disease status in 293 hyperlipidemic patients.** *Metabolism* 1999, **48:**1437.
11. Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, Bayazeed B, Baron AD: **Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation.** *J Clin Invest* 1997, **100:**1230-1239.
12. Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T: **Leptin is associated with increased risk of myocardial infarction.** *J Intern Med* 1999, **246:**409-418.
13. Tkac I, Kimball BP, Lewis G, Uffelman K, Steiner G: **The severity of coronary atherosclerosis in type 2 diabetes mellitus is related to the number of circulating triglyceride-rich lipoprotein particles.** *Arterioscler Thromb Vasc Biol* 1997, **17:**3633-3638.
14. Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N: **Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS).** *Circulation* 2001, **104:**3052-3056.

15. Ota T, Takamura T, Hirai N, Kobayashi K-i: **Preobesity in World Health Organization classification involves the Metabolic Syndrome in Japanese.** *Diabetes Care* 2002, **25:**1252-1253.
16. Ong PJL, Dean TS, Hayward CS, Monica PLD, Sanders TAB, Collins P: **Effect of fat and carbohydrate consumption on endothelial function.** *The Lancet*, **354:**2134.
17. Naderali EK, Williams G: **Effects of short-term feeding of a highly palatable diet on vascular reactivity in rats.** *Eur J Clin Invest* 2001, **31:**1024-1028.
18. Naderali EK, Williams G: **Prolonged endothelial-dependent and -independent arterial dysfunction induced in the rat by short-term feeding with a high-fat, high-sucrose diet.** *Atherosclerosis* 2003, **166:**253.
19. German JB, Dillard CJ: **Saturated fats: what dietary intake?** *Am J Clin Nutr* 2004, **80:**550-559.
20. Marsset-Baglieri A, Fromentin G, Tome D, Bensaid A, Makkarios L, Even PC: **Increasing the Protein Content in a Carbohydrate-Free Diet Enhances Fat Loss during 35% but Not 75% Energy Restriction in Rats.** *J Nutr* 2004, **134:**2646-2652.
21. Krauss RM, Blanche PJ, Rawlings RS, Fernstrom HS, Williams PT: **Separate effects of reduced carbohydrate intake and weight loss on atherogenic dyslipidemia.** *Am J Clin Nutr* 2006, **83:**1025-1031.
22. Keogh JB, Grieger JA, Noakes M, Clifton PM: **Flow-mediated dilatation is impaired by a high-saturated fat diet but not by a high-carbohydrate diet.** *Arterioscler Thromb Vasc Biol* 2005, **25:**1274-1279.
23. Naderali EK, Fatani S: **The effects of fenofibrate on metabolic and vascular changes induced by chocolate-supplemented diet in the rat.** *Eur J Pharmacol* 2005, **521:**99.
24. Vogel MDRA, Corretti MDMC, Plotnick MDGD: **Effect of a single high-fat meal on endothelial function in healthy subjects.** *Am J Cardiol* 1997, **79:**350.
25. Seo T, Qi K, Chang C, Liu Y, Worgall TS, Ramakrishnan R, Deckelbaum RJ: **Saturated fat-rich diet enhances selective uptake of LDL cholesteryl esters in the arterial wall.** *J Clin Invest* 2005, **115:**2214-2222.
26. Verhamme P, Quarck R, Hao H, Knaapen M, Dymarkowski S, Bernar H, Van Cleemput J, Janssens S, Vermylen J, Gabbiani G: **Dietary cholesterol withdrawal reduces vascular inflammation and induces coronary plaque stabilization in miniature pigs.** *Cardiovasc Res* 2002, **56:**135.
27. Pelkman CL, Fishell VK, Maddox DH, Pearson TA, Mauger DT, Kris-Etherton PM: **Effects of moderate-fat (from monounsaturated fat) and low-fat weight-loss diets on the serum lipid profile in overweight and obese men and women.** *Am J Clin Nutr* 2004, **79:**204-212.
28. Volek J, Feinman R: **Carbohydrate restriction improves the features of Metabolic Syndrome. Metabolic Syndrome may be defined by the response to carbohydrate restriction.** *Nutr Metab* 2005, **2:**31.
29. Appel LJ, Sacks FM, Carey VJ, Obarzanek E, Swain JF, Miller ER, III, Conlin PR, Erlinger TP, Rosner BA, Laranjo NM, et al: **Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids: results of the OmniHeart Randomized Trial.** *JAMA* 2005, **294:**2455-2464.

30. Suter PM: **Carbohydrates and dietary fiber.** *Handb Exp Pharmacol* 2005, **170**:231-261.
31. Bays H, Dujovne CA: **Adiposopathy is a more rational treatment target for metabolic disease than obesity alone.** *Curr Atheroscler Rep* 2006, **8**:144.
32. Marques-Lopes I, Forga L, Martinez JA: **Thermogenesis induced by a high-carbohydrate meal in fasted lean and overweight young men: insulin, body fat, and sympathetic nervous system involvement.** *Nutrition* 2003, **19**:25.
33. Barbagallo CM, Rizzo M, Noto D, Frasheri A, Pernice V, Rubino A, Pieri D, Pinto V, Cefalu AB, Giordano C: **Accumulation of apoE-enriched triglyceride-rich lipoproteins in patients with coronary artery disease.** *Metabolism* 2006, **55**:662.
34. Corrado E, Muratori I, Tantillo R, Contorno F, Coppola G, Strano A, Novo S: **Relationship between endothelial dysfunction, intima media thickness and cardiovascular risk factors in asymptomatic subjects.** *Int Angiol* 2005, **24**:52-58.
35. Kuno T, Hozumi M, Morinobu T, Murata T, Mingci Z, Tamai H: **Antioxidant vitamin levels in plasma and low density lipoprotein of obese girls.** *Free Radic Res* 1998, **28**:81-86.
36. Sato Ji, O'Brien T, Katusic ZS, Fu A, Nygren J, Singh R, Nair KS: **Dietary antioxidants preserve endothelium dependent vasorelaxation in overfed rats.** *Atherosclerosis* 2002, **161**:327.

Figure legends

Figure 1 - Arterial contractility

Figure 2 – Arterial relaxation

Table 1 Dietary breakdown of macronutrients (% of energy)

Diet	Total CHO	Complex CHO	Simple CHO	Total Fat	Saturated fat	MUFA	PUFA	Total protein
Chow	63.0	57.7	5.3	10.3	1.7	3.0	5.6	28.1
'Biscuit'	55.9	37.2	18.7	30.0	12.9	10.6	5.4	15.0
High-fat	35.0	16.9	16.9	45.0	39.1	0.0	5.5	20.0

Table 2 Body weight, energy intake and metabolic parameters

	Chow	Biscuit	High-fat
Daily energy intake (kJ/d)	361±15	459±14**	390±13
Body weight gain (g)	299±22	347±15	339±22
Epididymal fat mass (g)	4.5±0.6	7.7±0.8	6.6±2.2
Glucose (mM)	9.8±1.0	8.9±1.7	9.3±1.0
Insulin (µU/ml)	22.2±2.6	21.1±1.2	19.4±1.6
Leptin (ng/ml)	5.3±0.9	6.3±0.3	5.9±0.4
Triglycerides (mg/dL)	89.4±4.8	126.5±10.1*	77.7±3.6
NEFAs (mM)	0.29±0.01	0.34±0.01*	0.40±0.02**

*$p<0.05$; **$p<0.01$ *vs* chow

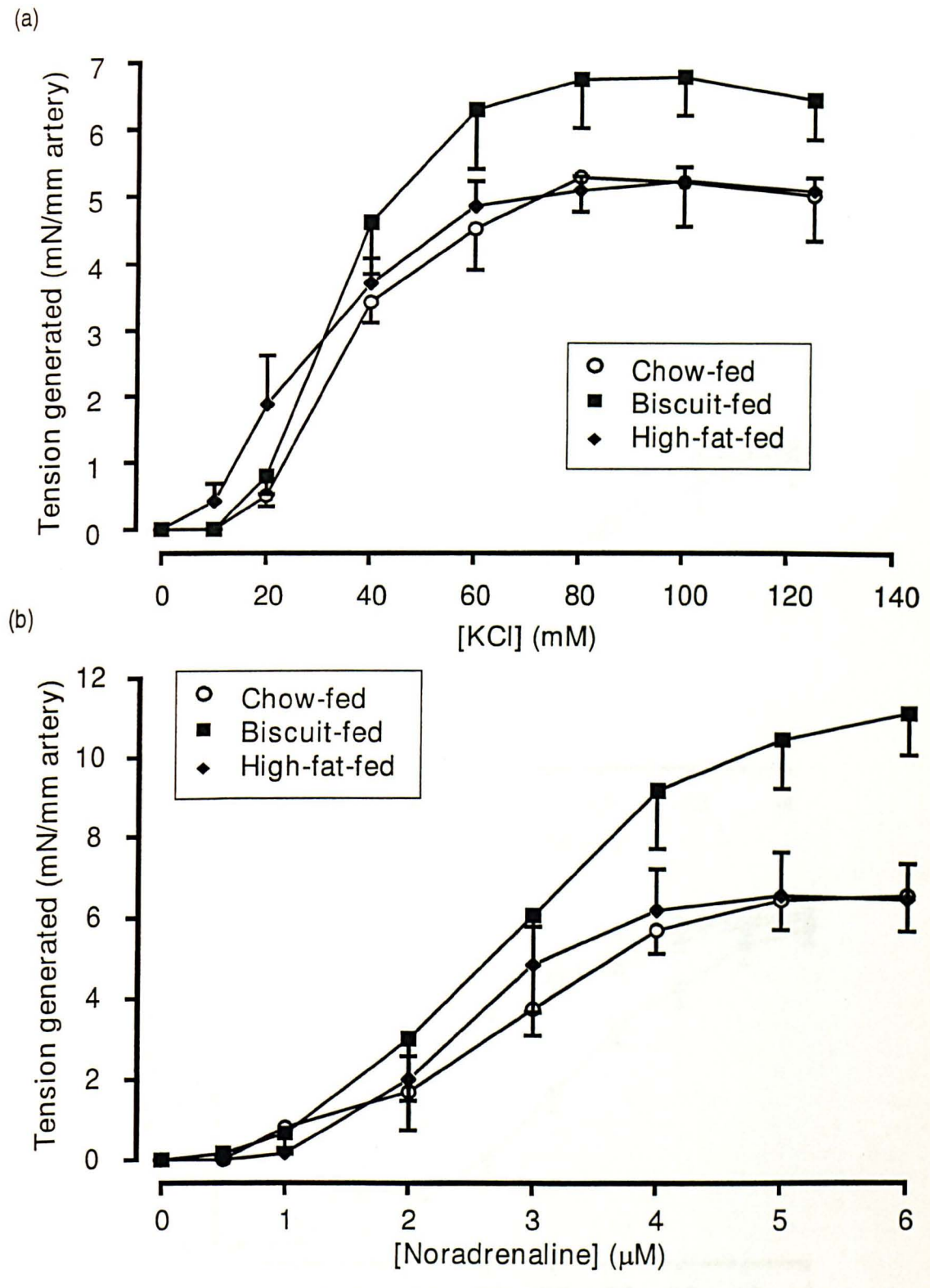

(a)
Tension generated (mN/mm artery)
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
[KCl] (mM)
Chow-fed
Biscuit-fed
High-fat-fed
(b)
Tension generated (mN/mm artery)
0
2
4
6
8
10
12
0
1
2
3
4
5
6
[Noradrenaline] (μM)
Chow-fed
Biscuit-fed
High-fat-fed

Figure 1

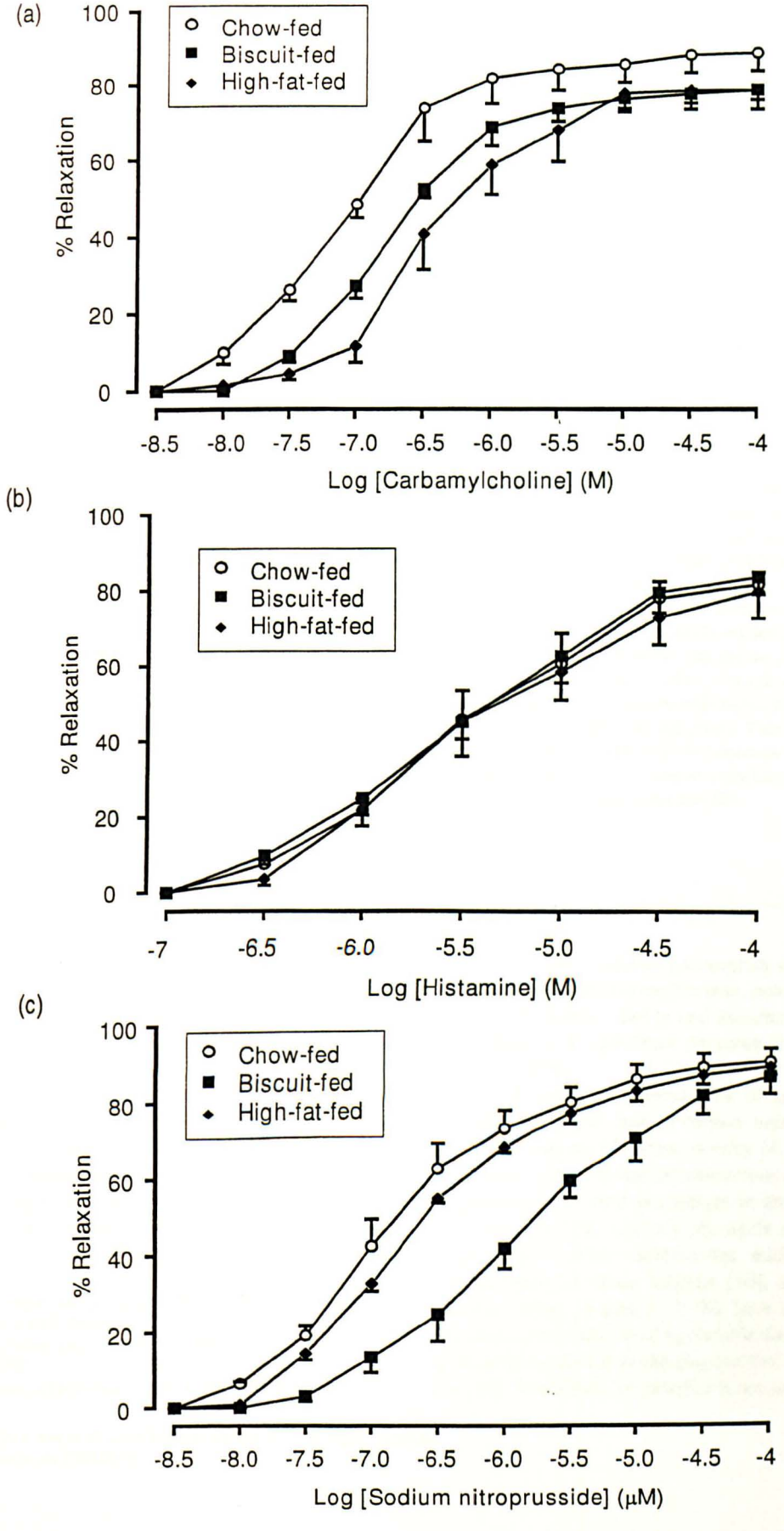

(a)
Chow-fed
Biscuit-fed
High-fat-fed
% Relaxation
100
80
60
40
20
0
-8.5 -8.0 -7.5 -7.0 -6.5 -6.0 -5.5 -5.0 -4.5 -4
Log [Carbamylcholine] (M)
(b)
Chow-fed
Biscuit-fed
High-fat-fed
% Relaxation
100
80
60
40
20
0
-7 -6.5 -6.0 -5.5 -5.0 -4.5 -4
Log [Histamine] (M)
(c)
Chow-fed
Biscuit-fed
High-fat-fed
% Relaxation
100
80
60
40
20
0
-8.5 -8.0 -7.5 -7.0 -6.5 -6.0 -5.5 -5.0 -4.5 -4
Log [Sodium nitroprusside] (μM)

Figure 2

ELSEVIER

Atherosclerosis 172 (2004) 63–69

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Chronic withdrawal of a high-palatable obesity-inducing diet completely reverses metabolic and vascular abnormalities associated with dietary-obesity in the rat☆

Ebrahim K. Naderali*, Sameer Fatani, Gareth Williams

Neuroendocrine and Obesity Biology Unit, Department of Medicine, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

Received 23 July 2003; received in revised form 2 September 2003; accepted 22 September 2003

Abstract

Chronic consumption of a high-palatable diet induces obesity and markedly impairs arterial relaxation. We have recently reported that endothelial function is only partially resorted after acute withdrawal of palatable diet. Therefore, this study was designed to investigate the effects of chronic withdrawal of high-palatable obesity-inducing diet on metabolic and vascular function in rats. Wistar rats were fed either standard laboratory chow throughout (controls) or given a highly-palatable diet (diet-fed) for 15 weeks; or fed the diet for 8 weeks and then returned to chow (diet-to-chow) for further 7 weeks before sacrifice. Diet-fed rats had higher body weight, fat mass, liver and heart weight than both chow-fed and diet-to-chow groups ($P < 0.01$ for all). Compared with chow-fed and diet-to-chow groups, diet-fed rats had significantly raised fasting plasma levels of insulin, leptin and triglycerides levels (each +180%; $P < 0.0001$), but not glucose or non-esterified fatty acids. There were no significant differences between any metabolic parameters between chow-fed and diet-to-chow groups. Mesenteric arteries showed no significant differences between any groups in KCl-induced tension generation, while diet-fed groups had significantly higher noradrenaline-induced vasoconstriction than both chow-fed and diet-to-chow groups. Maximum endothelial-dependent vasorelaxation responses to carbamylcholine (CCh) were significantly (by 23%; $P < 0.001$) attenuated in the diet-fed group. This defect was abolished in the diet-to-chow group. There were no significant differences in endothelium-independent vasorelaxation responses to sodium nitroprusside between the three groups. In conclusion, palatable diet induces obesity and metabolic abnormalities as well as a marked endothelial dysfunction. These abnormalities are completely reversed by chronic withdrawal of the obesity-inducing high-palatable diet.

Keywords: Diet; Obesity; Insulin resistance; Triglycerides; Vascular function

1. Introduction

Throughout the world, consumption of high-energy diets together with a general reduction in the levels of physical activity are causing an alarming health problem, namely obesity. Obesity is a major cardiovascular risk factor and the prevalence of atherosclerotic disease in obese humans has been recognised for more than two decades [1,2]. An association has been described between obesity, arterial hypertension, insulin resistance and dyslipidaemia, which comprise core features of the 'metabolic syndrome' or Syndrome X [3]. Many obese patients accomplish weight loss with diet, exercise and lifestyle modification, achieving substantial reductions in central obesity and associated disturbances corresponding to a significant decrease in cardiac events and mortality [4–6].

Obesity induces abnormalities of arterial function and structure in human and in certain animal models that reflect some aspects of human obesity [4,7–9]. These include alterations of the release of vasoactive substances from endothelial cells as well as changes in arterial wall elasticity. A low-caloric diet improves [9], while elevating the plasma lipid profile further deteriorates endothelium-dependent vasodilatation in obese subjects [10], and in patients with coronary artery disease [11]. We have also recently shown that acute consumption of a palatable diet causes endothelial dysfunction long before development of any significant obesity [12]. Moreover, endothelial function was only partially

☆ This study was supported by a grant from British Heart Foundation (FS/02/002). EKN is a BHF Research Fellow.

* Corresponding author. Tel.: +44-151-7064087; fax: +44-151-706-5797.

E-mail address: naderali@liverpool.ac.uk (E.K. Naderali).

0021-9150/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2003.09.021

corrected with short-term withdrawal of the palatable diet [13]. Thus, in this study we aimed to investigate whether diet- and obesity-induced endothelial abnormalities are fully reversible after chronic withdrawal of the high-energy, palatable diet.

2. Methods

2.1. Animals and experimental protocol

All procedures were approved by the Institutional Committee and accord with current UK legislations. Adult male Wistar rats ($n = 18$) were randomised and assigned to a control group ($n = 6$) and a test group ($n = 12$). All animals had free access to water and were housed individually under controlled environmental conditions (19–22 °C; 30–40% humidity) and a 12 h light–dark cycle (lights on at 07:00 h). Controls were fed a standard laboratory pelleted diet (chow-fed; CRM Biosure, Cambridge, UK), while test group had free access to a highly-palatable diet consisting of (by weight) 33% ground pellet diet, 33% Nestlë condensed milk, 7% sucrose and 27% water. Chow-fed controls remained on their prospective diet for 15 weeks, while after 8 weeks, palatable-diet-fed animals were subdivided into two groups. Palatable diet was removed from one group and the standard chow diet was reintroduced (diet-to-chow) for further 7 weeks, while the remaining subgroup was maintained on the palatable diet (diet-fed) for another 7 weeks. After 15 weeks from the start of the study, rats were killed by CO_2 inhalation after 2 h of fasting, and the total body fat mass was measured using the Bioimpedence method using the TOBEC® equipment (Biotech Instrument Ltd.).

2.2. Biochemical analyses

Trunk blood was removed into cold heparinized tubes and the gonadal and perirenal fat pads, as well as the gastrocnemius muscles and heart were dissected and weighed. The plasma was immediately separated by centrifugation before being frozen for later measurements of blood analytes (glucose, insulin, leptin, non-esterified free fatty acids (NEFA) and triglycerides (TG). Plasma glucose concentration was determined using a glucose oxidase method, and NEFA and TG concentrations were measured using commercial diagnostic kits (Boehringer Mannheim, and Sigma Diagnostics.). Insulin and leptin concentrations were measured by radioimmunoassay (RIA) kits (Pharmacia/Upjohn Diagnostics, and Linco Research, respectively).

2.3. Assessment of vascular contractility

Six third-order mesenteric arteries (>200 μm diameter and 2 mm lengths) were carefully dissected from each animal. Each artery was freed of fat and connective tissue and mounted on two 40 μm diameter stainless-steel wires in an automated myograph (Cambustion Ltd.). The vessels (in duplicate) were incubated in a 5 ml organ bath containing physiological salt solution (PSS; composition [in mM]: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5) gassed with 95% O_2 and 5% CO_2 at 37 °C.

After 30 min equilibration, the length–tension characteristics for each vessel were determined as described previously [14]. The computer also calculated the target tension that each vessel should develop in response to a maximal stimulus. Arteries were then allowed a further 30 min to equilibrate before being depolarised twice with a high-potassium physiological salt solution (KPSS, 125 mM) in which NaCl in normal PSS was replaced by an equimolar concentration of KCl. Cumulative concentration–response curves to either KCl (10–125 mM) or noradrenaline (NA, 0.5–6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded.

2.4. Assessment of vascular relaxation

To investigate defects in endothelial-dependent and -independent vasorelaxation, we measured relaxation induced in NA-preconstricted arteries following exposure to endothelium-dependent (carbamylcholine (CCh)) or endothelium-independent (sodium nitroprusside (SNP)) vasodilators. To eliminate interference of vasoactive prostanoids, 10 mM indomethacine was added to the organ bath prior to contraction with NA (8 μM). When contraction reached a plateau after 2 min, concentration–response curves were carried out to either CCh or SNP (for both, 10 nM to 100 μM).

2.5. Homeostasis model assessment (HOMA)

HOMA, an index of insulin resistance, which employs measures of fasting plasma concentrations of glucose and insulin, was calculated according to the method described previously [15].

2.6. Data interpretation and statistical analyses

Vasoconstriction in response to NA and KCl were expressed as absolute force generated. Relaxation in response to CCh and SNP was calculated as the percentage reduction from the maximal tension generated in response to the supramaximal concentration of NA (8 μM). Data are expressed as mean ± S.D. or where stated as S.E.M. for more clarity. An average response for all the vessels from a given animal was determined before group analysis. Data have normal distribution (Shapiro Wilk W-test). Statistical significance was tested using Student 't-test' or repeated-measures (ANOVA; Bonferroni t-test) or the Mann-Whitney U-test, as appropriate. Results were considered statistically significant at $P < 0.05$. Univariate correlation between maximal CCh-induced vasorelaxation and fasting terminal insulin,

leptin and TG levels were determined by linear regression followed by two-tailed analyses, using ARCUS PRO-STAT (version 3.23).

3. Results

3.1. Metabolic data

Animals giving palatable diet progressively gained more weight than their chow-fed counterparts. A significant difference in total body weight was observed after 5th week of feeding and further increased after 8th week where diet-fed animals had significantly (>10%, $P = 0.0059$) higher total body weight than chow-fed controls (Fig. 1). Following the next 7 weeks, average weekly weight gain of diet-fed animals was 1.8-fold greater than chow-fed controls (18.43 ± 0.50 g versus 10.34 ± 0.23 g control), while average weekly weight gain of diet-to-chow animals was significantly lower ($P < 0.001$, 10.04 ± 0.06 g versus 18.43 ± 0.50 g diet-fed) than their diet-fed counterparts (Fig. 1). However, at the end of the experiment, total body weight of diet-to-chow animals was still significantly ($P = 0.0431$) higher than control chow-fed but was markedly ($P < 0.0062$) lower than the diet-fed animals (Fig. 1).

At the end of the experiment, Bioimpedence measurements showed that diet-fed animals had 70 and 27% more total body fat than chow-fed and diet-to-chow groups, respectively. Diet-fed animals had significantly higher epididymal and perirenal fat pad mass than both chow-fed (by >180%, $P < 0.0001$) and diet-to-chow (by >175%, $P < 0.0002$) groups (Table 1). Diet-fed rats had significantly (for both, $P < 0.0230$) higher heart weights than both chow-fed and diet-to-chow rats (Table 1). There were neither significant differences between fat pad depots and heart weights of diet-to-chow and chow-fed groups, respectively; nor there were any significant differences in gastrocnemius muscle weights between the three groups (Table 1).

While fasting plasma glucose levels were comparable in all there groups (12.42 ± 1.12 mM chow-fed; 14.40 ± 0.362 mM diet-fed; 14.43 ± 0.42 mM diet-to-chow), diet-fed rats had significantly higher (>130%, $P < 0.0093$) fasting

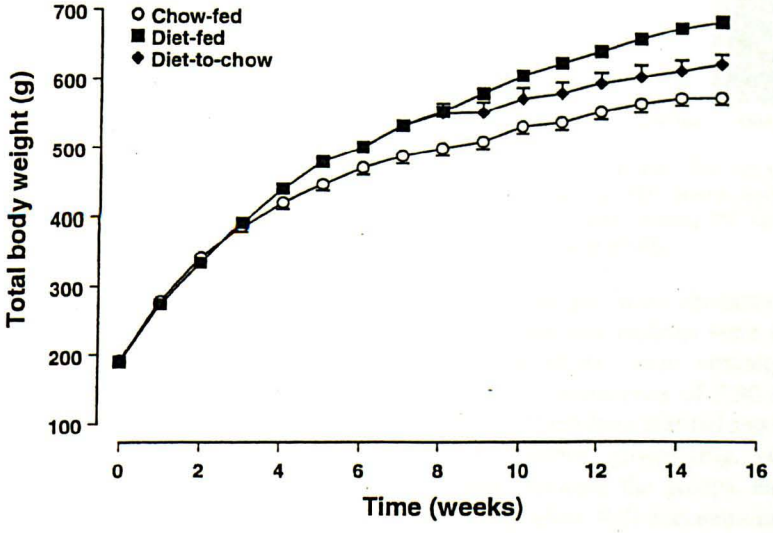


Fig. 1. The effect of a palatable-diet and its removal on total body weight in the rat. Animals were fed either standard chow (chow-fed) or a palatable diet (diet-fed) for 15 weeks, or fed the palatable diet for 8 weeks and then returned to standard chow for further 7 weeks (diet-to-chow). Diet-fed animals were significantly ($P < 0.00001$) heavier than both chow-fed and diet-to-chow groups. Diet-to-chow group was significantly heavier ($P < 0.04$) than chow-fed but lighter ($P < 0.006$) than diet-fed groups. Data are expressed as mean ± S.E.M.

Table 1
Physiological and metabolic characteristics of the three experimental groups at the end of the experiment

	Chow-fed ($n = 6$)	Diet-fed ($n = 6$)	Diet-to-chow-fed ($n = 6$)
Total body weight (g)	570.2 ± 9.7	680.2 ± 8.7[a,b]	619.2 ± 7.7[a]
Total fat pad mass (g)	21.0 ± 1.8	40.5 ± 2.9[a]	24.1 ± 2.4
Other fat mass (g)	73.8 ± 6.9	121.3 ± 11.9[a,b]	102.5 ± 6.6[a]
Gastrocnemius muscle mass (g)	3.2 ± 0.1	3.3 ± 0.1	3.2 ± 0.1
Heart weight (g)	1.7 ± 0.1	2.0 ± 0.1[a,b]	1.7 ± 0.1

Data are mean ± S.D. $P < 0.001$ for all.
[a] Diet-fed and diet-to-chow-fed vs. chow-fed.
[b] Diet-fed vs. diet-to-chow-fed.

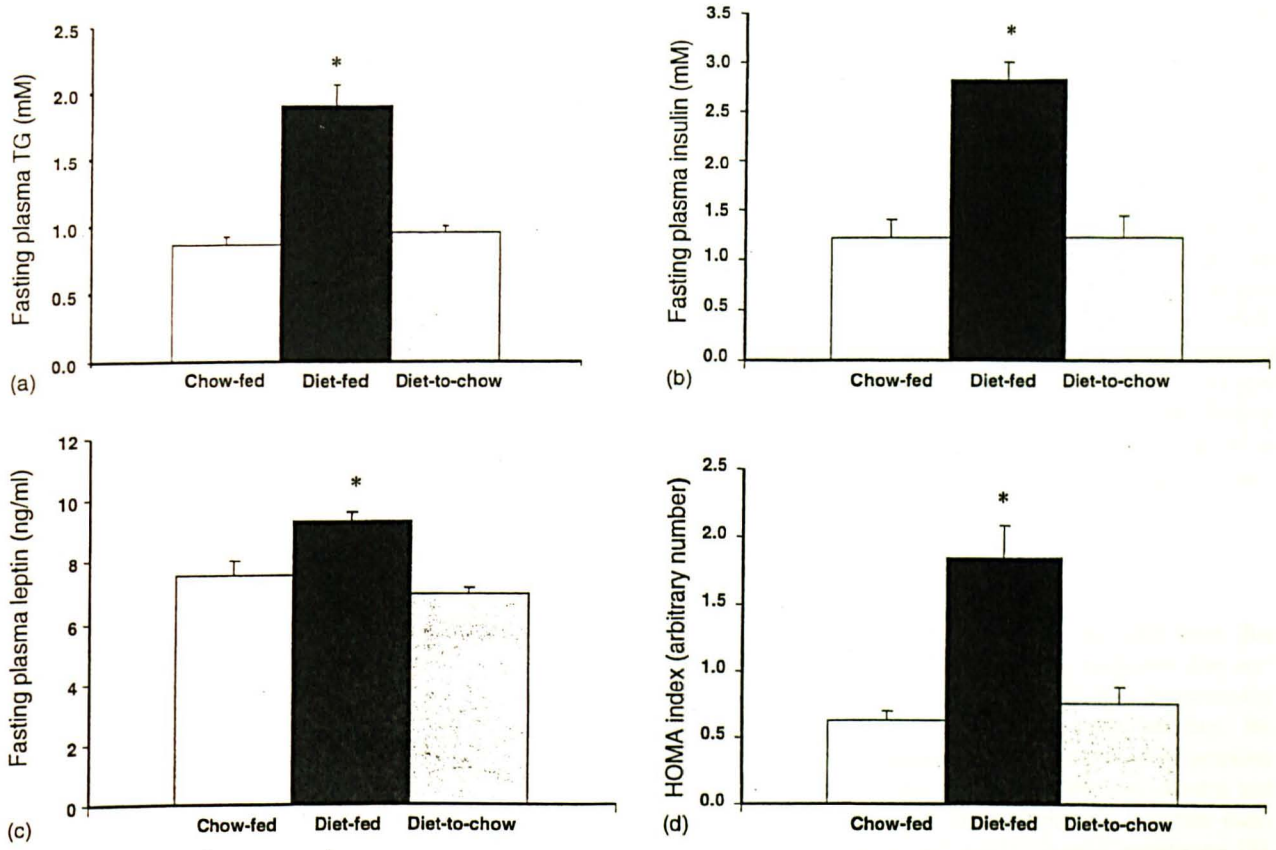


Fig. 2. Effects of highly-palatable diet feeding and its withdrawal on fasting plasma levels of: (a) triglyceride (TG), (b) insulin, (c) leptin, and (d) HOMA index in three experimental animals. Diet-fed rats had significantly (for all, $P < 0.01$) higher fasting (TG), insulin, leptin levels than both chow-fed and diet-to-chow groups. Diet-fed animals were also insulin resistance, indicated by a higher HOMA index. Fasting TG, insulin and leptin levels returned to normal levels by long-term removal of the palatable diet and insulin resistance was corrected subsequently.

terminal levels of plasma insulin than both chow-fed and diet-to-chow group. As a result, diet-fed rats had significantly (for both, $P < 0.001$) higher HOMA index, indicating development of insulin resistance in these animals (Fig. 2). Moreover, diet-fed rats had significantly higher (up to 35%, $P < 0.001$) plasma leptin levels than both chow-fed control and diet-to-chow groups (Fig. 2).

Fasting plasma levels of NEFA were comparable in all there groups (0.45 ± 0.24 mM chow-fed; 0.48 ± 0.41 mM diet-fed and 0.40 ± 0.24 mM diet-to-chow), while diet-fed rats had significantly (greater than two-fold, $P < 0.001$, for both) higher levels of fasting TG than both chow-fed and diet-to-chow groups (Fig. 2).

3.2. Vascular data

There were no significant differences in vessel diameter between the three groups (189 ± 20 μm chow-fed, 200 ± 15 μm diet-fed and 200 ± 10 μm diet-to-chow; $P > 0.5$ for all).

3.3. Agonist-induced vasoconstriction responses

Cumulative concentration response curves (CCRC) to KCl (10–125 mM) and NA (0.5–6 μM) in arteries from all three groups produced characteristic sigmoid relationship. KCl-induced contractions were similar in all three groups such that CCRC were virtually superimposable, giving maximum contractions of 5.90 ± 0.41 mN/mm chow-fed, 6.16 ± 0.31 mN/mm diet-fed and 6.32 ± 0.26 mN/mm artery for diet-to-chow groups (Fig. 3a), with no significant differences between the groups, either overall (by ANOVA) or at any given KCl concentration. However, arteries from the diet-fed group showed greater contractile reactivity to NA administration, reaching significance (>30%, $P < 0.01$ for all) at 3 and 4 μM NA than chow-fed and diet-to-chow groups (Fig. 3b).

3.4. Endothelium-dependent and -independent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM to 100 μM), achieving a maximum of $90 \pm 2\%$ at a CCh concentration of 100 μM, with EC_{50} values of 0.14 ± 0.02 μM. Arteries from diet-fed rats that were similarly exposed to CCh displayed a significant rightward shift of CCRC compared with chow-fed control arteries (EC_{50} diet-fed: 0.75 ± 0.03, $P = 0.0023$ versus chow-fed; Fig. 4a).

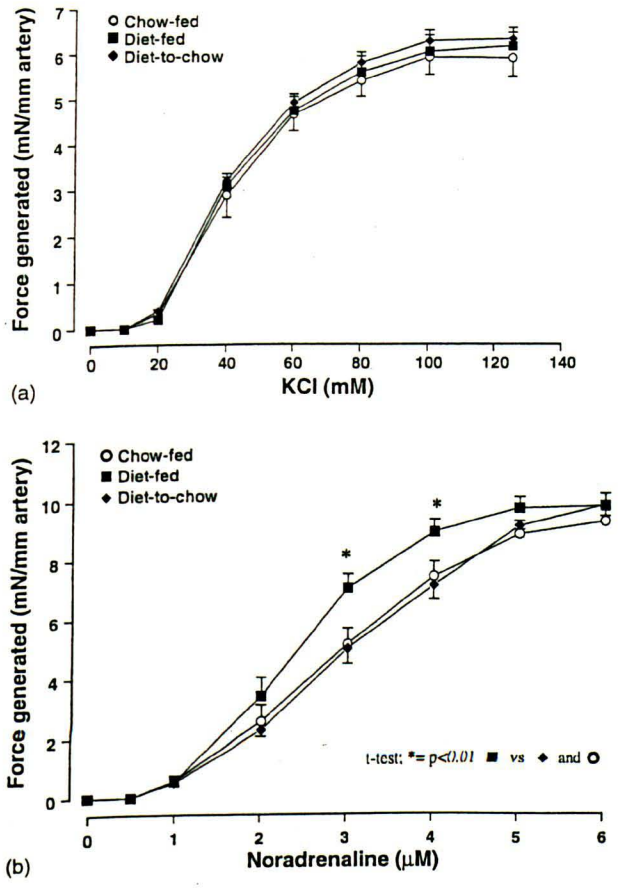


Fig. 3. Concentration–response curves for: (a) KCl (10–125 mM) and (b) noradrenaline (0.5–6 μM) in arteries from chow-fed, diet-fed and diet-to-chow-fed animals. Data represent mean ± S.E.M. for six animals in each group. KCl-induced vasoconstrictions were similar between all three groups. However, 3 and 4 μM NA-induced contractions significantly ((*) $P < 0.001$) were greater than both chow-fed and diet-to-chow-fed groups. Data represents mean and S.E.M. for $n = 6$ in each groups.

Maximal vasorelaxation to 100 μM CCh was reduced by 23% in diet-fed rats ($P = 0.0045$ versus chow-fed). However, CCh-induced vasorelaxation curves in arteries from diet-to-chow group were virtually identical to those in chow-fed group, achieving maximal relaxation of 95 ± 1% at 100 μM CCh, with EC_{50} values of 0.19 ± 0.04 μM.

SNP (10 nM to 100 μM) also induced concentration dependent vasorelaxation of arteries from all three groups. Concentration-relaxation–response curves were similar and superimposable in all three groups (Fig. 4b), with no statistically significant differences between the groups, either overall (by ANOVA) or at any given SNP concentration.

3.5. Metabolic determinants of vascular dysfunction

Of the various blood analytes measured, insulin, leptin and TG showed significant increases in diet-fed animals. Therefore, the relationship between endothelial dysfunction (measured as the percentage reduction of 100 μM CCh-induced vasorelaxation) and terminal plasma levels of insulin, leptin and TG were examined. In each case, univariate correlation analysis showed an inverse correlation between fasting terminal levels of TG ($r^2 = 0.4787$; $P = 0.0013$), insulin ($r^2 = 0.4237$; $P = 0.0023$) and leptin ($r^2 = 0.609$; $P = 0.0001$) and maximal CCh-induced vasorelaxation. We also analysed the relationship between total body weight and total fat pad mass with vascular reactivity. Univariate correlation analysis once again indicated an inverse correlation relationship between total body weight ($r^2 = 0.3751$; $P = 0.0043$) and fat pad mass ($r^2 = 0.3565$; $P = 0.0048$) and maximum carbamylcholine-induced vasorelaxation. Furthermore, analysis of blood analytes showed significant positive correlations between fasting plasma insulin ($r^2 = 0.3043$; $P = 0.0025$) and leptin ($r^2 = 0.4189$; $P = 0.0020$) with that of circulating triglycerides.

4. Discussion

The present study, demonstrates for the first time that chronic removal of an obesity-inducing palatable diet corrects all the metabolic abnormalities, cardiac hypertrophy and vascular dysfunctions associated with obesity. We have previously demonstrated that chronic consumption of a palatable diet induces significant level of obesity and markedly affects metabolic parameters and vascular reactivity in the absence of any visible insulin resistance [8]. Moreover, short-term feeding (2 days) of the same palatable diet significantly alters lipid profile and markedly attenuates endothelial function in the absence of any significant obesity [12]. These defects were only partially corrected by acute removal of the diet [13], indicating that diet per se may have a major role in the development of vascular abnormalities in animal models of obesity.

In this study, diet-fed animals developed marked obesity, which was characterised by more than two-fold increase in total body fat content as determined by Bioimpedence methods and measurements of fat pad mass. Obese animals had significantly higher heart weight and fasting plasma levels of insulin, suggesting cardiac hypertrophy and development of insulin resistance in dietary-obese animals, respectively. These findings are similar to those seen in human obesity [1–4]. In humans, obesity leads to dyslipidaemia and type 2 diabetes and hypertension [16–18], while changes in life style improve obesity-induced abnormalities to a certain degree [4,6,9].

A number of experimental studies have reported severe detrimental outcome of excessive high-energy diet on vascular reactivity. For example, high-fat diet impairs endothelium-dependent vasorelaxation [8,12–14,19,20] while antioxidant therapy improves endothelial function [21,22], suggesting a diet-induced oxidative stress in vasculature. In obesity, plasma antioxidant levels are decreased [21–23], whereas myocardial oxidative stress is increased [24,25]. These changes may, at least in part, explain

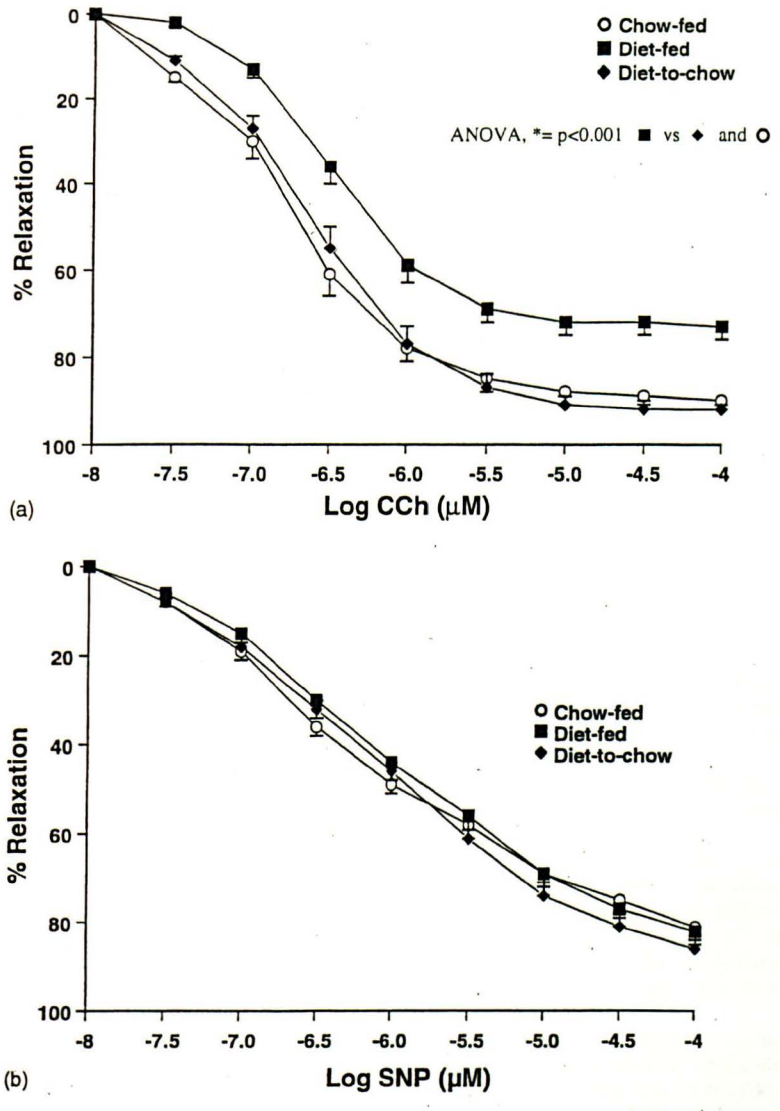


Fig. 4. Concentration-relaxation–response curves (mean ± S.E.M.) of: (a) carbamylcholine (CCh), (b) sodium nitroprusside (SNP) in the three groups. Arteries were contracted with 8 μM noradrenaline. When contractions reached a plateau cumulative concentration responses to CCh or SNP were carried out. CCh-induced vasorelaxation was significantly reduced in diet-fed animals (ANOVA $P < 0.001$) in comparison to chow-fed group. This abnormality was completely abolished by the chronic removal of the palatable diet. SNP-induced vasorelaxations were similar in all three groups.

the increased risk of vascular disease and the abnormal endothelium-dependent vasorelaxation found in obese subjects and animals. In obese patients with hypertension, ACh-induced forearm blood flow (FBF) is significantly reduced compared to healthy individuals. Caloric restriction decreases body weight and the mean blood pressure, fasting plasma insulin, total cholesterol, triglycerides, low-density lipoproteins (LDL) as well as enhancing ACh-induced FBF, while nitric oxide synthase inhibitors mask increase in ACh-induced FBF, indicating that reduction in energy intake improves endothelial function possibly by increasing endogenous NO production in obese hypertensive patients [9]. Lipid lowering also reduces the risk for coronary events, cardiovascular morbidity and mortality in a broad range of patients [26]. Similar observations have also been reported in animal studies. In miniature pigs, 6–9 month of cholesterol-rich diet induces LDL-hypercholesterolaemia and advanced coronary atherogenesis. Withdrawal of dietary cholesterol reverses hypercholesterolaemia and stabilizes coronary plaque in miniture pigs [27] and partially restores endothelial function in rats [13].

In the present study, diet-fed obese rats had significant vascular abnormalities, in particular a marked endothelial dysfunction. The potential factors for vascular abnormalities are obesity per se [1], insulin resistance [3], increased leptin levels [28], and higher level of plasma triglyceride [8,12,13], as well as total body weight and an increase in fat-pad mass. In fact, there were negative correlations between endothelial function and plasma levels of insulin, leptin and triglycerides. Previous studies reporting occurrence

of changes in vascular function in the absence of obesity or rise in plasma leptin levels and insulin resistance [12–14] argue against major roles of obesity per se, insulin and leptin resistance in vascular dysfunction. Moreover, in this study diet-to-chow rats were significantly obese compared with chow-fed controls, yet their vascular function was similar to that of chow-fed group, further arguing, at least in the rat, against the role of obesity per se in the genesis of vascular abnormalities. On the other hand, recent human and animal studies point to the role of circulating triglyceride in the genesis of vascular dysfunction [8,11–13,29,30]. In this study, circulating triglyceride levels were raised more than 115% and a significant negative correlation was seen between fasting plasma triglyceride levels and endothelium-dependent vasorelaxation. The plasma TG levels of chow-fed and diet-to-chow groups were similar as were CCh-induced vasorelaxation between the two groups, suggesting that changing diet (i.e. removing palatable diet and replacing it with the standard chow) resulted in lowering plasma triglyceride levels and thereby improving vascular function.

Overall, this study indicates that prolonged consumption of a high-palatable, high-energy diet induces metabolic and cardiovascular disorders. Nonetheless, these abnormalities are completely reversible by chronic withdrawal of the palatable diet.

References

[1] Andres R. Effects of obesity on total mortality. Int J Obes 1980;4:381–6.

[2] Barret-Conner EL. Obesity, atherosclerosis and coronary heart disease. Ann Int Med 1985;103:1010–9.

[3] Reaven GM. Role of insulin resistance in human disease. Diabetes 1988;37:1595–607.

[4] Singh RB, Rastogi V, Rastogi SS, Niaz MA, Beegom R. Effects of diet and moderate exercise on central obesity and associated disturbances, myocardial infarction and mortality in patients with and without coronary artery disease. J Am Coll Nutr 1996;15:592–601.

[5] Jen CJ, Chan HP, Chen HI. Chronic exercise improves endothelial calcium signalling and vasodilatation in hypercholesterolemic rabbit femoral artery. Arterioscler Thromb Vasc Biol 2002;22:1219–24.

[6] Gutin B, Barbeau P, Owens S, Lemmon CR, Bauman M, Allison J, et al. Effects of exercise intensity on cardiovascular fitness, total body composition, and visceral adiposity of obese adolescents. Am J Clin Nutr 2002;75:818–26.

[7] Cowan GS, Defibaugh N, White T, Hiler ML, Somes G. Aorta and iliac arterial size in pre-operative morbidity obese patients: a preliminary report. Obese Surg 1991;1:155–9.

[8] Naderali EK, Brown MJ, Pickavance LC, Wilding JP, Doyle PJ, Williams G. Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role for triglycerides. Clin Sci 2001;101:499–506.

[9] Sasaki S, Higashi Y, Nakagawa K, Kimura M, Noma K, Sasaki S, et al. A low-caloric diet improves endothelium-dependent vasodilataion in obese patients with essential hypertension. Am J Hypertens 2002;15:302–9.

[10] Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, et al. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. J Clin Invest 1997;100:1230–9.

[11] Zhao SP, Liu L, Gao M, Zhou QC, Li YL, Xia B. Impairment of endothelial function after a high-fat meal in patients with coronary artery disease. Coron Artery Dis 2001;12:561–5.

[12] Naderali EK, Williams G. Effects of short-term feeding of a highly-palatable diet on vascular reactivity in rats. Eur J Clin Invest 2001;31:1024–8.

[13] Naderali EK, Williams G. Prolonged endothelial-dependent and -independent arterial dysfunction induced in the rat by short-term feeding with a high-fat, high-sucrose diet. Atherosclerosis 2003;166:253–9.

[14] Naderali EK, Pickavance LC, Wilding JPH, Williams G. Diet-induced endothelial dysfunction in the rat is independent of the degree of increase in total body weight. Clin Sci 2001;100:635–41.

[15] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeaostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.

[16] Carey VJ, Walters EE, Colditz GA, Solomon CG, Willett WC, Rosner BA, et al. Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women: the nurses' health study. Am J Epidemiol 1997;145:614–9.

[17] Lind L, Berne C, Lithell H. Prevalence of insulin resistance in essential hypertension. J Hypertens 1995;12:1456–62.

[18] Parker L, Lamont DW, Unwin N, Pearce MS, Bennett SM, Dickinson HO, et al. A lifecourse study of risk for hyperinsulin-aemia. Diabet Med 2003;20:406–15.

[19] Naderali EK, Pickavance LC, Wilding JP, Doyle PJ, Williams G. Troglitazone administration corrects metabolic abnormalities but not vascular dysfunction in rat with dietary obesity. Eur J Pharmacol 2001;416:133–9.

[20] Vogel RA, Corretti MC, Plotnick GD. Effects of a single high-fat mean on endothelial function in healthy subjects. Am J Cardiol 1997;79:350–4.

[21] Sato J, O'Brien T, Katusic ZS, Fu A, Nygren J, Singh R, et al. Dietary antioxidant preserves endothelium dependent vasorelaxation in overfed rats. Atherosclerosis 2002;161:327–33.

[22] Plotnick GD, Correti MC, Vogel RA. Effect of antioxidant vitamins on the transient impairment of endothelium-dependent brachial artery vasoactivity following a single high-fat mean. JAMA 1997;278:1682–6.

[23] Kuno T, Hozumi M, Morinobu T, Murata T, Mingci Z, Tamai H. Antioxidant vitamin levels in plasma and low-density lipoprotein of obese girls. Free Radic Res 1998;28:81–6.

[24] Vincent HK, Powers SK, Stewart DJ, Shanely RA, Demirel H, Naito H. Obesity is associated with increased myocardial oxidative stress. Int J Obes Relat Metab Disord 1999;23:67–74.

[25] Moor de Burgos A, Wartanowics M, Zeimlanski S. Blood vitamins and lipid levels in overweight and obese women. Eur J Clin Nutr 1992;46:803–8.

[26] Bucher HC, Griffith LE, Guyatt GH. Systemic review on the risk and benefits of different cholesterol-lowering interventions. Arterioscler Thromb Vas Biol 1999;19:187–95.

[27] Verhamme P, Quarck R, Hao H, Knaapen M, Dymarkowski S, Bernar H, et al. Dietary cholesterol withdrawal reduces vascular inflammation and induces coronary plaque stabilization in miniature pigs. Cardiovasc Res 2002;56:135–44.

[28] Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, et al. Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). Circulation 2001;104:3052–6.

[29] Tka I, Kimball BP, Lewis G, Uffelman K, Steiner G. The severity of coronary athersoclerosis in type 2 diabetes mellitus is related to the number of circulating triglyceride-rich lipoprotein particles. Atheroscler Thromb Vasc Biol 1997;17:3633–8.

[30] Krauss RM. Triglycerides and atherogenic lipoproteins: rationale for lipid management. Am J Med 1998;105:805–12.

Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 521 (2005) 99–104

www.elsevier.com/locate/ejphar

The effects of fenofibrate on metabolic and vascular changes induced by chocolate-supplemented diet in the rat

Ebrahim K. Naderali *, Sameer Fatani

Neuroendocrine & Obesity Biology Unit, Department of Medicine, University of Liverpool, UCD, Daulby Street, Liverpool L69 3GA, UK

Received 28 April 2005; received in revised form 13 June 2005; accepted 21 July 2005
Available online 13 September 2005

Abstract

Current evidences suggest that diet per se plays an important role in genesis of metabolic and vascular function abnormalities. We have investigated the effects of addition of a high-fat diet (chocolate) in the presence or absence of short-term (7 days) administration of fenofibrate on metabolic and vascular changes in adult male Wistar rats. Despite similarities in total body weight in all groups, compared with control fed groups, chocolate-supplemented animals had significantly higher plasma triacylglyceride and non-esterified fatty acids and leptin (for all, $P<0.01$), but not glucose or insulin levels. Fenofibrate treatment corrected metabolic changes. In the mesenteric arteries, responses to KCl and noradrenaline were significantly (for both, $P<0.01$) higher in chocolate-supplemented group. Furthermore, vasorelaxant responses to carbamylcholine, but not to sodium nitroprusside, were significantly ($P<0.01$) reduced in the chocolate-supplemented group. Although fenofibrate failed to improve noradrenaline and KCl responses, it was effective in improving carbamylcholine-induced vasorelaxation. These data suggest that high-fat diet has a profound effect on plasma lipid profile and vascular function. Furthermore, fenofibrate treatment may ameliorate high-fat diet effects on vascular function and metabolic changes.

Keywords: High-fat diet; Vascular contractility; Endothelial dysfunction; Fenofibrate; Hypertriglycerideamia

1. Introduction

Acceleration in the rate of diet-induced obesity throughout the world is becoming a major health problem. Excess energy intake in the form of refined carbohydrates and fatty acids is one of the main causes of human obesity. Obesity is a major risk factor for cardiovascular disorders, which include hypertension, atherosclerosis and ultimately myocardial infarction (Andres, 1980; Lakka et al., 2002). Arterial function and structural abnormalities which include alterations of the release of vasoactive substances from endothelial cells as well as changes in arterial wall elasticity have been seen in obese human subjects and certain animal models that reflect some aspects of human obesity (Cowan et al., 1991; Dobrian et al., 2000). Moreover, human and animal studies have shown various degrees of endothelial dysfunction in obesity, namely a reduction to vasorelaxant's response (Abram, 1997, Naderali et al., 2001; Perticone et al., 2001; Steinberg et al., 1996).

Increasing reports suggest that diet and its variant component play important roles in arterial function. Studies have shown that consumption of a specific diet of high fat or low protein causes endothelial abnormalities in humans and animals, while a low fat diet has a protective effect on vascular reactivity (Vogel et al., 1997; Verhamme et al., 2002; Naderali et al., 2004). Moreover, pharmacological lowering of plasma lipids by fenofibrate, retards atheroma developments (Renier et al., 2000), indicating a need to control circulating plasma lipids. Therefore, the aim of this study was to investigate the effects of supplementation of a high-fat diet in the presence or absence of short-term (7 days) administration of fenofibrate on metabolic changes and vascular function.

* Corresponding author. Tel.: +44 151 706 4087; fax: +44 151 706 5626.
E-mail address: naderali@liverpool.ac.uk (E.K. Naderali).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.07.027

2. Materials and methods

2.1. Animals and experimental protocol

Adult male Wistar rats ($n=21$) were randomized and assigned to two groups which were fed on either a standard pelletted laboratory chow (CRM Biosure, Cambridge, UK; which provided 60% of energy as carbohydrate, 30% as protein and 10% as fat) throughout ($n=7$; control), or given chow plus Galaxy smooth and creamy milk chocolate (Mars) (which provided 42.2% of energy as carbohydrate, 5.1% as protein and 52.7% as fat) ($n=14$; chocolate-supplemented). After 14 weeks, half of the chocolate-supplemented group was given fenofibrate (50 mg/kg/day) and the remainder were given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, UK) by oral gavage daily for 7 days, before being sacrificed. All animals had free access to their respective diets and water, and were housed in groups of 2 under controlled environmental conditions (19–22 °C; 30–40% humidity) and a 12-h light/dark cycle (lights on at 07:00 h). Daily energy intake was determined in all three groups for the last 7 days of the study.

The rats were killed by CO_2 inhalation, and the epididymal and perirenal fat pads and the gastrocnemius and cardiac muscles were dissected and weighed. Blood was collected for haematocrite studies and for the measurement of glucose, insulin, leptin, non-esterified fatty acids and triacylglycerol. Plasma glucose concentration was determined by the glucose oxidase method, while non-esterified fatty acids and triacylglycerol concentrations were determined by commercial diagnostic kits (Boehringer Mannheim, U.K and Sigma, U.K, respectively). Insulin and leptin concentrations were measured by radioimmunoassay (RIA) kits (Pharmacia/Upjohn Diagnostics, U.K. and Linco Research, U.K, respectively).

2.2. Assessment of vascular function

Six third-order mesenteric arteries from each animal (150–180 μM diameter, 2 mm lengths) were carefully dissected from each animal and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK). Pairs of arteries were incubated in a 5-ml organ bath containing physiological salt solution (PSS; composition [in mM]: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5) gassed with 95% O_2 and 5% CO_2 at 37 °C.

After 30 min equilibration, the length–tension characteristics for each vessel were determined (Naderali et al., 2000). Arteries were allowed a further 30 min to equilibrate before being depolarised twice with high-potassium physiological salt solution (KPSS) in which NaCl in normal PSS was replaced by an equimolar concentration of KCl (125 mM). Cumulative concentration–response curves to either KCl (10–125 mM) or noradrenaline (0.5–6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to KCl (125 mM) was discarded. We have also measured the effects of selected endothelium-dependent vasodilator, carbamylcholine, and endothelium-independent vasodilator, sodium nitroprusside on arteries preconstricted with 8 μM noradrenaline.

2.3. Reagents

Noradrenaline, carbamylcholine, sodium nitroprusside, and fenofibrate were all obtained from Sigma Chemicals. All solutions were prepared freshly in distilled water prior to use.

2.4. Data interpretation and statistical analyses

KCl- and noradrenaline-induced vasoconstriction responses were expressed as absolute force generated. Relaxation in response to carbamylcholine and sodium nitroprusside were calculated as the percentage reduction from the maximal tension generated in response to a supramaximal concentration of noradrenaline (8 μM). An average response for all the vessels from a given animal was determined before group analysis. Data are presented as mean±S.E.M. Statistical significance was tested using repeated-measures analysis of variance (ANOVA) or the Mann–Whitney test, as appropriate. Differences were considered statistically significant at values of $P \leq 0.05$.

3. Results

3.1. Metabolic data

Supplementation of chocolate did not alter total body weight gain. In fact throughout the experiment there were no significant differences in total body weight at any given time

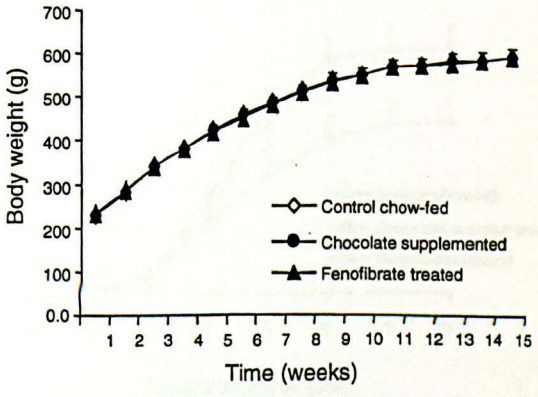


Fig. 1. The effect of chocolate supplementation and fenofibrate treatment on total body weight in the rat. Control animals were fed standard chow while chocolate-supplemented groups was given chow plus chocolate (Galaxy) for 15 weeks. Fenofibrate-treated rats were given fenofibrate (50 mg/kg/day) daily for 7 days. At the end of 15 weeks, there were no significant changes in total body weight among the three groups. In fact weight increase in all three groups were superimposable. Data are expressed as mean±S.E.M.

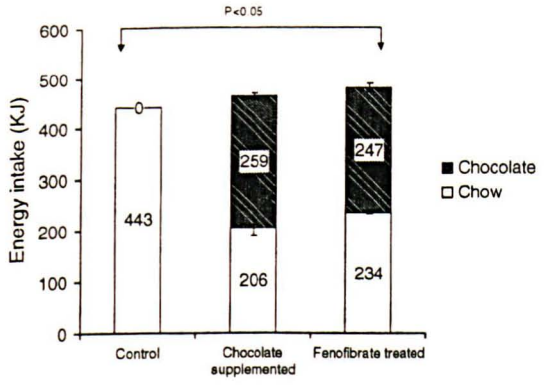


Fig. 2. Cumulative weekly energy intake of animals from all three groups during fenofibrate (50 mg/kg/day) treatment period. The increase in total energy intake of animals on chocolate supplementation was not statistically significant ($P=0.346$), however fenofibrate treatment further increased energy intake, which was significantly ($P<0.05$) higher than chow-fed controls.

between chow-fed control and chocolate-supplemented test groups (Fig. 1). Furthermore, the epididymal and perirenal fat pad masses as well as gastrocnemius muscle mass were comparable in all three groups, indicating the absence of any obesity. Analysis of food intake during the last 7 days showed that compared with control chow-fed rats, increase in total weekly energy intake of chocolate-supplemented group was insignificant (Fig. 2). However, fenofibrate-treated rats had significantly ($P<0.05$) higher energy intake than control chow-fed groups (Fig. 2). Nonetheless, this increase in energy intake did not translate to a significant increase in total body weight (Table 1).

While supplementation of chocolate had no significant effect on cardiac muscle mass or haematocrit values, fenofibrate-treated animals have significantly ($P<0.01$) higher cardiac muscle mass and reduced haematocrit values (Table 1). At the end of the experiment, fasting plasma glucose, insulin levels were comparable between control chow-fed and chocolate-supplemented groups, however, fenofibrate-treated animals had significantly ($P<0.01$) lower glucose and insulin levels than control counterparts. Furthermore, chocolate-supplemented animals had significantly higher fasting concentrations of plasma leptin, triacylglycerol and non-esterified fatty acids than chow-fed controls. These changes were almost completely reversed by fenofibrate treatment (Table 1).

Table 1
Physiological and metabolic characteristics of the 3 experimental groups

	Chow-fed controls ($n=7$)	Chocolate-supplemented ($n=7$)	Fenofibrate-treated ($n=7$)
Body weight (g)	598±23	606±19	611±21
Epididymal fat pad mass (g)	6.56±0.80	7.20±0.59	6.56±0.64
Perirenal fat pad mass (g)	6.20±0.94	7.24±0.66	6.69±0.84
Gastrocnemius muscle mass (g)	3.27±0.15	3.06±0.11	3.16±0.11
Heart muscle mass (g)	1.57±0.09	1.54±0.05	1.70±0.08[a,b]
Haematocrit	49.1±0.6	47.5±0.8	46.4±0.5[a]
Plasma glucose (mM)	11.3±0.9	13.5±1.0	9.3±0.4[a,b]
Plasma insulin (μU/ml)	1.52±.38	1.31±0.33	1.12±0.21[a]
Plasma leptin (ng/ml)	15.1±2.2	21.6±4.0[a]	14.2±6.9[b]
Plasma triglycerides (mM)	1.50±0.06	1.96±0.12[a]	0.96±0.08[a,b]
Plasma NEFA (mM)	0.28±0.02	0.39±0.02[a]	0.23±0.01[b]

Data are mean±SEM.
[a] $P<0.01$ vs controls.
[b] $P<0.01$ vs chocolate-supplemented.

3.2. Agonist-induced contractile responses

KCl (10–125 mM)- and noradrenaline (0.5–6 μM)-induced vasoconstriction in arteries from all three groups

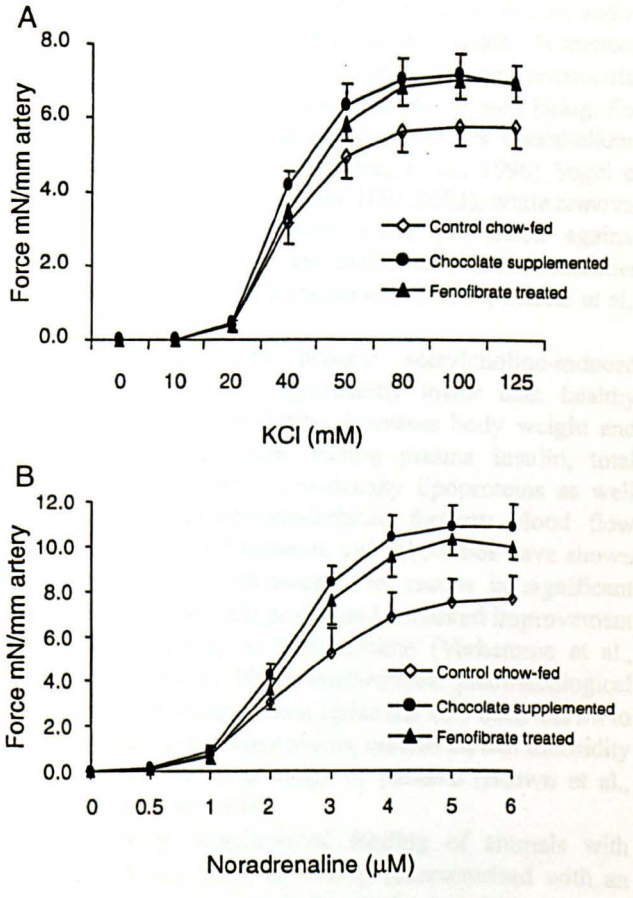


Fig. 3. Vasoconstriction responses to cumulative concentration of (A) KCl (10–125 mM) and (B) noradrenaline (0.5–6 μM) in arteries from chow-fed control, chocolate-supplemented and fenofibrate-treated animals. Both KCl and noradrenaline-induced contractions were significantly (ANOVA, $P<0.01$) augmented in arteries from chocolate-supplemented animals. Data represent mean±S.E.M.

produced a characteristic sigmoid relationship. The contractile responses to KCl and noradrenaline in arteries from chocolate- supplemented animals (with or without fenofibrate treatment) were significantly ($P<0.001$) increased in comparison to control groups (Fig. 2). The maximal KCl-induced contractions were 5.78±0.54 control, 7.14±0.57 chocolate-supplemented, and 7.07±0.52 mN/mm artery fenofibrate-treated, respectively (Fig. 3A). A similar picture was also seen with noradrenaline-induced vasoconstriction giving maximal contraction of 7.68±1.02 control, 10.91±0.96 supplemented, and 10.34±0.66 mN/mm artery fenofibrate-treated, respectively (Fig. 3B).

3.3. Endothelium-dependent and -independent vasorelaxation

Noradrenaline preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of carbamylcholine (10 nM–100 μM), achieving a maximum of 87±2% at a carbamylcholine concentration of 100 μM. Arteries from test and fenofibrate-treated groups that were similarly exposed to carbamylcholine displayed similar responses with maximal relaxation of 85±6% and 92±2% (Fig. 4A). However, arteries from chocolate-supplemented rats had significantly ($P<0.01$) higher EC_{50} than control chow-fed animals (EC_{50}: 1.86±0.02 chocolate-supplemented, vs EC_{50}: 0.28±0.01 μM chow-fed). Fenofibrate treatment significantly ($P<0.05$) improved EC_{50} compared with chocolate-supplemented group (EC_{50}: 0.68±0.01 μM fenofibrate treatment vs EC_{50}: 1.86±0.02 μM chocolate-supplemented).

Endothelium-independent vasodilator (sodium nitroprusside; 10 nM–100 μM) also induced concentration dependent vasorelaxation of arteries from all three groups with no significant differences in arterial vasorelaxation between the three groups achieving a maximal relaxation of 85±3% control, 82±2% chocolate-supplemented, and 84±3% fenofibrate-treated groups, respectively (Fig. 4B).

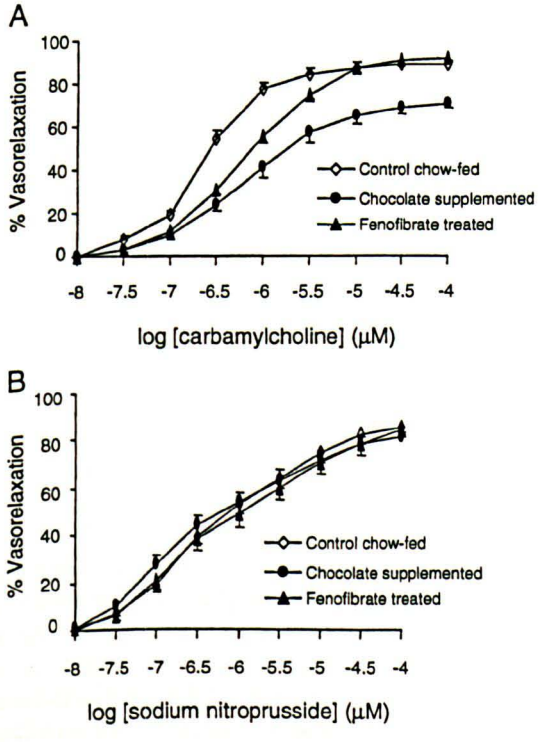


Fig. 4. The effects of (A) carbamylcholine, and (B) sodium nitroprusside on NA-precontracted (8 μM) arteries from three groups of rats. When contraction reached a plateau after 2 min, concentration–response curves to carbamylcholine or sodium nitroprusside were carried out. Data represent mean±S.E.M. The concentration–response curves to carbamylcholine but not to sodium nitroprusside, were significantly reduced in chocolate-supplemented group (ANOVA, $P<0.01$), while fenofibrate treatment significantly ($P<0.01$) improved carbamylcholine-induced vasorelaxation in chocolate-supplemented animals.

4. Discussion

Excessive availability of high-energy diet in many industrialised societies, is producing a health hazard, namely obesity and obesity related secondary disorders, which ultimately leads to the deterioration of quality of life, and in many cases to premature and sudden death. Numerous experimental studies have reported severe detrimental outcome of excessive high-energy diets on well being. For example, high-fat, high-energy diet impairs endothelium-dependent vasorelaxation (Steinberg et al., 1996; Vogel et al., 1997; Naderali and Williams, 2001,2003), while removal of high-energy diet provides some protection against obesity-induced metabolic and cardiovascular abnormalities (Verhamme et al., 2002; Naderali et al., 2004; Sasaki et al., 2002).

In hypertensive obese patients, acetylcholine-induced forearm blood flow is significantly lower than healthy individuals. Caloric restriction decreases body weight and the mean blood pressure, fasting plasma insulin, total cholesterol, triglycerides, low-density lipoproteins as well as enhancing acetylcholine-induced forearm blood flow (Sasaki et al., 2002). Moreover, animal studies have shown that withdrawal of high-energy diet results in significant reduction of plasma lipid profile and a marked improvement of vascular reactivity to acetylcholine (Verhamme et al., 2002; Naderali et al., 2004). Furthermore, pharmacological lowering of circulating plasma lipids has also been shown to reduce the risk for coronary events, cardiovascular morbidity and mortality in a broad range of patients (Brown et al., 1993; Bucher et al., 1999).

In this study, supplemental feeding of animals with chocolate did not result in obesity (characterised with an excessive increase in total body weight and fat pad mass). This may be due to comparable caloric intake between chow-fed control and chocolate-supplemented groups. However, there were significant metabolic abnormalities, namely higher plasma lipids of triacylglycerol and non-esterified fatty acids, in chocolate-supplemented animals. In agreement

with previous reports (Despres et al., 2002; Capell et al., 2003), fenofibrate treatment in the presence of chocolate diet completely reversed lipid profile. Higher plasma lipids seen in this study were similar to those achieved by chronic consumption of high-fat, high-energy diets and obesity (Naderali et al., 2001, 2004; Perticone et al., 2001; Steinberg et al., 1996; Verhamme et al., 2002) indicating that adipose tissue accumulation is not a pre-requisite for abnormal lipid profile (Vogel et al., 1997; Naderali and Williams, 2003).

Examination of contractile and relaxation properties of arteries showed striking differences in responses to various stimuli between the three groups. In contrast to previous reports (Walker et al., 1997; Fontes et al., 1998; Naderali et al., 2001, 2004), contractile responses to KCl and noradrenaline were significantly augmented in chocolate-supplemented diet. Although from our study it is not possible to contemplate the mechanism of hyperreactivity to KCl and noradrenaline, nonetheless, one possibility is that long-term consumption of chocolate may have a direct effect on the activity of the contractile apparatus, or it may have increased/eased the availability of the calcium for contraction required by KCl and noradrenaline. The latter theory is more plausible as chocolate has been shown to markedly increase calciuria (Nguyen et al., 1994). However, these hypotheses merit further investigation.

Numerous studies have shown that excessive high-energy diet impairs endothelium-dependent arterial function (Sasaki et al., 2002; Brown et al., 1993; Bucher et al., 1999; Naderali et al., 2001, 2004; Walker et al., 1997; Fontes et al., 1998). Moreover, defects in vasorelaxation can also be seen with short-term consumption of high-energy diet in the absence of any obesity (Vogel et al., 1997; Naderali and Williams, 2001, 2003). In agreement with previous reports, in this study there was a marked attenuation of endothelial mediated carbamylcholine-induced vasorelaxation in the absence of any obesity, suggesting a significant defect in either carbamylcholine-induced NO production via eNOS activity (Boulanger, 1999) or the release of endothelium-derived hyperpolarizing factor (Gerber et al., 1998). On the other hand, vasorelaxant effect of sodium nitroprusside is due to its direct stimulatory action on cGMP and thereby induction of NO generation (Azula et al., 1996) in vascular smooth muscle cells. Failure of the chocolate-supplemented diet in altering sodium nitroprusside-induced vasorelaxation, argues against any detrimental changes in arterial smooth muscle activity.

A number of various hypotheses have been put forward as the mechanism(s) of action of high-fat diet and obesity related vascular dysfunction, which include a rise in plasma non-esterified fatty acids (Steinberg et al., 1997) hypertriglycerideamia (Tka et al., 1997; Lewis et al., 1999; Naderali et al., 2001). The detrimental effects of increased circulating plasma lipids has been, at least partly, attributed to inhibition of eNOS activity and thereby reduction in NO synthesis (Liao et al., 1995), ultimately resulting in a reduced endothelial-dependent vasorelaxation. Raised non-esterified fatty acids and triacylglycerols levels in chocolate-supplemented animals seen in this study, argue for the role of plasma non-esterified fatty acids and triacylglycerols as the possible agents effecting vasorelaxation. These hypotheses are further strengthened by the finding that fenofibrate treatment in our study improved carbamylcholine-induced responses. Although the mechanism of action of fenofibrate on vascular function is not yet fully known, the protective effects of fenofibrate on vascular function may be due to correction of lipid profile (Despres et al., 2002; Capell et al., 2003) or increasing formation, availability and duration of NO action (Haak et al., 1998; Playford et al., 2002), indicating improvement of endothelial function in fenofibrate-treated animals. Overall, this study confirms that consumption of a high-fat diet attenuates arterial relaxation in the absence of any obesity in the rat. The arterial dysfunction may be due to an induction of hyperlipidaemia in general or hypertriglyceridaemia in particular. Moreover, lowering circulating plasma non-esterified fatty acids and/or triacylglycerols by fenofibrate treatment may have beneficial effects on endothelial function.

Acknowledgement

This study is supported by a grant from British Heart Foundation (FS/02/002). EKN is a BHF Research Fellow. The authors would like to express their gratitude to Dr. I. S. Wood for his valuable comments on the manuscript.

References

Abram, J., 1997. Role of endothelial dysfunction in coronary artery disease. Am. J. Cardiol. 79, 2–9.

Andres, R., 1980. Effects of obesity on total mortality. Int. J. Obes. 4, 381–386.

Azula, F.J., Alzola, E.S., Conde, M., Trueba, M., Macarulla, J.M., Marino, A., 1996. Thrombin-stimulated phospholipase C activity is inhibited without visible delay by a rapid increase in the cyclic GMP levels induced by sodium nitroprusside. Mol. Pharmacol. 50, 367–379.

Boulanger, C.M., 1999. Secondary endothelial dysfunction: hypertension and heart failure. J. Mol. Cell. Cardiol. 31, 39–49.

Brown, B.G., Zhao, X.Q., Sacco, D.E., Albers, J.J., 1993. Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical events in coronary disease. Circulation 87, 1781–1791.

Bucher, H.C., Griffith, L.E., Guyatt, G.H., 1999. Systemic review on the risk and benefits of different cholesterol-lowering interventions. Arterioscler. Thromb. Vasc. Biol. 19, 187–195.

Capell, W.H., DeSouza, C.A., Poirier, P., Bell, M.L., Stauffer, B.L., Weil, K. M., Hernandez, T.L., Eckel, R.H., 2003. Short-term triglyceride lowering with fenofibrate improves vasodilator function in subjects with hypertriglyceridemia. Arterioscler. Thromb. Vasc. Biol. 23, 307–313.

Cowan, G.S., Defibaugh, N., White, T., Hiler, M.L., Somes, G., 1991. Aorta and iliac arterial sizes in pre-operative morbidity obese patients: a preliminary report. Obes. Surg. 1, 155–159.

Despres, J.P., Lemieux, I., Salomon, H., Delaval, D., 2002. Effects of micronized fenofibrate versus atorvastatin in the treatment of

dyslipidaemic patients with low plasma HDL-cholesterol levels: a 12-week randomized trial. J. Intern. Med. 251, 490–499.

Dobrian, A.D., Davies, M.J., Prewitt, R.L., Lauterio, T.J., 2000. Development of hypertension in a rat model of diet-induced obesity. Hypertension 35, 1009–1015.

Fontes, R.C.A., Almeida, L., Paiva, I., Tavares, P., Cabrita, S., Wulfroth, P., Matos, B.M.L., Teixeira, F., 1998. Influence of 0.1% or 0.2% cholesterol-enriched diets on the induction of atherosclerosis and aorta reactivity in vitro. J. Cardiovasc. Pharmacol. 3, 690–699.

Gerber, R.T., Anwar, M.A., Poston, L., 1998. Enhanced acetylcholine induced relaxation in small mesenteric arteries from pregnant rats: an important role for endothelium-derived hyperpolarizing factor (EDHF). Br. J. Pharmacol. 125, 455–460.

Haak, T., Haak, E., Kusterer, K., Weber, A., Kohleisen, M., Usadel, K.H., 1998. Fenofibrate improves microcirculation in patients with hyperlipidemia. Eur. J. Med. Res. 3, 50–54.

Lakka, H.M., Lakka, T.A., Tuomilehto, J., Salonen, J.T., 2002. Abdominal obesity is associated with increased risk of acute coronary events in men. Eur. Heart. J. 23, 706–713.

Lewis, T.V., Dart, A.M., Chin-Dusting, J.P., 1999. Endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridemic humans with normal levels of plasma LDL cholesterol. J. Am. Coll. Cardiol. 33, 805–812.

Liao, J.K., Shin, W.S., Lee, W.Y., Clark, S.L., 1995. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. J. Biol. Chem. 270, 319–324.

Naderali, E.K., Williams, G., 2001. Effects of short-term feeding of a highly palatable diet on vascular reactivity in rats. Eur. J. Clin. Invest. 31, 1024–1028.

Naderali, E.K., Williams, G., 2003. Prolonged endothelial-dependent and -independent arterial dysfunction induced in the rat by short-term feeding with a high-fat, high-sucrose diet. Atherosclerosis 166, 253–259.

Naderali, E.K., Doyle, P., Williams, G., 2000. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea-pigs. Clin. Sci. 98, 537–543.

Naderali, E.K., Brown, M.J., Pickavance, L.C., Wilding, J.P.H., Doyle, P.J., Williams, G., 2001. Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role for triglycerols. Clin. Sci. 101, 499–506.

Naderali, E.K., Fatani, S., Williams, G., 2004. Chronic withdrawal of a high-palatable obesity-inducing diet completely reverses metabolic and vascular abnormalities associated with dietary-obesity in the rat. Atherosclerosis 172, 63–69.

Nguyen, N.U., Henriet, M.T., Dumoulin, G., Widmer, A., Regnard, J., 1994. Increase in calciuria and oxaluria after a single chocolate bar load. Horm. Metab. Res. 26, 383–386.

Perticone, F., Cervolo, R., Mafald, C., Ventura, G., Iacopino, S., Sinopoli, F., Mattioli, P.L., 2001. Obesity and body fat distribution induce endothelial dysfunction by oxidative stress. Diabetes 50, 159–165.

Playford, D.A., Watts, G.F., Best, J.D., Burke, V., 2002. Effect of fenofibrate on brachial artery flow-mediated dilatation in type 2 diabetes mellitus. Am. J. Cardiol. 90, 1254–1257.

Renier, G., Desfaits, A.C., Serri, O., 2000. Effect of gliclazide on monocyte–endothelium interactions in diabetes. J. Diabetes Its Complicat. 14, 215–223.

Sasaki, S., Higashi, Y., Nakagawa, K., Kimura, M., Noma, K., Sasaki, S., Hara, K., Matsuura, H., Goto, C., Oshima, T., Chayama, K., 2002. A low-caloric diet improves endothelium-dependent vasodilatation in obese patients with essential hypertension. Am. J. Hypertens. 15, 302–309.

Steinberg, H.O., Chacker, H., Leaming, R., Johnson, A., Brechtel, G., Baron, A.D., 1996. Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance. J. Clin. Invest. 97, 2601–2610.

Steinberg, H.O., Tarshoby, M., Monestel, R., Hook, G., Cronin, J., Johnson, A., Bayazeed, B., Baron, A.D., 1997. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. J. Clin. Invest. 100, 1230–1239.

Tka, I., Kimball, B.P., Lewis, G., Uffelman, K., Steiner, G., 1997. The severity of coronary atherosclerosis in type 2 diabetes mellitus is related to the number of circulating triglyceride-rich lipoprotein particles. Arterioscler. Thromb. Vasc. Biol. 17, 3633–3638.

Verhamme, P., Quarck, R., Hao, H., Knaapen, M., Dymarkowski, S., Bernar, H., Van Cleemput, J., Janssens, S., Vermylen, J., Gabbiani, G., Kockx, M., Holvoet, P., 2002. Dietary cholesterol withdrawal reduces vascular inflammation and induces coronary plaque stabilization in miniature pigs. Cardiovasc. Res. 56, 135–144.

Vogel, R.A., Corretti, M.C., Plotnick, G.D., 1997. Effects of a single high-fat mean on endothelial function in healthy subjects. Am. J. Cardiol. 79, 350–354.

Walker, A.B., Dores, J., Buckingham, R.E., Savage, M.W., Williams, G., 1997. Impaired insulin-induced attenuation of noradrenaline-mediated vasoconstriction in insulin-resistant obese Zucker rats. Clin. Sci. 93, 235–241.

ELSEVIER

Atherosclerosis 177 (2004) 307–312

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Fenofibrate lowers adiposity and corrects metabolic abnormalities, but only partially restores endothelial function in dietary obese rats

Ebrahim K. Naderali*, Sameer Fatani, *Gareth Williams*

Neuroendocrine and Obesity Biology Unit, Department of Medicine, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

Received 8 March 2004; received in revised form 24 June 2004; accepted 22 July 2004
Available online 11 September 2004

Abstract

In humans, dietary-induced obesity markedly increases plasma lipid profile and impairs vascular function leading to increased incidence of cardiovascular events. We have recently reported that chronic withdrawal of obesity-inducing diet attenuates obesity and completely corrects endothelial function. The aim of this study was to investigate whether fenofibrate-induced decrease in adiposity would also correct vascular function in the presence of obesity-inducing diet. Wistar rats were fed with either standard laboratory chow (lean, $n = 9$) or given a highly palatable diet (diet-fed, $n = 18$) for 15 weeks. After 7 weeks, half of the diet-fed group was treated with fenofibrate (fenofibrate-treated, $n = 9$) for 8 weeks before being sacrificed. Untreated diet-fed ($n = 9$) rats had significantly higher body weight, total fat mass (by up to two-fold, $p < 0.001$ for both), and raised fasting plasma levels of insulin, leptin and triglycerides (up to 110%; $p < 0.001$), but not glucose or nonesterified fatty acids (NEFA) than both lean control and fenofibrate-treated groups. Resistance mesenteric arteries responses to KCl- and noradrenaline-induced vasoconstriction were similar in all three groups. However, compared with lean controls, endothelium-dependent vasorelaxation responses were shifted to the right in both untreated and fenofibrate-treated diet-fed groups. Fenofibrate treatment improved endothelium-dependent vasorelaxation at only high carbamycholine concentrations (10 μM). There were no differences in endothelium-independent vasorelaxation between the three groups. These results indicate that, in the presence of obesity-inducing diet, fenofibrate markedly reverses obesity and corrects insulin resistance and lipid profile, but it only has a limited beneficial effect on vascular function. Therefore, it seems that diet component rather than obesity per se plays a key role in the genesis of vascular abnormalities.

Keywords: Diet; Obesity; Insulin resistance; Fenofibrate; Endothelial function

1. Introduction

Obesity and type 2 diabetes are strong risk factors for the development of atherosclerosis. The underlying pathophysiological mechanisms are not fully understood, but may include hyperlipidaemia and increased oxidation of low-density lipoproteins [1], hypertriglyceridaemia [2], insulin resistance [3] and hyperleptinaemia [4]. Obesity, type 2 diabetes and the above risk factors are also associated with marked defects in vascular function. Endothelial dysfunction in particular is prominent and may contribute to atherogenesis [1,2,5,6]. The lipid-lowering fibrates, such as fenofibrate and ciprofibrate, have been shown to impede the development of atheroma. These drugs bind to peroxisome proliferator-activated receptor α (PPARα), leading to enhanced lipid catabolism in the liver [7] and reductions in total body fat as well as the circulating plasma lipids.

Fenofibrate has been reported to retard angiographic progression of coronary atherosclerosis in diabetic patients [8], and improve the microcirculation in patients with hyperlipidaemia [9]. The mechanism(s) of the beneficial effects of fenofibrate on vascular function has not been fully elucidated yet, but direct activation of PPARα in arterial wall [10], correction of lipid abnormalities [11,12], and increasing the formation, availability and action of NO [13,14] have all been postulated.

In this study we aimed to evaluate further the effect of fenofibrate on vascular abnormalities in the presence of obesity-inducing diet in obese rats.

* Corresponding author. Tel.: +44 151 706 4087; fax: +44 151 706 5797.
E-mail address: naderali@liverpool.ac.uk (E.K. Naderali).

0021-9150/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2004.07.029

2. Material and methods

2.1. Animals

Adult male Wistar rats ($n = 27$) were randomised and assigned to a control group ($n = 9$, 191 ± 3 g) and a test group ($n = 18$, 190 ± 1 g) that was fed a highly palatable diet for 15 weeks.

All animals had free access to water and were housed individually under controlled environmental conditions (19–22 °C; 30–40% humidity) and a 12-h light/dark cycle (lights on at 07:00 h). Controls were fed a standard laboratory pelleted diet (CRM Biosure, Cambridge, UK), which provides 60% of energy as carbohydrate, 30% as protein and 10% as fat. The test group had free access to a highly palatable diet consisting of 33% (by weight) ground pellet diet, 33% Nestlë condensed milk, 7% sucrose and 27% water. This provided 65% of energy as carbohydrate, 19% as protein and 16% as fat. After 7 weeks, half of the test group were given fenofibrate (50 mg/kg/day) and the remainder were given vehicle (1% carboxymethyl cellulose at 1 ml/kg body weight; Sigma, Pool, UK) by oral gavage, daily for 8 weeks.

On the day of experiment, the rats were killed by CO_2 inhalation after 2 h of fasting. The total body fat mass was measured immediately by bioimpedance method using the TOBEC® equipment (Biotech. Instruments Ltd., Kimpton, UK). Blood was removed by cardiac puncture into cold heparinized tubes, and the gonadal and perirenal fat pads as well as the gastrocnemius and soleus muscles were dissected and weighed. The plasma was immediately separated by centrifugation before being frozen for later measurements of glucose, insulin, leptin, nonesterified free fatty acids (NEFA) and triglycerides (TG). Plasma glucose concentration was determined using a glucose oxidase method, and NEFA and TG concentrations using commercial diagnostic kits (Boehringer Mannheim, Milton Keynes, Bucks and Sigma Diagnostics, Poole, UK). Insulin and leptin concentrations were measured by radioimmunoassay (RIA) kits (Pharmacia/Upjohn Diagnostics, Lewes, Sussex and Linco Research, Biogenesis, Poole, Dorset, UK, respectively).

2.2. Homeostasis model assessment (HOMA)

HOMA, an index of insulin resistance, which employs measures of fasting plasma concentrations of glucose and insulin, was calculated according to the method described previously [15].

2.3. Assessment of vascular function

Six third-order mesenteric arteries (>200 μm diameter, 2-mm length) were carefully dissected from each animal. Each artery was freed of fat and connective tissue and mounted on two 40-μm diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, UK), based on the principle of the Mulvany myograph which measures isometric tension generated in response to various stimuli. Arterial segments (two at a time) were incubated in a 5-ml organ bath containing physiological salt solution (PSS; composition [in mM]: NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.026; and glucose 5.5) gassed with 95% O_2 and 5% CO_2 at 37 °C.

After 30 min equilibration, the length–tension characteristics for each artery were determined as described previously [16]. The computer also calculated the target tension that each vessel should develop in response to a maximal stimulus. Arteries were then allowed a further 30 min to equilibrate before being depolarised twice with high-potassium physiological salt solution (KPSS, 125 mM) in which the NaCl in normal PSS was replaced by an equimolar concentration of KCl. Cumulative concentration–response curves to either KCl (10–125 mM) or noradrenaline (NA, 0.5–6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded.

2.4. Assessment of defect in vascular relaxation

Endothelium-dependent and -independent defects were investigated by exposing NA-precontracted arteries to carbamylcholine (CCh) or sodium nitroprusside (SNP), respectively. To eliminate possible interference by vasoactive prostanoids, 10 mM indomethacin was added to the organ bath, prior to contraction with NA (8 μM). When contraction reached a plateau (after 2 min), concentration–response curves were performed to either CCh or SNP (for both, 10 nM–100 μM).

2.5. Reagents

Noradrenaline (NA), carbamylcholine (CCh), sodium nitroprusside (SNP), indomethacin, carboxymethyl cellulose and fenofibrate were all obtained from Sigma (Poole, Dorset, UK). NA, CCh and SNP were dissolved in double distilled water while indomethacin was dissolved in ethanol. NA and SNP were kept away from light throughout the experiment. All water-soluble solutions were freshly made on the day of experiment.

2.6. Data interpretation and statistical analyses

Vasoconstriction in response to NA and KCl were expressed as absolute force generated (mN/mm artery). Relaxation in response to CCh and SNP was calculated as the percentage reduction from the maximal tension generated at the supramaximal concentration of NA (8 μM). The average response for all the vessels from a given animal was used in the group analysis. EC_{50} values were determined to evaluate changes in agonist-induced responses.

Data showed a normal distribution (Shapiro–Wilkes W test) and are therefore expressed as mean ± S.E.M. Statistical significance was tested using Student 't-test' or repeated-

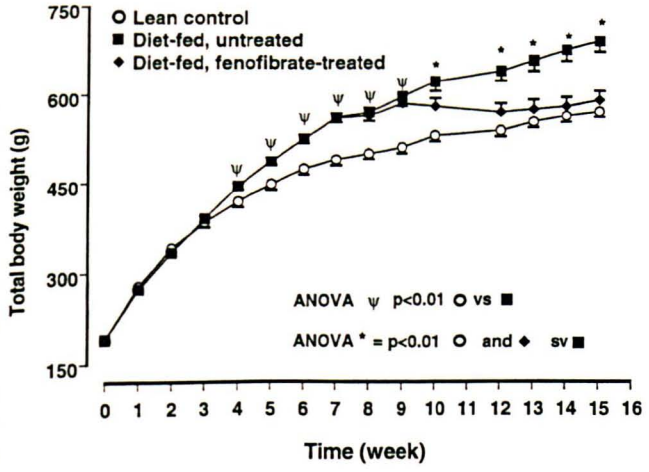


Fig. 1. The effects of palatable diet and fenofibrate on total body weight in male Wistar rats. Lean control animals were fed a standard laboratory chow while diet-fed groups was given a palatable diet for 15 weeks. Fenofibrate-treated rats were given fenofibrate (50 mg/kg/day) daily for 8 weeks. At the end of 15 weeks, untreated diet-fed animals were significantly ($p < 0.0001$) heavier than lean control and fenofibrate-treated groups. Data are expressed as mean ± S.E.M.

measures (ANOVA; Bonferroni *t*-test) or the Mann–Whitney test, as appropriate. Differences were considered statistically significant at the $p < 0.05$ levels.

3. Results

3.1. Effects of fenofibrate on body weight and adiposity

As shown in Fig. 1, rats fed palatable diet progressively gained more weight than chow-fed controls. This difference became significant after the third week of feeding and was maintained thereafter. The final body weight of the diet-fed animals was significantly higher than that of chow-fed controls (>21%; 695 ± 17 versus 572 ± 8 g, $p < 0.0001$). Overall, the weekly weight gain of diet-fed animals was significantly greater than that of chow-fed controls (diet-fed: 20.1 ± 4.77 versus control: 11.98 ± 4.65 g, $p = 0.0135$).

Administration of fenofibrate significantly reduced weekly weight gain of diet-fed animals and final total body weight (Fig. 1). After 8 weeks of treatment, total body weight of the diet-fed fenofibrate-treated animals was not significantly different from that of chow-fed controls (fenofibrate treated: 593 ± 16 versus chow-fed: 572 ± 8 g, $p = 0.2738$), but it was significantly lower ($p = 0.00071$) than that of untreated diet-fed animals (Fig. 1).

After 15 weeks of study, gastrocnemius and soleus muscle masses were comparable between all three groups. However, diet-fed animals had significantly higher total white adipose tissue (sum of epididymal and perirenal fat pads) mass than both chow-fed (by >2-fold, $p < 0.0002$) and fenofibrate-treated (by >1.5-fold, $p = 0.0114$) groups (Table 1). However, fenofibrate-treated animals had significantly (by 1.4-fold; $p = 0.0264$) heavier white adipose tissue than chow-fed controls. Bioimpedence measurements indicated higher percentage of body fat in diet-fed animals compared with chow-fed and fenofibrate-treated groups. Conversely, diet-fed rats had significantly lower percentage of lean mass than chow-fed and fenofibrate-treated animals (Table 1).

3.2. Effects of diet and fenofibrate on blood metabolites

Palatable diet feeding did not affect fasting plasma glucose levels (11.7 ± 1.2 chow-fed; 14.3 ± 0.3 mM diet-fed; $p = 0.0625$). However, diet-fed rats had significantly higher fasting terminal insulin (3.8 ± 0.8 diet-fed versus 1.6 ± 0.3 chow-fed; $p = 0.0025$) than the chow-fed group, with a significantly higher HOMA index value (2.40 ± 0.52 diet-fed versus 0.73 ± 0.10 chow-fed; $p = 0.0086$) indicating development of insulin resistance in diet-fed animals. Fenofibrate treatment significantly lowered levels of both glucose (10.8 ± 0.3 mM; $p < 0.0001$ versus untreated diet-fed group) and insulin (1.4 ± 0.2 mM; $p = 0.0014$ versus untreated diet-fed group) levels. Consequently, HOMA index was also reduced

Table 1
The effects of fenofibrate on physiological and metabolic profiles of dietary-induced obese rats

	Lean controls ($n = 9$)	Diet-fed, untreated ($n = 9$)	Diet-fed, fenofibrate-treated ($n = 9$)
Epididymal fat-pad mass (g)	5.4 ± 0.6	11.2 ± 1.0[a]	7.2 ± 0.7[b]
Perirenal fat pad mass (g)	6.1 ± 0.5	10.9 ± 1.4[a]	6.8 ± 0.8[b]
% Lean mass	83.6 ± 2.0	76.3 ± 1.6[c]	80.7 ± 1.8
% Fat mass	16.4 ± 2.0	23.7 ± 1.6[c]	19.3 ± 1.8
Gastrocnemius muscle mass (g)	3.2 ± 0.1	3.4 ± 0.1	3.1 ± 0.1
Soleus muscle mass (g)	0.21 ± 0.01	0.22 ± 0.01	0.21 ± 0.01
HOMA index	0.73 ± 0.10	2.40 ± 0.52[a]	0.67 ± 0.10[b]
Plasma glucose (mM)	11.7 ± 1.2	14.3 ± 0.3	10.8 ± 0.3[b]
Plasma insulin (μg/l)	1.6 ± 0.3	3.8 ± 0.8[a]	1.4 ± 0.2[b]
Plasma leptin (ng/ml)	6.9 ± 0.5	9.4 ± 0.7[a]	8.5 ± 0.4[a]
Plasma triglycerides (mM)	0.79 ± 0.04	1.68 ± 0.17[a]	0.80 ± 0.06[b]
Plasma NEFA (mM)	0.44 ± 0.02	0.45 ± 0.04	0.31 ± 0.02[a,b]

Data are mean ± S.E.M.
[a] $p < 0.001$ vs. lean control.
[b] $p < 0.001$ vs. untreated diet-fed.
[c] $p < 0.01$ vs. lean control and fenofibrate treated diet-fed.

(0.67 ± 0.10; $p = 0.0073$ versus untreated diet-fed group), indicating correction of insulin resistance (Table 1).

Diet-fed rats had significantly higher plasma leptin levels than chow-fed control (9.4 ± 0.7 diet-fed versuss 6.9 ± 0.5 ng/ml chow-fed; $p = 0.0131$). The rise in letpin levels was not affected by fenofibrate treatment (8.5 ± 0.4 ng/ml; $p = 0.03612$ versus chow-fed). Fasting plasma levels of NEFA were comparable in diet-fed and chow-fed groups (0.44 ± 0.02 chow-fed; 0.45 ± 0.04 mM diet fed). However, diet-fed rats had significantly higher (>110%) levels of fasting TG than chow-fed (chow-fed: 0.79 ± 0.04; diet-fed: 1.68 ± 0.17 mM; $p = 0.0004$). Fenofibrate treatment significantly lowered fasting plasma levels of both NEFA (0.31 ± 0.02 mM; $p = 0.0012$ versus chow-fed and untreated diet-fed groups) and TG (0.80 ± 0.06 mM; $p = 0.0001$ versus untreated diet-fed group) below those of chow-fed group (Table 1).

3.3. Vascular data

Diet-induced obesity did not alter vessel diameter, and there were no significant differences in arterial diameter between the three groups (200 ± 15 in chow-fed; 205 ± 15 in diet-fed; 200 ± 10 μm in fenofibrate-treated).

3.4. Agonist-induced vasoconstriction responses

Cumulative concentration–response curves of KCl (10–125 mM) and NA (0.5–6 μM) in arteries from all three groups showed the characteristic sigmoid form. KCl-induced contraction was similar in all three groups, and the concentration–response curves were virtually superimposable, reaching maximum force of 5.68 ± 0.44 (chow-fed), 6.07 ± 0.33 (diet-fed) and 5.35 ± 0.37 (fenofibrate-treated) mN/mm artery (Fig. 2a). There was no significant differences between the groups, either overall (by ANOVA) or at any given KCl concentration. A similar pattern was also seen with NA-induced contractions, giving maximum force of 9.14 ± 0.63 (chow-fed), 9.94 ± 0.43 (diet-fed) and 9.70 ± 0.84 (fenofibrate-treated) mN/mm artery (Fig. 2b).

3.5. Endothelium-dependent and -independent vasorelaxation

NA-preconstricted arteries from chow-fed rats demonstrated progressive relaxation to cumulative addition of CCh (10 nM–100 μM), achieving a maximum of 88 ± 2% at a CCh concentration of 100 μM, with EC_{50} values of 0.28 ± 0.03 μM. Arteries from diet-fed rats that were similarly exposed to CCh displayed a significant rightward shift of the concentration–response curve, as compared with chow-fed control arteries (EC_{50} diet-fed: 0.94 ± 0.04 μM, $p = 0.0023$ versus chow-fed) (Fig. 3a). Maximal vasorelaxation to 100 μM CCh was reduced by 17% in diet-fed rats ($p = 0.0069$ versus chow-fed). Fenofibrate treatment did not improve EC_{50} values (0.86 ± 0.04 μM, $p = 0.0025$ versus chow-fed), but it enhanced maximal CCh-induced vasorelaxation at higher concentrations (>10 μM), achieving 86 ± 3% relaxation at 100 μM CCh (Fig. 3a).

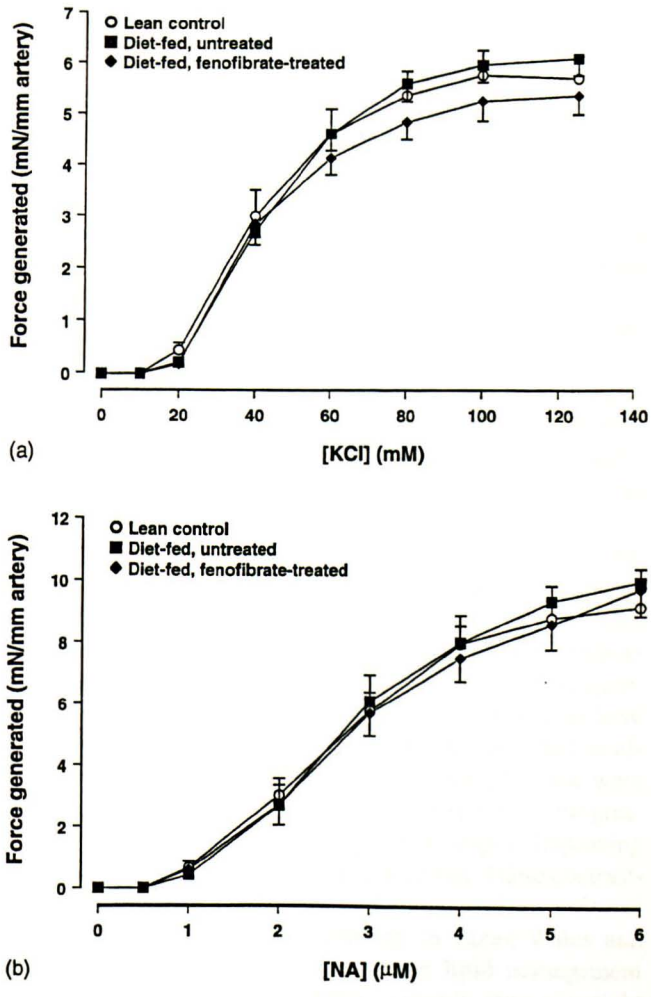


Fig. 2. Vasoconstriction responses to cumulative concentration of (a) KCl (10–125 mM) and (b) noradrenaline (0.5–7 μM) in arteries from lean control, untreated diet-fed and fenofibrate-treated diet-fed animals. Data represent mean ± S.E.M. for nine animals in each group. There were no significant differences in agonist-induced vasoconstriction between the three groups.

Feeding the high-palatable diet did not affect concentration-dependent vasorelaxation induced by SNP (10 nM–100 μM). The concentration–relaxation response curves were identical and virtually superimposable in all three groups, with EC_{50} values of 2.08 ± 0.07 (chow-fed), 1.73 ± 0.12 (untreated obese) and 1.46 ± 0.10 μM (fenofibrate-treated) (Fig. 3b). There were no statistically significant differences between all three groups, either overall (by ANOVA) or at any given SNP concentration.

4. Discussion

Recent studies in animal models and in subjects with diabetes have shown that PPARα activation reduces diet-induced weight gain and visceral fat mass and improving insulin

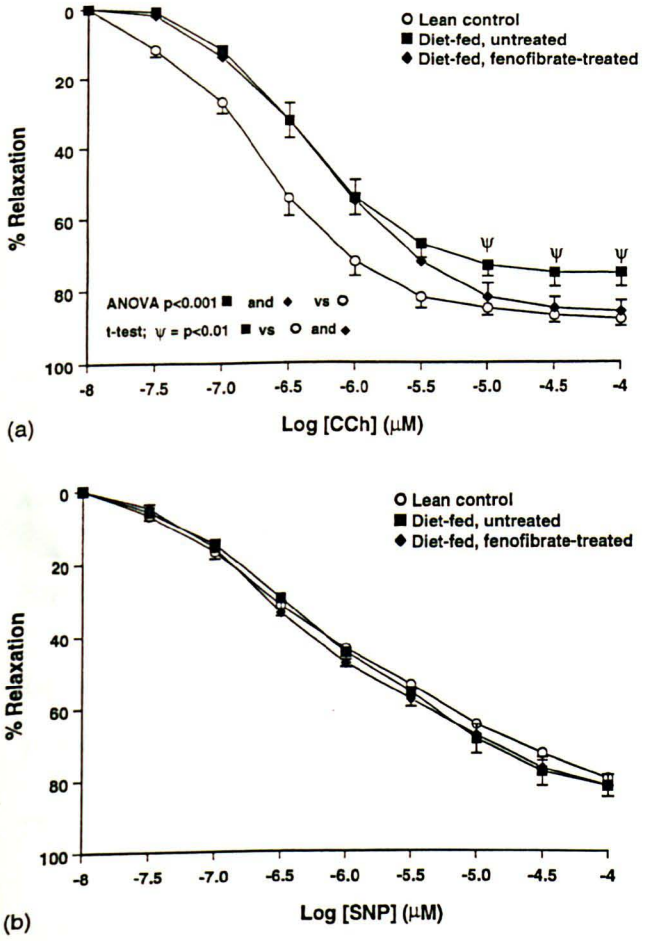


Fig. 3. The effects of (a) carbamylcholine (CCh), (b) sodium nitroprusside (SNP) on arteries from lean control, untreated diet-fed and fenofibrate-treated diet-fed rats. Noradrenaline (8 μM) preconstricted arteries challenged with cumulative concentrations of CCh or SNP. CCh-induced vasorelaxation was significantly reduced in diet-fed animals (ANOVA, $p < 0.001$) in comparison to lean control group. Moreover, vasorelaxation curves were shifted to the right in both diet-fed groups. Fenofibrate treatment only partially restored CCh-induced vasorelaxation. Data represent mean ± S.E.M. for nine animals in each group.

resistance [17,18]. The precise mechanism(s) by which PPARα of improves insulin resistance is not fully known yet, but increasing fatty acid catabolism in liver, decreasing skeletal muscle TG content or decreasing production of cytokines associated with insulin resistance pathways [19], counteracting hypertriglyceridaemia by increasing hepatic oxidation of fatty acids and reduced synthesis and secretion of TG [20] have all been postulated as possible mechanisms by which PPARα activation improves insulin resistance. Moreover, The mechanism of PPARα-induced reduction in weight gain also remains controversial. There is a suggestion that a reduction in fat mass and consequential decrease in leptin production may ultimately induce hypophagia and the resultant decrease in weight gain [21], while another study reported lack of any association between plasma levels of leptin, food intake and inhibition of weight gain by PPARα agonist, fenofibrate treatment [18].

We and others [2,6,22] have shown that dietary obesity not only causes metabolic abnormalities but also markedly affects vascular function, in particular endothelium-dependent agonist-induced vasorelaxation. The vascular abnormalities induced by dietary obesity are complex and multifactorial. Possible factors include fat mass per se, insulin resistance [3], increased NEFA levels [22] hypertriglyceridaemia [2] decrease in plasma HLD-c levels [11] and hyperleptinaemia [4]. The key players could, in theory, be identified through interventions that aim to correct these abnormalities and findings, which leads to the amelioration of vascular dysfunction.

In this study, in agreement with previous reports [16,23] 8-week treatment of fenofibrate reduced total body weight, weekly weight gain and adiposity. Moreover, fenofibrate treatment completely corrected dietary-induced insulin-resistance, hyperinsulinaemia and hypertriglyceridaemia but not hyperletinaemia.

By contrast, the beneficial effects of fenofibrate on endothelial function were relatively minor and only appeared at higher concentration of carbamylcholine (>31.6 μM CCh). The failure of fenofibrate to completely correct dietary-induced endothelial dysfunction seen in our study is in agreement with various previous reports. Studies by others have not shown any significant changes in endothelial function after acute treatment with 250 mg fenofibrate [24], nor were there any beneficial effects on blood pressure or progression of atherosclerosis process [23,25] despite improving insulin sensitivity and hypertriglyceridaemia. These contrasting findings could be explained by differences in experimental conditions. For example, subjects in Idzior-Walus and colleagues' [26] study had been under lipid management for at least 12 months, establishing a steady state of weight and lipid parameters and those in Malik and colleagues' [27] study did not include obese or insulin-resistant subjects. Another recent study [12] reported that fenofibrate treatment (2 weeks) significantly reduced fasting TG levels and improved both endothelium-dependent and -independent vasorelaxation. Once again, these patients underwent specific dieting for at least 2 weeks before any vascular analysies were carried out. Taking these observations together, it is possible to hypothesise that withdrawal of a fat-enriched diet attenuated vascular dysfunction in these subjects and that this may have further improved by administration of fenofibrate, as seen in our study. Consistent with an effect of diet per se, we have recently reported that acute withdrawal of an obesity-inducing diet partially restores normal endothelial function [28] while chronic withdrawal completely corrects dietary-obesity-induced vascular dysfunction [29], indicating that diet per se rather than dietary-induced obesity plays a key role in vascular abnormalities. However, it is also possible to argue that hyperletinaemia [4] seen in diet-fed rats (both vehicle- and fenofibrate-treated rats) may, at least in part, explain the limited beneficial effects of fenofibrate on vascular function. Nonetheless, we have previously shown that diet-induced vascular dysfunction occurs irrespective of circulating plasma leptin levels [30].

In conclusion, our study indicates that fenofibarte treatment in the presence of obesity-inducing diet decreases adiposity, lowers plasma TG and NEFA levels and improves insulin resistance in dietary-obese animals, but has little effect on vascular function. The lack of marked beneficial effects of fenofibrate on vascular function may be, at least in part, due to the continuous presence of detrimental factors in obesity-inducing diet, of which high sucrose and/or fats may play important roles. Furthermore, findings from this study indicate that pharmacological attenuation of adiposity alone does not alleviate obesity-induced endothelial dysfunction, and that removal of obesity-inducing diet is an important step in correcting vascular dysfunction.

Acknowledgments

This study was supported by a grant from British Heart Foundation (FS/02/002). EKN is a BHF Research Fellow.

References

[1] Renier G, Desfaits AC, Serri O. Gliclazide decreases low-density lipoprotein oxidation and monocyte adhesion to the endothelium. Metabolism 2000;49:17–22.
[2] Naderali EK, Brown MJ, Pickavance LC, Wilding JP, Doyle PJ, Williams G. Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role for triglycerides. Clin Sci 2001;101:499–506.
[3] Reaven GM. Role of insulin resistance in human disease. Diabetes 1988;37:1595–607.
[4] Wallace AM, McMahon AD, Packard CJ, et al. Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). Circulation 2001;104:3052–6.
[5] Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000;101:948–54.
[6] Naderali EK, Pickavance LC, Wilding JP, Doyle PJ, Williams G. Troglitazone administration corrects metabolic abnormalities but not vascular dysfunction in rat with dietary obesity. Eur J Pharmacol 2001;416:133–9.
[7] Mancini FP, Lanni A, Sabatino L, et al. Fenofibrate prevents and reduces body weight gain and adiposity in diet-fed obese rats. FEBS Lett 2001;491:154–8.
[8] Steiner G, Hamsten A, Hoskin J, et al. Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes. Lancet 2001;357:905–10.
[9] Liang B, McMaster JC, Kroeger EA, et al. The effect of fenofibrate treatment on endothelium-dependent relaxation induced by oxidative modified low density lipoprotein from hyperlipidemic patients. Mol Cell Biochem 2000;207:123–9.
[10] Fruchart JC, Duriez P, Staels B. Molecular mechanism of action of the fibrates. J Soc Biol 1999;193:67–75.
[11] Desprēs JP, Lemieux I, Salomon H, Delaval D. Effects of micronized fenofibrate versus atrovastatin in the treatment of dyslipidaemic patients with low plasma HDL-cholestrol levels: a 12-week randomised trial. J Intern Med 2002;251:490–9.
[12] Capell WH, DeSouza CA, Poirier P, et al. Short-term triglyceride lowering with fenofibrate improves vasodilator function in subjects with hypertriglycerideamia. Arterioscler Thromb Vasc Biol 2003;23:307–13.
[13] Haak T, Haak E, Kusterer K, Weber A, Kohleisen M, Usadel KH. Fenofibrate improves microcirculation in patients with hyperlipidemia. Eur J Med Res 1998;3:50–4.
[14] Playford DA, Watts GF, Best JD, Burke V. Effects of fenofibrate on brachial artery flow-mediated dilatation in type 2 diabetes mellitus. Am J Cardiol 2002;90:1254–7.
[15] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeaostasis model assessment: insulin resistance and b-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.
[16] Naderali EK, Doyle O, Williams G. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea pigs. Clin Sci 2000;98:537–43.
[17] Lee HJ, Choi SS, Park MK, et al. Fenofibrate lowers abdominal and skeletal adiposity and improves insulin sensitivity in OLETF rats. BBRC 2002;296:293–9.
[18] Guerre-Millo M, Gervois P, Raspe E, et al. Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. J Biol Chem 2000;275:16638–42.
[19] Wang PR, Guo Q, Ippolito M. High fat fed hamster, a unique animal model for treatment of diabetic dyslipideamia with peroxisome proliferator activated receptor alpha selective agonists. Eur J Pharmacol 2001;427:285–93.
[20] Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088–93.
[21] Kubota N, Terauchi Y, Miki H, et al. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol Cell 1999;4:597–609.
[22] Steinberg HO, Chacker H, Leaming R, Johnson A, Brechtel G, Baron D. Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance. J Clin Invest 1996;97:2601–10.
[23] Furuhashi M, Ura N, Murakami H, et al. Fenofibrate improves insulin sensitivity in connection with intramuscular lipid content, muscle fatty acid-binding protein, and beta-oxidation in skeletal muscle. J Endocrinol 2002;174:321–9.
[24] Bae JH, Bassenge E, Lee HJ, et al. Impact of postprandial hypertriglyceridaemia on vascular responses in patients with coronary artery disease: effects of ACE inhibitors and fibrates. Atherosclerosis 2001;158:165–71.
[25] Wilmink HW, Twickler MB, Banaga JD, et al. Effects of statin versus fibrate on post-prandial endothelial dysfunction: role of remnant-like particles. Cardiovasc Res 2001;50:577–82.
[26] Idzior-Walus B, Sieradzki J, Rostworowski W, et al. Effects of comicronised fenofibrate on lipid and insulin sensitivity in patients with polymetabolic syndrome X. Eur J Clin Invest 2000;30:871–8.
[27] Malik J, Melenovsky V, Wichterle D, et al. Both fenofibrate and atrovastatin improve vascular reactivity in combined hyperlipidaemia (fenofibrate versus atrovastatin trial-FAT). Cardiovasc Res 2001;52:290–8.
[28] Naderali EK, Williams G. Prolonged endothelial-dependent and -independent arterial dysfunction induced in the rat by short-term feeding with a high-fat, high-sucrose diet. Atherosclerosis 2003;166:253–9.
[29] Naderali EK, Fatani S, Williams G. Chronic withdrawal of a high-palatable obesity-inducing diet completely reverses metabolic and vascular abnormalities associated with dietary-obesity in the rat. Atherosclerosis 2004;172:63–9.
[30] Naderali EK, Pickavance LC, Wilding JPH, Williams G. Diet-induced endothelial dysfunction is the rat is independent of the degree of increase in total body weight. Clin Sci 2001;100:635–41.