8-2011

Genetic and nutritional studies to elucidate the role of adipose tissue in the pathogenesis of metabolic syndrome

Nishan Sudheera Kalupahana
nkalupah@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Nishan Sudheera Kalupahana entitled "Genetic and nutritional studies to elucidate the role of adipose tissue in the pathogenesis of metabolic syndrome." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

Naima Moustaid-Moussa, Major Professor

We have read this dissertation and recommend its acceptance:

David R. Bassett Jr., Brynn Voy, Guoxun Chen

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

GENETIC AND NUTRITIONAL STUDIES TO ELUCIDATE THE ROLE OF ADIPOSE TISSUE IN THE PATHOGENESIS OF METABOLIC SYNDROME

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Nishan Sudheera Kalupahana
August, 2011

DEDICATION

To my parents, Wimala and Weerasena Kalupahana, for their guidance;

My wife Anuradhi for her patience, love and support;

And Chaithri for making everything worthwhile

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Naima Moustaid-Moussa, for giving me the opportunity to work in her lab. She epitomized mentorship at the highest level, by not only guiding me in my research, but also providing me numerous opportunities to participate in training programs, develop collaborations, grant and research writing skills, enabling me to become an independent researcher.

I would like to thank Dr. Shamila Rajaratne and Dr. Jay Wimalasena for helping me in starting my graduate work at the University of Tennessee and for their continuous support throughout. I really appreciate the guidance and encouragement extended for the research by my current dissertation committee members, Dr. David Bassett Jr., Dr. Brynn Voy and Dr. Guoxun Chen and former committee member Dr. Jung Han Kim.

The work in this dissertation research was a collaborative effort. In this regard, I would like to acknowledge the following current and past members of the Moustaid-Moussa lab for their kind support: Dr. Morvarid Soltani-Bejnood, Sarah Fletcher, Suzanne Booker, Taryn Stewart, Rachael Hadidsaz, Lorin Hall, Christina Raney, Wenting Xin, Dr. Nalin Siriwardhana, Richard J. Giannone, Hyoung-Yon Kim, Allison Stewart, Kristelle Levenez and Brian Cohen. I also extend my gratitude to the following collaborators: Dr. Shelly Newman and Dr. Arnold Saxton form the University of Tennessee Institute of Agriculture, Dr. David Wasserman from Vanderbilt University, Dr. Kate Claycombe from USDA-ARS, Dr. Alice H. Lichtenstein and Dr. Nirupa Matthan from Tufts University and Dr. Florence Massiera, Dr. Annie Quignard-Boulange and Dr. Gérard Ailhaud from France.

Finally, I would like to thank the American Heart Association, American Society for Nutrition, USDA, University of Tennessee Obesity Research Center, Department of Nutrition,

Department of Animal Science, UT AgResearch and the College of Education, Health and Human Sciences for their funding support.

ABSTRACT

Obesity is a major health problem in the United States and worldwide. It increases the risk for type-2 diabetes and cardiovascular diseases. A chronic low-grade inflammation occurring in white adipose tissue (WAT) is causally linked to the development of insulin resistance (IR), metabolic syndrome and obesity-associated chronic diseases. The aim of this dissertation research was to elucidate the WAT function in metabolic syndrome using genetic (overexpression of an adipose pro-inflammatory hormone, angiotensinogen) and nutritional manipulations/approaches (caloric restriction and omega-3 fatty acids), with specific emphasis on the role of inflammation.

Previous research indicates that WAT renin-angiotensin system (RAS) is overactivated in obesity. However, its role in the pathogenesis of IR is hitherto unknown. Using mice overexpressing angiotensinogen (Agt), the only precursor for the hypertensive hormone angiotensin (Ang) II, in WAT, we showed that adipose-specific RAS overactivation leads to systemic IR. This is at least in part due to Ang II, NADPH oxidase and NF-kB-dependent increases in WAT inflammation.

Caloric restriction is the main dietary intervention to treat obesity-associated metabolic disorders. While most health agencies recommend a low-fat diet, energy-restricted high-fat diets (HFR) are also claimed to be effective in this regard. Here, we show that weight loss due to HFR is accompanied by improvements of IR but only partial resolution of WAT inflammation. Further, this diet negatively impacted the adipokine profile supporting the current recommendations for low-fat diets.

Dietary interventions targeted at reducing WAT inflammation have not been explored in detail. Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid of marine origin

with anti-inflammatory properties. We show that EPA is able to both prevent and reverse high-fat diet-induced IR and hepatic steatosis via modulation of WAT inflammation.

In conclusion, primary changes occurring in WAT, such as overexpression of Agt, can lead to WAT inflammation and systemic IR. Moreover, nutritional interventions targeting at reducing adiposity (caloric restriction) and inflammation (EPA) can both lead to improvements in systemic IR. Our findings support the current recommendation of low-fat diets for improvement in metabolic profile and show that dietary modulation of WAT function can be used to improve metabolic derangements in obesity.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

ABBREVIATIONS

[2-^{3}H]DG	[^{3}H]-labeled 2-deoxyglucose
2-D DIGE	2 dimensional difference gel electrophoresis
AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ACEI	ACE inhibitor
Agt	Angiotensinogen
ALA	α-linolenic acid
AMPK	AMP-activated protein kinase
Ang	Angiotensin
ANOVA	Analysis of variance
Ap2	Adipocyte protein 2
ARB	AT_1 blocker
AT_1	Angiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
AT_4	Angiotensin type 4 receptor
ATM	Adipose tissue macrophage
AUC	Area under the glucose curve
BMI	Body mass index
BSA	Bovine serum albumin
Ccr	C-C motif chemokine receptor
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
ChREBP	Carbohydrate-responsive element-binding protein
COX	Cyclooxygenase
DAG	Diacylglycerol
DGAT	DAG acyltransferase
DHA	Docosahexaenoic acid
DIO	Diet-induced obesity
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FAS	Fatty acid synthase

FDR	False discovery rate
FOXO	Forkhead box O
FTO	Fat mass and obesity associated
GH	Growth hormone
GHRKO	Growth-hormone receptor knockout
Glut	Glucose transporter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GO	Gene ontology
GPR	G protein-coupled receptor
Grb	Growth factor receptor-bound protein
GSK	Glycogen synthase kinase
GTT	Glucose tolerance test
GWAT	Gonadal white adipose tissue
HDL	High-density lipoprotein
HF	High saturated fat
HF-EPA-P	High saturated fat EPA prevention
HF-EPA-R	High saturated fat EPA reversal
HFR	Energy-restricted high-fat diet
HOMA-IR	Homeostasis model assessment of insulin resistance
IKK	Inhibitor of kB kinase
IMTG	Intramyocellular triglyceride
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase
LA	Linoleic acid
LDL	Low-density lipoprotein
LF	Low-fat
LPS	Lipopolysaccharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MGL	Macrophage galactose N-acetyl-galactosamine specific lectin
MGLL	Monoglyceride lipase
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic acid
mTOR	Mammalian target of rapamycin
(n-3) LC-PUFA	Long-chain (n-3) PUFA
NADPH	Nicotinamide adenine dinucleotide phosphate

NCoR	Nuclear receptor corepressor
NF-kB	Nuclear factor kB
NGF	Nerve growth factor
NHANES	National health and nutrition examination survey
PAI	Plasminogen activator inhibitor
PDK	3-phosphoinositide-dependent protein kinase
PG	Prostaglandin
PH	Pleckstrin-homology
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PS	Penicillin / streptomycin
PSGL	P-selectin glycoprotein ligand
PUFA	Polyunsaturated fatty acid
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RAS	Renin angiotensin system
RBP	Retinol binding protein
Rg	Tissue-specific index of glucose metabolism
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Significance analysis of microarrays
SCD	Stearoyl-CoA desaturase
SD	Sprague-Dawley
SH	Src-homology
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
SPARC	Secreted protein acidic and rich in cysteine
SREBP	Sterol regulatory element-binding protein
SVF	Stromal vascular fraction
TAB	TAK-1 binding protein
TAG	Triacylglycerol
TAK	TGF-β activated kinase
TCA	Tricarboxylic acid
Tg	Transgenic
TGF	Transforming growth factor
Th	Helper T cell
TIMP	Tissue inhibitors of MMP
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization
Wt	Wild-type
ZF	Zucker fatty

CHAPTER I
INTRODUCTION AND OVERVIEW

Obesity is a major health problem in the United States and worldwide. Obesity increases the risk of premature death and several co-morbidities such as Type 2 diabetes, cardiovascular disease and several forms of cancer. It is also a feature of the metabolic syndrome which is characterized by abdominal obesity, hypertension, dyslipidemia and hyperglycemia. Individuals with the metabolic syndrome are at a higher risk of developing the obesity-associated co-morbidities. Recent evidence has causally linked obesity and increased adiposity to the pathogenesis of metabolic syndrome.

Adipose tissue is an endocrine organ which secretes numerous bioactive peptides collectively known as adipokines. Further, obesity is characterized by a chronic low-grade inflammation and a dysregulation of adipokine secretion in the adipose tissue, which can lead to insulin resistance and metabolic syndrome. Angiotensin II, generated from its precursor angiotensinogen, is among the adipokines of interest to us. We have used a series of genetic and dietary manipulations to study the role of adipose tissue in the pathogenesis of the metabolic syndrome. First, we used a mouse model which overexpresses angiotensinogen to dissect the role of adipose tissue renin angiotensin system overactivation in the pathogenesis of insulin resistance in obesity. Next we used different dietary approaches to alleviate the adipose tissue inflammation and metabolic dysfunctions caused by high-fat feeding. In this regard we studied the role of high-fat reduced energy diets and high-fat diets supplemented with omega-3 fatty acids to study the dietary modulation of adipose tissue dysfunction in obesity.

The findings of this work will help in furthering the understanding of adipose tissue dysfunction in obesity and help in development of dietary and pharmacological interventions to prevent and treat obesity-associated co-morbidities.

CHAPTER II
LITERATURE REVIEW

Obesity and Type-2 diabetes - trends in the United States and the world

Obesity is a major health problem in the Unites States (US) with an estimated 68% of the adult population being overweight or obese [1]. The classification of overweight and obesity are based on the calculation of body mass index (BMI), which is defined as weight in kilograms divided by the square of the height in meters. Overweight is defined as a BMI of 25 to 29.9 kg/m^2 and obesity as a BMI of greater than or equal to 30 kg/m^2 [2]. According to the National Health and Nutrition Examination Survey (NHANES) data [1], the prevalence of overweight and obesity in the US was 44.9% in 1960-62 which increased to 67.9% in 2007-08 (Figure 1). The increasing prevalence of obesity is also a problem worldwide. According to the World Health Organization (WHO), approximately 1.6 billion and 400 million adults were overweight and obese respectively in the world in 2005 [3].

According to NHANES data, the prevalence of obesity among children and adolescents (defined as BMI greater than or equal to 95^{th} percentile from the CDC growth charts) aged 2-19 years in US was 16.9% in 2007-2008 [4]. This is a significant increase from the reported value of 5.5% for this group in 1976-1980.

Obesity increases the risk of premature death [5] as well as several co-morbidities. These include cardiovascular diseases [6], type-2 diabetes [7], several forms of cancer [8], obstructive sleep apnea [9], osteoarthritis [10], asthma [11], depression [12] and gallbladder disease [13]. Thus, parallel to the increase in obesity rates, the prevalence

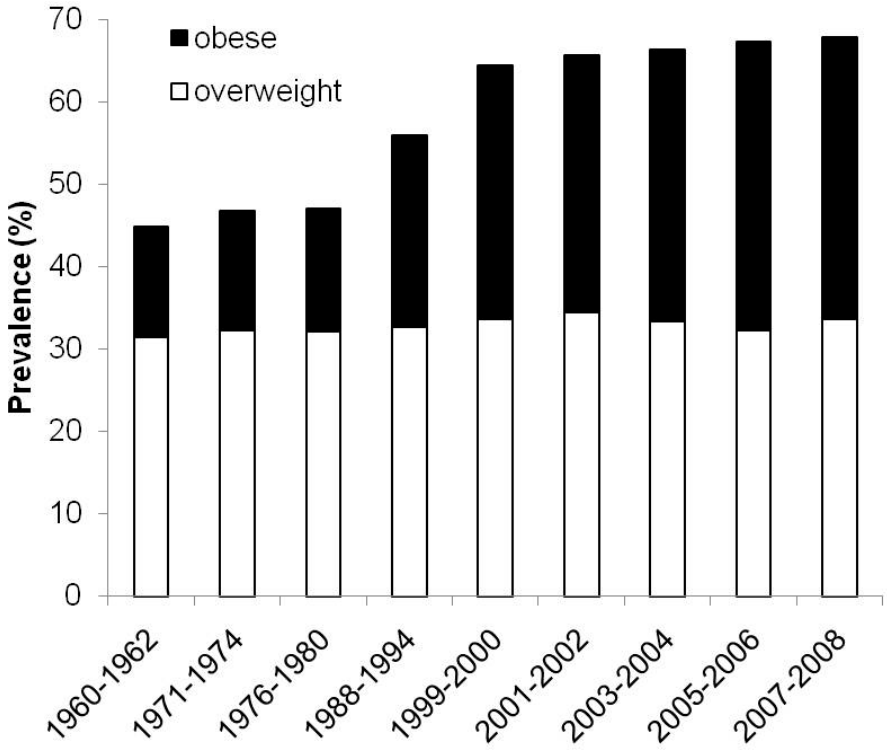

Figure 1. Prevalence of overweight and obesity in the United States

Change in prevalence of overweight and obesity in US adults from 1960 to 2008 is shown. Based on NHANES data from Flegal et al, 2010 [1]

of diabetes and other co-morbid conditions have also increased during the last few decades. For example, between 1980 and 2008, the number of US adults with type 2 diabetes has more than tripled (5.5 to 18.0 million) [14]. Worldwide, 150 million people had type 2 diabetes in 2001, which was projected to increase to 220 million in 2010 and 300 million by 2025 [15].

Etiology of obesity

While obesity is a manifestation of chronic positive energy balance, its exact etiology can be complex. Genes, environmental factors and gene-environment interactions are important factors in this regard. Twin and family studies have shown that genetic factors can account for 45–75% of the variation in BMI [16]. The two conventional approaches for identifying genes associated with a trait of interest are association studies and linkage analysis [17]. Using the former approach, where associations between candidate genes and phenotypes are identified, several monogenic forms of obesity have been discovered. These include mutations in leptin, leptin receptor, pro-opiomelanocortin and melanocortin 4 receptor and syndromic forms of obesity including Bardet-Biedl and Prader-Willi syndromes [18]. These single-gene syndromic and non-syndromic forms account for about 5% of obese individuals. More recently, large-scale genome-wide association studies have identified common genetic variations associated with differences in adiposity across populations. Single nucleotide polymorphisms (SNPs) of the fat mass and obesity associated (*FTO*) gene have been identified in this manner. However, such loci with risk alleles for obesity only account for

around 2% of the variation of BMI. Thus, it is likely that alleles with very low frequencies might explain at least part of the missing genetic contribution [19].

Although genetic factors are responsible for several forms of obesity, they alone are unlikely to be responsible for the recent increase in obesity trends. In this regard, environmental factors and gene-environment interactions become important factors for the recent rise in obesity rates. Indeed, physical activity is decreasing in the society especially among children [20]. Moreover, food availability has also become plentiful during this period. In this context, it is possible that the 'thrifty genotype' in some individuals interacts with these environmental conditions to manifest as obesity.

Obesity and metabolic syndrome

Obesity is associated with the metabolic syndrome, which is characterized by hyperglycemia, abdominal obesity, hypertension, elevated plasma triglycerides and reduced plasma high-density lipoprotein cholesterol (HDL) levels [21]. Individuals with the metabolic syndrome frequently have pro-inflammatory and pro-thrombotic metabolic profiles and are at a higher risk of developing type-2 diabetes and cardiovascular disease. Recent evidence has causally linked obesity and increased adiposity to the pathogenesis of metabolic syndrome and type 2 diabetes. Furthermore, adipose tissue dysfunction and inflammation play major roles in these disorders.

Adipose tissue dysfunction in obesity

Disclosure: The work described in this section has been submitted for the following publication, with minor modifications in the numbering of tables and figures:

"Kalupahana NS, Claycombe KJ and Moustaid-Moussa N. Omega-3 fatty acids alleviate adipose tissue inflammation and insulin resistance: mechanistic insights. Submitted to *Advances in Nutrition"*

Structure and function of adipose tissue

White adipose tissue is the major site for storage of excess energy in the body. It is composed of adipocytes, an extra-cellular matrix, vascular and neural tissues and other cell types [22]. These other cell types include preadipocytes, fibroblasts, stem cells and immune cells such as macrophages and T cells. Adipose tissue secretes numerous bioactive peptides collectively known as adipokines [23]. Examples include hormones involved in energy homeostasis such as leptin, peptides involved in glucose homeostasis such as adiponectin, resistin, apelin and visfatin, chemokines such as monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8, pro-inflammatory cytokines such as IL-6, IL-1, angiotensin (Ang)-II and tumor necrosis factor (TNF)-α, and anti-inflammatory peptides such as IL-10. Thus, adipose tissue is now considered to be an endocrine organ which plays a major role in energy balance, glucose homeostasis, blood pressure regulation and immune function [24]. A detailed list of adipokines and their functions are given in Table 1.

Distribution of body fat

The distribution of white adipose tissue throughout the body can be divided into two major divisions, viz. subcutaneous and visceral adipose tissue [25]. Subcutaneous fat

is mainly present in the gluteofemoral region, back and anterior abdominal wall, while visceral fat is present intra-abdominally. Since visceral fat is present around internal organs, and because it can drain directly into the liver via the portal vain, it is considered to be more metabolically active than subcutaneous fat. Moreover, the adipokine profile is different between the two fat depots, with the visceral depot having a more pro-inflammatory adipokine profile [26]. Thus, in contrast to subcutaneous fat, excessive accumulation of visceral fat is considered to be detrimental to metabolic health [27]. Indeed, epidemiological studies have shown that central obesity (increased visceral fat), is associated with overall mortality, insulin resistance, type 2 diabetes mellitus and cardiovascular disease [5]. Rodent models are frequently used to study mechanisms of obesity and its co-morbidities. In these animals, the inguinal and gonadal fat pads are considered to be comparable to subcutaneous and visceral fat depots respectively in humans.

Adipose tissue inflammation in obesity

Excessive triglyceride accumulation within adipocytes, as a result of positive energy balance, leads to adipocyte hypertrophy and a dysregulation of adipokine secretory patterns. This has been primarily linked to an unbalance between secretions of pro- vs. anti-inflammatory adipokines. Specifically, adipocyte hypertrophy is characterized by increased secretion of pro-inflammatory adipokines such as TNF-α [28],

Table 1. Major adipokines and their functions

Adipokine	Physiological effects	Ref.
Leptin	Reduces energy intake and increases expenditure, angiogenesis and hematopoiesis, immune functions	[29]
Adiponectin	Improves insulin sensitivity, anti-inflammatory, anti-atherogenic, promotes fatty acid oxidation	[30]
Resistin	Promotes insulin resistance	[31]
Angiotensin II	Vasoconstriction, sodium and water retention, increases blood pressure, promotes insulin resistance	[32]
MCP-1	Promotes macrophage infiltration and insulin resistance, pro-inflammatory, chemotaxic	[33]
TNF-α	Pro-inflammatory, promotes insulin resistance	[34]
PAI-1	Pro-thrombotic, pro-inflammatory	[35]
IL-6	Pro-inflammatory	[36]
IL-10	Anti-inflammatory	[37]
Visfatin	Insulin-mimetic actions, cell proliferation	[38]
Apelin	Promotes glucose uptake, angiogenesis	[39]
RBP-4	Promotes insulin resistance	[40]
VEGF	Angiogenesis	[41]
NGF	Neuronal development	[42]
IL-1	Pro-inflammatory	[43]
IL-1Ra	Anti-inflammatory	[43]
Vaspin	Insulin-sensitizing effects	[44]
Omentin	Regulates insulin action	[45]
Neuropeptide Y	Energy homeostasis, proliferation of preadipocytes	[46]
Hepcidin	Pro-inflammatory	[47]
IL-8	Pro-inflammatory, chemotaxic	[48]
IL-18	Pro-inflammatory	[49]
Thrombospondin-1	Pro-inflammatory	[50]
Chemerin	Impairs glucose tolerance	[51]

MCP-1 – monocyte chemotactic protein-1, TNF-α – tumor necrosis factor-α, PAI-1 – plasminogen activator-1, IL – interleukin, RBP-4 – retinol binding protein-4, VEGF - vascular endothelial growth factor, NGF – nerve growth factor; IL-1Ra – IL-1 receptor antagonist

IL-6 [26], angiotensinogen, Ang II [52], leptin [53] and MCP-1 [54] and reduced secretion of anti-inflammatory adipokines such as adiponectin [55] and IL-10.

Thus, obesity is associated with a chronic low-grade inflammation in the adipose tissue [56, 57]. Both adipocytes and macrophages are considered to be the source of proinflammatory cytokines in obesity [23, 58]. Major cellularity and immune changes between lean and obese adipose tissue is illustrated in Figure 2.

While the exact trigger for the onset of adipose tissue inflammation is hitherto unknown, several possible mechanisms have been suggested. In a state of positive energy balance, adipose tissue expands to accommodate the storage of excess triglycerides. Adipose tissue remodeling via degradation of the extracellular matrix (ECM) and adipogenesis are two key processes in this expansion. Matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP) play important roles in ECM degradation and adipose tissue remodeling [59, 60]. Defective adipose tissue expansion as a result of dysregulation of any of the above factors could lead to adipocyte injury, death and inflammation. For example, factors that promote adipose tissue fibrosis such as secreted protein acidic and rich in cysteine (SPARC) are associated with obesity and adipose tissue inflammation [61].

Rodent studies show that increasing adipose tissue mass without a similar magnitude increase in supporting vasculature could lead to tissue hypoxia, triggering the expression of hypoxia-inducible factor-1 and inflammatory genes [62]. Similarly, oxygen partial pressure in subcutaneous adipose tissue negatively correlates with adiposity in humans [63]. Thus, hypoxia could be a trigger for adipose tissue inflammation.

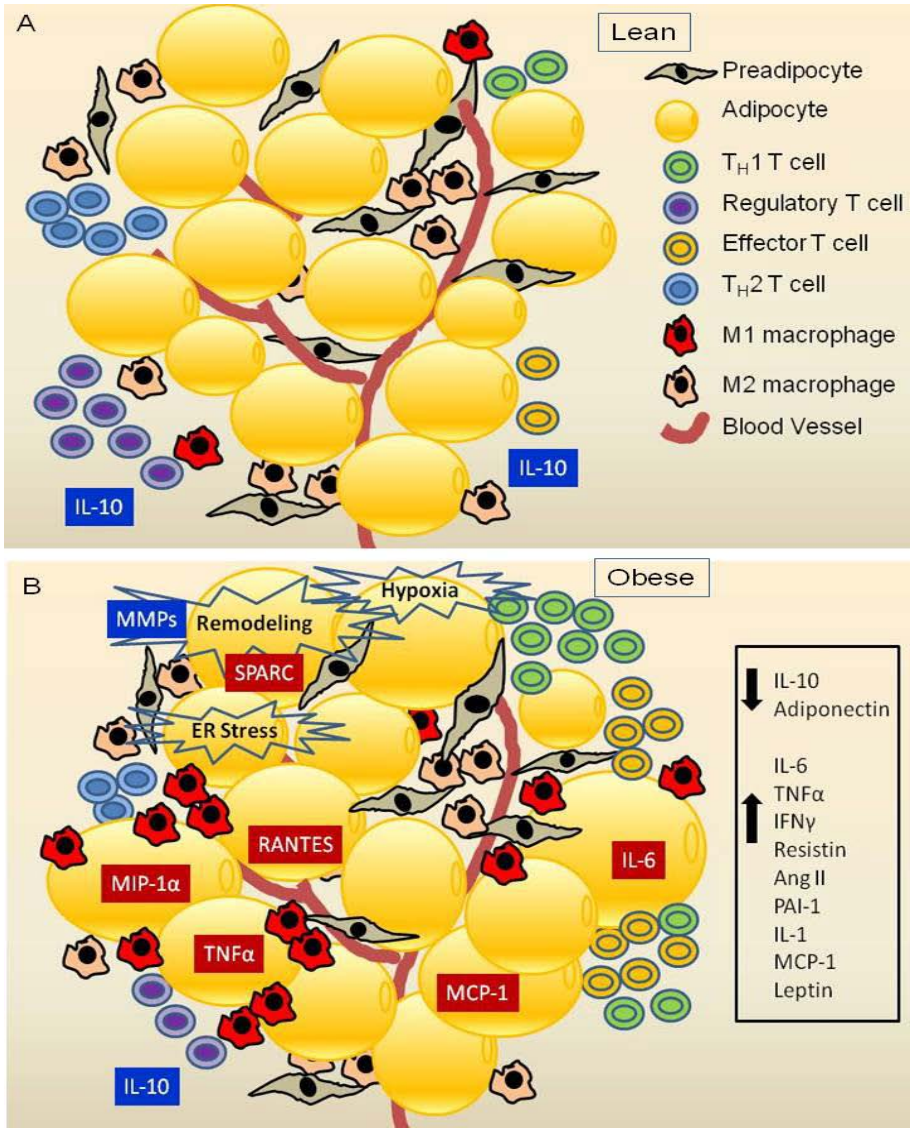

Figure 2. Immune cell infiltration of adipose tissue in obesity

Lean individuals have higher M2/M1 macrophage, T_H2/T_H1 T cell and Regulatory / Effector T Cell ratios (A). Excessive triglyceride accumulation leads to adipose tissue remodeling, relative hypoxia and ER stress which trigger production of chemokines and changes in the above cell ratios culminating in increased production of pro-inflammatory adipokines and reduced production of anti-inflammatory adipokines (B). MMPs-Matrix metalloproteinases, SPARC- secreted protein acidic and rich in cysteine

Further, several animal and human studies have suggested that adipose tissue endoplasmic reticulum (ER) stress is another important triggering event for subsequent inflammation in obesity [64-67].

While there is evidence that adipose tissue expansion *per se* is an important initiator of the inflammatory processes during the development of obesity, other lines of evidence suggest that dietary fats could also trigger this process. Indeed, the nuclear factor kappa B (NF-kB) pathway in the visceral adipose tissue is activated 2 hours after consumption of a meal rich in saturated fatty acids in rodents [68]. Other studies have shown similar chronic effects of fatty acids (saturated fatty acids and conjugated linoleic acid) in triggering inflammation in the adipose tissue [69-71]. Given that these fatty acids are ligands for Toll-like receptors (TLR) 2 and 4, and since TLR 2 and 4 are expressed in human adipocytes [72], it is likely that the effect of saturated fats on adipose tissue inflammation is mediated via these receptors. Indeed, obesity-induced adipose tissue inflammation is attenuated in mice with a mutation in TLR-4 [73].

Other pattern recognition receptors of the innate immune system such as Nod-like receptors are also implicated in obesity and high saturated fat-associated adipose tissue inflammation [74]. Conversely, there are studies showing that adipose tissue inflammation can be reduced without changing adipose mass [75, 76]. This is also consistent with our recent findings that omega-3 polyunsaturated fatty acids reverse high-fat induced metabolic disorders and adipose inflammation [77]. Taken together, the current research suggests a role of dietary fat in the onset of adipose tissue inflammation.

Adipose tissue inflammation in obesity is characterized by macrophage infiltration [56, 78] (Figure 2). Adipose tissue macrophages (ATM) are classified into two main types. M1 or classically activated macrophages are stimulated by interferon (IFN)-γ and lipopolysaccharide (LPS) and produce pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 and reactive oxygen species such as NO (Figure 2). M2 or alternatively activated macrophages are activated by IL-4 and IL-13 and express anti-inflammatory factors IL-10, transforming growth factor (TGF)-β, IL-1 receptor antagonist-a, IL-4 and arginase [79, 80]. Phenotypically, murine ATM express F4/80 antigen. The murine M1 ATM highly express cluster of differentiation (CD) 11c, while the M2 express macrophage galactose N-acetyl-galactosamine specific lectin 1 (MGL1) [81]. In contrast, human ATM express CD14, while CD11c is only poorly expressed. Human ATM also express CD206, CD209 and CD163 [81].

Obesity induces an M2 to M1 shift in ATM populations, characterized by a reduction in anti-inflammatory IL-10 and arginase production and an increase in pro-inflammatory TNF-α production [82] (Figure 2). This increase in M1 ATM could be due to either a ‘phenotypic switch’ from M2 to M1, or due to additional recruitment of M1 macrophages from blood vessels. Lipotoxicity of macrophages seems to play a major role in the phenotypic switch of M2 to M1 [83]. Detailed mechanisms of the M2 to M1 switch have previously been reviewed by Olefsky et al. [84]. Briefly, TLR4 ligands such as saturated fatty acids activate NF-kB and activator protein 1 transcription factors, leading to increased production of proinflammatory cytokines such as TNF-α, IL-6 and IL-1 giving rise to the M1 phenotype. In the lean adipose tissue, this is prevented by

repression of TLR4-responsive genes by nuclear receptor corepressor (NCoR) complexes. Peroxisome proliferator-activated receptor (PPAR) γ along with IL-4 and IL-13 prevent the signal-dependent turnover of NCoR and thus maintain the M2 phenotype.

Evidence for M1 recruitment originated from studies showing increased $MGL1^{-}$ C-C motif chemokine receptor $(Ccr)^{+}$ macrophages recruited around necrotic adipocytes in high-fat diet-fed mice; while the MGL^{+} ATM levels remain unchanged [85]. Adipose tissue from obese animals expresses high levels of chemokines such as MCP-1, Macrophage inflammatory protein-1α (MIP-1α) and regulated upon activation, normal T-cell expressed and secreted (RANTES), chemokine receptors such as Ccr2 and Ccr5 [56] and adhesion molecules such as P-selectin glycoprotein ligand-1 (PSGL-1) [86]. The expression of these chemokines, chemokine receptors and adhesion molecules play a major role in recruitment of macrophages to adipose tissue in obesity. Indeed, mice overexpressing MCP-1 in the adipose tissue have higher macrophage infiltration of adipose tissue, while MCP-1 knockout mice are protected from high-fat diet-induced adipose tissue macrophage infiltration [87]. Similarly, Ccr2 deficient mice also have lower ATM numbers [88], while PSGL-1 knockout mice are protected from HF diet-induced adipose inflammation [86]. In contrast, however, MIP-1α-deficient mice are not protected against high-fat diet induced adipose tissue macrophage infiltration [89]. Thus, MCP-1 is a key mediator of the initiation of adipose tissue inflammation in obesity. However, the exact mechanism of MCP-1 induction in obesity is not known. It is likely that adipocyte hypertrophy plays a role in this, since adipocyte size positively correlates with MCP-1 expression in humans [90, 91].

Recent evidence also points towards involvement of T cells in the adipose tissue inflammation in obesity [92]. Nishimura et al. showed that CD8 (+) effector T cells infiltrate the WAT in high-fat fed mice, with a concurrent reduction in CD4 (+) helper (Th) and regulatory (Treg) T cells [93]. Moreover, these changes occur before the adipose tissue infiltration with macrophages. The adipose tissue infiltration of macrophages is prevented by genetic depletion of CD8 (+) T cells. Feuerer et al. showed that the number of Treg cells in the WAT of obese mice is significantly lower than in lean ones [94]. Winer et al. showed that obese mice have a higher Th1/Th2 ratio promoting IFN-γ secretion from adipose tissue [95] (Figure 2). Taken together, this suggests that T cells are early modulators of adipose tissue inflammation in obesity. The cytokine profile of these T cells could play an important role in determining the M1/M2 phenotype of ATMs.

Role of adipose tissue in insulin resistance

Insulin resistance is defined as an inadequate response by insulin-sensitive tissues (liver, skeletal muscle and adipose tissue) to normal circulating levels of insulin [96]. At physiological levels, insulin inhibits hepatic glucose production, promotes skeletal muscle glucose uptake and inhibits lipolysis. Thus, insulin resistance leads to impairments in insulin-mediated suppression of hepatic glucose production, skeletal muscle glucose disposal and inhibition of lipolysis, leading to relative hyperglycemia and increased plasma levels of non-esterified fatty acids (NEFAs). In response to the relative hyperglycemia, there is a compensatory response by the pancreatic β cells, which secrete

more insulin. This hypersecretion of insulin in turn increases skeletal muscle glucose uptake and inhibits hepatic glucose production to maintain normoglycemia. Thus, insulin resistant individuals maintain normoglycemia through overproduction of and secretion of higher insulin levels. Long term insulin resistance and hypersecretion of insulin eventually leads to pancreatic β cell failure. These events results initially in prediabetes and glucose intolerance and later progresses to hyperglycemia and type-2 diabetes [97].

Insulin resistance can be either genetic or acquired. But in most instances it is due to an interaction of environmental / lifestyle factors with genetic factors. Obesity, sedentary lifestyle and aging are known causes of insulin resistance [96]. The contribution of adipose tissue to the pathogenesis of insulin resistance and metabolic syndrome is reviewed in detail below.

Molecular mechanisms of insulin resistance

Insulin exerts its physiological actions on insulin-sensitive tissues via activation of a cascade of intracellular signaling events, all of which have been previously reviewed [98, 99]. Insulin binds to the insulin receptor, which induces autophosphorylation as well as phosphorylation of downstream substrates including the insulin receptor substrates (IRS) at tyrosine residues. The family of IRS proteins contains 6 members. IRS-1 and IRS-2 are widely distributed, while IRS-3 is present mainly in adipocytes and brain. IRS-4 is expressed in embryonic tissue. Tyrosine phosphorylation of the IRS leads to its binding to Src-homology-2 domain (SH2 domain) of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K). This leads to activation of the catalytic subunit of

PI3K, which in turn catalyses the formation of lipid second messenger PIP_3. This allows proteins with pleckstrin-homology domains (PH domains) to bind to PIP_3, leading to their activation. Activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1), leads to activation of Akt / protein kinase B (PKB). Akt/PKB is a serine/threonine kinase which targets several downstream proteins. Activation of Rab small GTPases via phosphorylation and inactivation of AS160 by Akt, leads to cytoskeletal reorganization and translocation of glucose transporter-4 (Glut-4) into the cell membrane. This leads to increased glucose entry into cells.

Akt also phosphorylates and deactivates glycogen synthase kinase 3 (GSK3), which leads to activation of glycogen synthase and subsequent glycogen synthesis. Akt also regulates transcription of several genes involved in gluconeogenesis and lipogenesis via control of winged helix or forkhead (FOXO) class of transcription factors. For example, Akt inhibits the FOXO1-mediated activation of hepatic guconeogenic genes in the liver [98]. Thus, the net effect of these signaling cascades is an increased glucose entry into cells as well as increased flux of glucose into intra-cellular metabolic pathways (skeletal muscle and adipose tissue) and reduced gluconeogenesis (liver).

Downregulation of insulin receptor protein level, as seen in obesity, can result in insulin resistance [98]. Defective insulin signaling at various levels of the above cascade is also known to be associated with insulin resistance. A reduction in IRS protein levels is also associated with insulin resistance. Hyperinsulinemia itself can reduce IRS protein via transcriptional regulation [99]. Suppressor of cytokine signaling-3 (SOCS-3) sterically blocks the interaction between insulin receptor and IRS and contributes to insulin

resistance [100]. Serine phosphorylation of IRS by free fatty acids, cytokines [101] and activation of NF-kB-mediated inflammatory pathways [102] is also known to induce insulin resistance. SOCS1 and 3 are also known to induce degradation of IRS [103]. Further downstream, higher expression of the regulatory subunit of PI3K is also associated with insulin resistance [104].

Proposed mechanisms for obesity-induced insulin resistance

Obesity induces insulin resistance in skeletal muscle, liver and adipose tissue [84]. Several models have been put forward to explain mechanisms of obesity-induced insulin resistance. The chronic low-grade inflammation occurring in adipose tissue is considered to be a major factor in the pathogenesis of obesity-induced insulin resistance. There are several lines of evidence to support this model. First, adipose specific overexpression of proinflammatory cytokines such as MCP-1 or Agt induces whole-body insulin resistance [87, 105]. Second, neutralization or knock down of inflammatory mediators such as TNF-α, MCP-1, Ccr-2 and PSGL-1 protects rodents from HF diet-induced insulin resistance [34, 86-88]. Finally, overexpression of anti-inflammatory adipokines such as adiponectin protects rodents from HF diet-induced insulin resistance [106].

Increased pro-inflammatory cytokines can induce insulin resistance by several mechanisms. As outlined earlier, pro-inflammatory cytokines can induce SOCS3 expression, which in turn can inhibit insulin signaling by inhibiting IRS action [100]. Pro-inflammatory cytokines also activate numerous intracellular serine kinases such as jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK). These serine kinases can

also inhibit insulin signaling at various levels [102]. Indeed, JNK1 or IKK-β knockout mice and mice with adipose specific JNK inactivation are protected from insulin resistance [107-109]. Finally, increased circulating free fatty acid levels due to adipose tissue insulin resistance can in turn inhibit insulin signaling via serine phosphorylation of IRS [101] and lead to insulin resistance in skeletal muscle and liver.

While the imbalance of pro- and anti-inflammatory adipokines can induce insulin resistance via paracrine effects, the endocrine effects of these adipokines are especially important in the development of insulin resistance in skeletal muscle and liver [34]. For example, circulating levels of adiponectin, an adipokine exclusively secreted by the adipose tissue, are positively correlated with insulin sensitivity in both humans and rodents [110]. Moreover, individuals with high plasma adiponectin levels have a lower risk of developing type-2 diabetes [111]. Finally, abdominal adiposity correlates with plasma C-reactive protein (an acute-phase protein) levels, indicating that systemic markers of inflammation are also increased with obesity [112]. While adipose tissue inflammation in obesity plays a key role in the development of insulin resistance, adipose inflammation in the absence of obesity does not seem to induce insulin resistance [113]. Thus, it is important to use mouse models with at least some degree of obesity when studying the contribution of individual inflammatory mediators to insulin resistance.

Increased lipid deposition in skeletal muscle and liver is also considered to be a factor linked to the pathogenesis of insulin resistance [96]. Indeed, obese insulin sensitive individuals have lower skeletal muscle and liver lipids than obese insulin resistant individuals [114]. This ectopic lipid deposition is attributed to the inability of the adipose

tissue (mainly subcutaneous) to store excess energy due to reduced differentiation / remodeling capacity. Insulin resistance in adipocytes leading to increased lipolysis and plasma free fatty acid levels also contributes to lipid accumulation in these tissues. This is also characterized by increased visceral fat mass. While the exact mechanism of these defects in adipogenesis / remodeling is not known, pro-inflammatory cytokines such as TNF-α are implicated because of their known inhibitory effects on adipogenesis [115], in a PPARγ-dependent manner. Conversely, PPARγ agonists such as thiazolidinediones are known to increase both adipogenesis and insulin sensitivity [116].

While it was initially hypothesized that increased fatty acid availability to skeletal muscle inhibits glucose utilization via inhibition of key glycolytic steps (Randle hypothesis), current evidence suggests that this is related to impairments in glucose transport due to defective insulin signaling [117]. Indeed, increased lipid accumulation in the liver and skeletal muscle is associated with increased fatty acid flux, which leads to excessive accumulation of fatty acid intermediates such as ceramide [96]. These lipid intermediates activate intracellular serine kinases which can lead to inhibition of insulin signaling. Ceramide can also directly inhibit Akt [118]. Indeed, pharmacological inhibition of ceramide synthesis protects rodents from obesity-associated insulin resistance.

Role of the renin angiotensin system in the pathogenesis of insulin resistance in obesity

Disclosure: The work described in this section has been submitted for the following publication, with minor modifications in the numbering of tables and figures:

"Kalupahana NS and Moustaid-Moussa N. The renin-angiotensin system: a link between obesity and insulin resistance. Submitted to *Obesity Reviews"*

The renin angiotensin system (RAS) is traditionally known for its role in regulation of blood pressure, fluid and electrolyte balance [119]. Angiotensinogen (Agt), the main precursor peptide of this system, undergoes enzymatic cleavage by renin and angiotensin-converting enzyme (ACE) to form angiotensin II (Ang II), the main effector peptide of this system. Ang II exerts its physiological effects via two G-protein coupled receptors, viz. Ang II type 1 (AT_1) and type 2 (AT_2) receptors. In addition to the systemic RAS, several local ones also exist in organs such as brain, pancreas, heart and adipose tissue [32]. Because Ang II increases blood pressure through AT_1, ACE inhibitors (ACEI) and AT_1 blockers (ARB) are clinically used as anti-hypertensive agents.

Interestingly, epidemiological studies have shown that patients on ACEI or ARB have a lower risk of developing type 2 diabetes compared to ones treated with other anti-hypertensive medications [120]. Subsequent randomized controlled trials have also shown that RAS blockade improves glycemic control [121] and lowers the risk of developing type 2 diabetes [122]. Because there is evidence for RAS overactivation in obesity, and because RAS blockade improves insulin resistance, it is possible that RAS is implicated in the pathogenesis of insulin resistance in obesity. Evidence for this hypothesis, with specific emphasis on the role of adipose RAS on the pathogenesis of insulin resistance is reviewed.

Components of the RAS

Components of the classical RAS are well characterized. The common precursor of all bioactive angiotensin peptides is Agt (Figure 3). It is mainly secreted by the liver in lean individuals. Adipose tissue is another important source of Agt, especially in obese individuals [123]. Agt is cleaved by the enzyme renin to form angiotensin I (Ang I). Renin is mainly produced by the kidneys, and is a regulatory step in the RAS. Renin can also bind to the renin receptor, and increase the catalytic efficiency of Ang I formation [124]. Ang I is subsequently cleaved by ACE, present mainly in the lung and vascular endothelium, to produce Ang II. Alternatively, Ang II can also be formed by the action of cathepsins and chymase, especially in local RAS [119].

Ang II is the main effector peptide of the RAS, which exerts its effect via two G-protein-coupled receptors (GPR) AT_1 or AT_2. Stimulation of AT_1 induces vasoconstriction and aldosterone secretion from the adrenal cortex, resulting in increased blood pressure and sodium and water retention. Stimulation of AT_2 generally exerts blood pressure lowering effects. Ang I and Ang II can be cleaved to angiotensin (1-9) and (1-7) respectively by the action of recently discovered enzyme ACE2 (Figure 3). Angiotensin (1-7) can act on another GPR, the Mas receptor. Ang II is subsequently degraded by aminopeptidases to produce angiotensin III and IV. Angiotensin IV acts on the AT_4 receptor. Most components of the systemic RAS are also found in the adipose tissue [125]. Additionally, Agt can be cleaved by cathepsins and chymase to produce Ang II, bypassing the renin-ACE axis in the adipose tissue [125]. Thus, Agt production is a regulatory step in the adipose RAS.

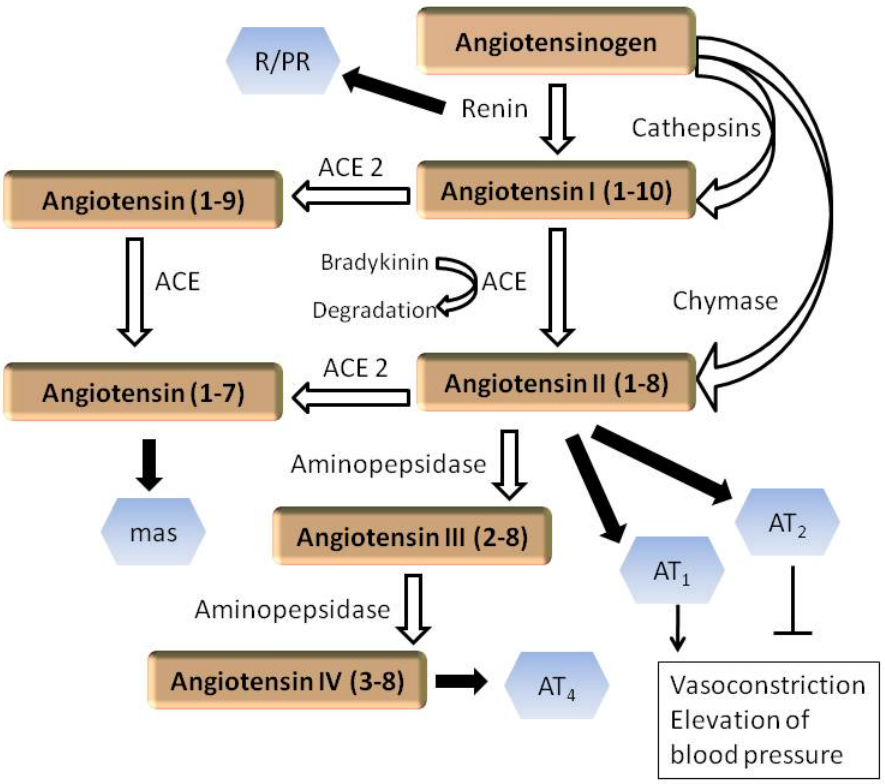

Figure 3: Components of the renin-angiotensin system

Angiotensinogen is cleaved by renin and angiotensin converting enzyme (ACE) to form angiotensin (Ang) I and II respectively. Ang II acts via Ang II type 1 (AT_1) or type 2 (AT_2) receptors to exert its physiological actions. Ang I and II can also be cleaved by ACE2 to form Ang (1-9) and (1-7), which in turn can act on the Mas receptor. Ang II is degraded to Ang III and IV. The latter can act on the AT_4 receptor. Renin can also act on the renin / pro-renin receptor (R/PR).

Association between RAS and obesity

Several polymorphisms of RAS genes are associated with body weight and adiposity. For example, the insertion/ deletion polymorphism (I/D) of *ACE* is associated with overweight and abdominal adiposity in Italian men [126]. Further, the M235T polymorphism of *AGT* is associated with visceral obesity in Japanese [127] and subcutaneous adipocyte size in French [128] women.

Obesity is also associated with overactivation of both systemic and adipose RAS in humans and animals (Table 2). In humans, obesity is associated with increases in plasma Agt, renin, ACE and Ang II (Table 2). The elevation of plasma Ang II following beta-adrenergic stimulation is also greater in obese than lean individuals [129]. Subcutaneous adipose tissue renin, ACE and AT_1 expression is also increased in obesity. Most, but not all studies show that adipose Agt expression is also higher in obese humans (Table 2). Moreover, weight-loss leads to reductions in plasma Agt, renin, ACE and adipose Agt levels [130]. Animal studies, in contrast, show that the direction of change in RAS components in obesity is strain-dependent (Table 2). Similar to humans, most diet-induced obese rodent models show overexpression of both systemic and adipose RAS components. When genetic models of obesity are considered, ob/ob and db/db mice show activation of systemic and adipose RAS, while the obese (fa/fa) Zucker rat, viable yellow mouse and Wistar fatty rat exhibit lower expression of systemic and adipose RAS components compared to lean littermates (Table 2). Taken together, this highlights the importance of selecting the correct animal model to study the role of systemic and adipose RAS in obesity.

Table 2. Association of RAS components with obesity

Subjects	RAS component	Association with obesity		
		Positive	No association	Negative
Humans	Plasma Agt	[131], [130], [132], [133], [134], [135]	[129], [136]	
	Plasma Renin	[130], [137], [138], [139]		
	Plasma ACE	[130], [135]		
	Plasma Ang II	[130]	[129]	
	Plasma Ang II (sympathetic stimulated)	[129]		
	Adipose Agt	[129], [52], [140], [141]	[128], [136]	[130], [142]
	Adipose AT_1	[136], [142]		
	Adipose Renin	[142]		
	Adipose ACE	[142]		
Animals	Plasma Agt	DIO-SD [143], DIO-B6 [123]		ZF [144]
	Plasma Ang II	DIO-SD [143], SF-Wistar rat [145]		
	Plasma renin	SD DIO [146], dogs [147]		ZF [148], [149]
	Adipose Agt	Ob/ob, Db/db [150], ZF [151], DIO-SD [143], DIO-B6 [152], [123] FF-SD [153]	FF-SD [154]	ZF, Viable yellow [32], Wistar fatty rat [155]
	Adipose AT_1	FF-SD [154]		ZF [156]
	Liver Agt		ZF, Viable yellow [32], [151], DIO-SD [143], DIO-B6 [123, 152]	

DIO – diet-induced obese; SD- Sprague-Dawley rat; B6- C57BL/6J mouse; ZF – Zucker fatty rat; SF – sucrose-fed; FF- fructose-fed

While there are discrepancies concerning adipose Agt expression in animal models of obesity, these studies consistently report no change in hepatic Agt production in obese compared to lean animals (Table 2). Considering that plasma Agt levels are increased in obesity, with relatively unchanged hepatic Agt production, this highlights the potential contribution of adipose-derived Agt to systemic levels. Indeed, adipose tissue may contribute up to 30% of plasma Agt level in obesity [157]. Further, studies in transgenic mice overexpressing Agt in adipose tissue demonstrate that overproduction by only about 20% can drive both adipocyte hypertrophy and high blood pressure [157].

Several studies have reported effects of overactivation of various RAS components or blockade on body weight and adiposity. Surprisingly, systemic RAS overactivation via chronic Ang II infusions or renin overproduction induces weight loss, rather than weight gain, in rodents (Table 3). This is attributed to initial reduction in energy intake and subsequent increase in energy expenditure [158, 159]. In contrast, adipose specific RAS overactivation via increased expression of Agt leads to increased adiposity [157]. Thus, it appears that paracrine/autocrine actions of Ang II in adipose tissue maybe important for fat mass expansion associated with RAS overexpression.

Pharmacological RAS blockade via ACE inhibitors or ARB reduce adiposity in rodents [160, 161], but not in humans. RAS blockade via genetic deletion of Agt, renin, ACE, AT_1 or AT_2 also protect rodents from diet-induced obesity (Table 3), suggesting a role of RAS in the development of obesity. Deficiency of Mas receptor, however, increases adiposity in rodents, suggesting a potential beneficial role for the Ang (1-7)-mas axis on adiposity [162].

Table 3: Effects of RAS manipulation in humans and animals

Manipulation	Body weight/ adiposity	Insulin sensitivity	Adipocyte size	Adipocyte number	Reference
Ang II infusion (acute – humans)		+			[163], [164], [165], [166]
Ang II infusion (chronic – rodents)	-	-			[167], [168], [158], [169], [159]
Renin overexpression	-	-			[170], [171]
Ang (1-7) infusion (rodents)		+			[172]
Renin knockout	-	+	-		[173]
Agt knockout	-		-		[174]
ACE knockout	-	+			[175]
AT_1 knockout	-	+	-		[176], [177]
AT_2 knockout	-	+	-	+	[178]
Adipose Agt overexpression	+		+	-	[157], [179]
Mas genetic deletion	+	-			[162]
Adipose Agt overexpression + AT_2 knockout	-		-		[179]

+ positive association; - negative association

RAS and insulin resistance

Numerous genetic studies have shown associations between polymorphisms in RAS genes and glucose homeostasis. The DD genotype of the ACE I/D polymorphism is associated with glucose intolerance and insulin resistance in several adult [180, 181] and infant [182] populations. Further, the AGT T174M and M235T polymorphisms are significantly associated with metabolic syndrome in aboriginal Canadians [183] and glycated hemoglobin in neonates [184], respectively. The plasma level of insulin sensitizer adiponectin is also associated with the AT_1 A1166C polymorphism in young women [185]. Having multiple risk genotypes of RAS polymorphisms significantly increases the risk of type 2 diabetes [186].

The strongest clinical evidence for an association between systemic RAS and insulin resistance originates from clinical trials which have shown that RAS blockade reduces the risk of developing type 2 diabetes [119, 187]. For example, there was a 14% lower risk of developing type 2 diabetes for patients on ACEI vs. conventional treatment (diuretics / beta blockers) in the in the Captopril Primary Prevention Project (CAPPP) [122]. In the Heart Outcomes Prevention Evaluation (HOPE) trial, there was a 34% risk reduction in the Ramipril (ARB) group compared to the placebo control group [187]. Pharmacological RAS blockade also improves insulin sensitivity in several rodent models of obesity or insulin resistance [188, 189] Moreover, rodents with genetic deletions of Agt, renin, ACE, AT_1 or AT_2 show improvements in insulin sensitivity and/or resistance to HF diet-induced insulin resistance (Table 3).

Conversely, chronic overactivation of systemic RAS induces whole body insulin resistance in rodents (Table 3). However, acute RAS overactivation via short-term Ang II infusions increases glucose disposal, and improves insulin sensitivity in humans [163] and rodents [190]. This latter phenomenon is attributed to acute haemodynamic adaptations in the form of redistribution of blood flow to skeletal muscle in response to increased Ang II levels [163, 164]. Since experimental chronic Ang II infusions are not feasible in human studies, animal models have been used to study the effects of chronic RAS overactivation. In these studies, chronic Ang II infusion induces skeletal muscle and hepatic insulin resistance, giving rise to whole-body insulin resistance [167]. The TG(mREN2)27 rat, another model of chronic systemic RAS overactivation, also develops skeletal muscle and systemic insulin resistance [170]. The insulin resistance in these animals is improved by either direct renin inhibition [191] or AT_1 blockade [192].

The mechanisms of Ang II-mediated skeletal muscle insulin resistance have been studied extensively. A summary is given in Figure 4. Muscle glucose uptake depends upon glucose delivery, glucose transport across the cell membrane and glucose utilization [193]. Of these processes, Ang II mainly impairs glucose transport and glucose utilization by the skeletal muscle [194] (Figure 4). Ang II impairs glucose transport mainly via inhibition of insulin signaling. Specifically, Ang II abolishes the insulin-mediated tyrosine phosprorylation of IRS-1, activation of Akt and translocation of Glut 4 in L6 myocytes *in vitro,* in a NADPH oxidase and AT_1-dependent manner [195]. This is also dependent upon activation of the NF-kB pathway in skeletal muscle [196].

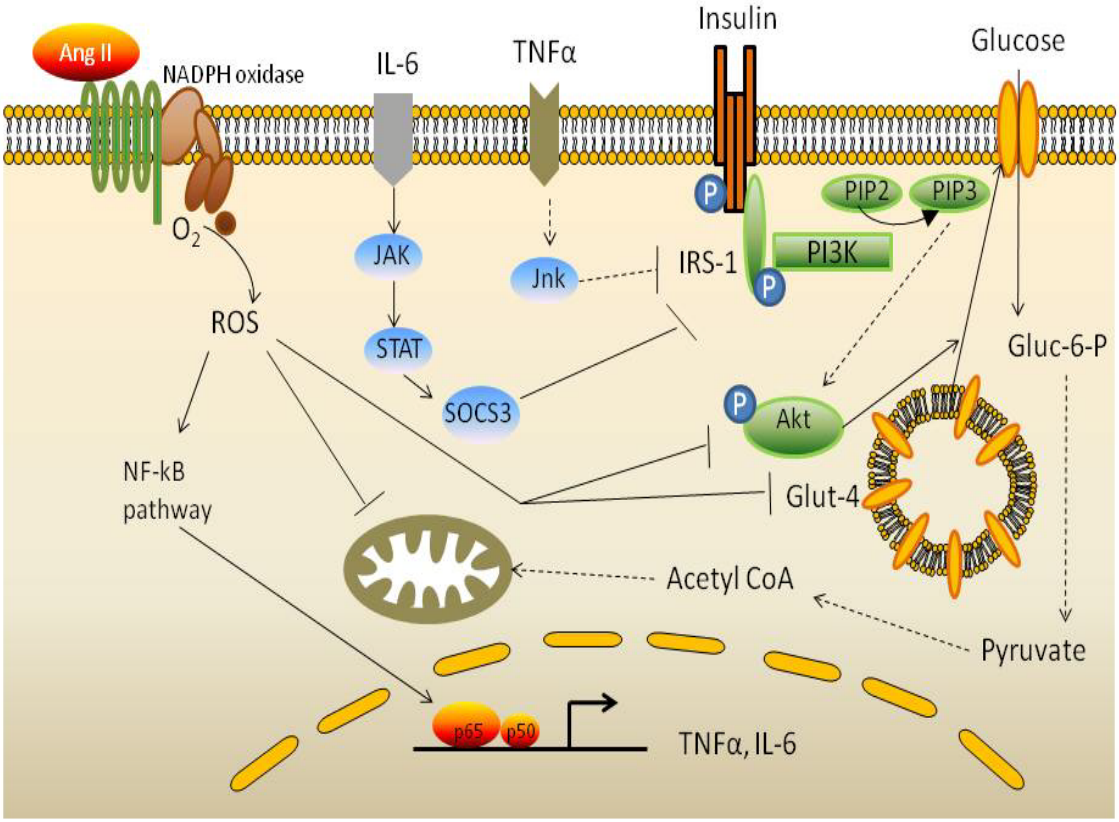

Figure 4. Mechanisms of Ang-II mediated skeletal muscle insulin resistance

Ang II activates NADPH oxidase via AT_1. This leads to generation of reactive oxygen species (ROS), which induce and activate nuclear translocation of NF-kB pathway. The latter mediates transcription of cytokines such as TNF-α and IL-6 and subsequent binding to their receptors. This binding induces serine kinases and SOCS3 expression, further inhibiting the tyrosine phosphorylation of IRS-1. This leads to deactivation of downstream insulin signaling and Glut-4 translocation, resulting in reduced glucose entry in to the cell. ROS also inhibit mitochondrial biogenesis leading to reduced glucose utilization.

Similarly, defective insulin-stimulated phosphorylation of IRS-1, Akt and glycogen synthase kinase-3beta [170] was reported in isolated skeletal muscle of the TG(mREN2)27 rat. The Ang-II mediated inhibition of IRS-1 is also due to its inactivation by serine phosphorylation or due to activation of protein tyrosine phosphatase-1B [198]. It is likely that Ang II activates NADPH oxides via AT_1, which leads to increased production of reactive oxygen species (ROS). This activates the NF-kB pathway, which increases transcription of cytokines such as TNF-α and IL-6. These cytokines acting in a paracrine fashion, increase SOCS3 expression [199], which further inhibits insulin signaling (Figure 4). In terms of glucose utilization, Ang II reduces skeletal muscle mitochondrial content in an AT_1 and AT_2-dependent manner in rodents both in *vitro and vivo* [200], an effect proposed to be mediated via ROS [200].

ACEI and ARB prevent these Ang II effects on insulin signaling and utilization and improve skeletal muscle insulin sensitivity. An additional mechanism of ACEI-mediated improvement in insulin sensitivity is via prevention of the degradation of bradykinin, a potent vasodilator and potentiator of insulin signaling [201]. This is explained by the ability of ACE to degrade bradykinin (Figure 3), which is prevented by ACE inhibition.

Additional mechanisms contributing to Ang II-mediated insulin resistance include the ability of Ang II to increase hepatic glucose production [202], which can contribute to whole-body insulin resistance. However, the exact mechanism responsible for this is not known, although recent evidence suggests that Ang II might be implicated in the development of hepatic steatosis [203]. Ang II's actions on the endocrine pancreas may

also play a role in defective glucose homeostasis associated with RAS overexpression. Indeed, the endocrine pancreas expresses a local RAS which is involved in the regulation of glucose-stimulated insulin secretion, insulin synthesis and pancreatic blood flow [204]. Further, RAS blockade improves islet morphology and function [205] and transfection of ACE2 into pancreatic islets of db/db mice also improves glycemic control [206].

Unlike in skeletal muscle, Ang II does not induce insulin resistance in adipose tissue [190]. Indeed, Ang II potentiates insulin-stimulated glucose uptake by adipocytes [190] via activation of insulin signaling molecules *in vitro* [207]. However, adipose tissue RAS could be important in the pathogenesis of systemic insulin resistance for several reasons. First, adipose-derived Agt and Ang II contribute to systemic levels of these hormones [208], which are increased in obesity [123]. Next, paracrine effects of Ang II on adipose tissue alter the adipokine profile toward a pro-inflammatory phenotype, which can then lead to skeletal muscle insulin resistance. For example, Ang II infusions reduce plasma adiponectin levels in an AT_1-dependent manner [209]. Finally, similar to several pro-inflammatory cytokines, Agt is expressed higher in visceral compared to subcutaneous adipose tissue [210]. Thus, it is important to discuss the adipose RAS in the context of obesity and insulin resistance.

Role of Adipose tissue RAS in regulating adipose tissue function

In the adipose tissue, Agt is synthesized and secreted by adipocytes. Most of the other RAS components necessary to produce Ang II are also present in the adipose tissue. Thus, the presence of an adipose RAS is well established. In addition to the classical

regulatory steps, Adipose RAS appears to be regulated at the level of Agt production, which is controlled by hormones such as insulin, androgens and dexamethasone and cytokines such as TNF-α [32, 210]. Indeed, insulin [211] and cytokine [212] response elements have been reported in the Agt promoter.

Functionally, Ang II plays a role in energy sensing, as well as modulating fat mass expansion via its effect on adipogenesis, lipogenesis and lipolysis. In genetically obese mice, feeding increases adipose Agt expression, while fasting reduces it [150], suggesting a role of Agt in energy sensing, possibly via the hexosamine pathway [213, 214]. It is plausible that in a state of acute energy influx to the adipose tissue, Agt production leads to increased local Ang II levels, which in turn induces local vasoconstriction resulting in lower lipolytic rates [215]. Conversely, in fasting conditions, due to lower local Ang II levels, vasodilatation occurs, leading to increased rates of lipolysis. These Ang II effects are mediated via AT_1.

Ang II also increases lipogenesis via AT_2 [216]. This is through induction of key lipogenic enzymes such as glycerol-3-phosphate dehydrogenase. Consistent with these effects, Ang II also potentiates insulin-stimulated glucose uptake by adipocytes [190]. Therefore, unlike in skeletal muscle, Ang II appears to enhance insulin action in adipocytes *in vitro*. Taken together, the net paracrine effect of Ang II is to reduce lipolysis and promote lipogenesis, ultimately increasing lipid storage and inflammation in adipose tissue (Figure 5).

While acute changes in energy availability modulate adipose RAS activity, the effects of chronic energy excess on it is inconsistent. While most studies report Agt

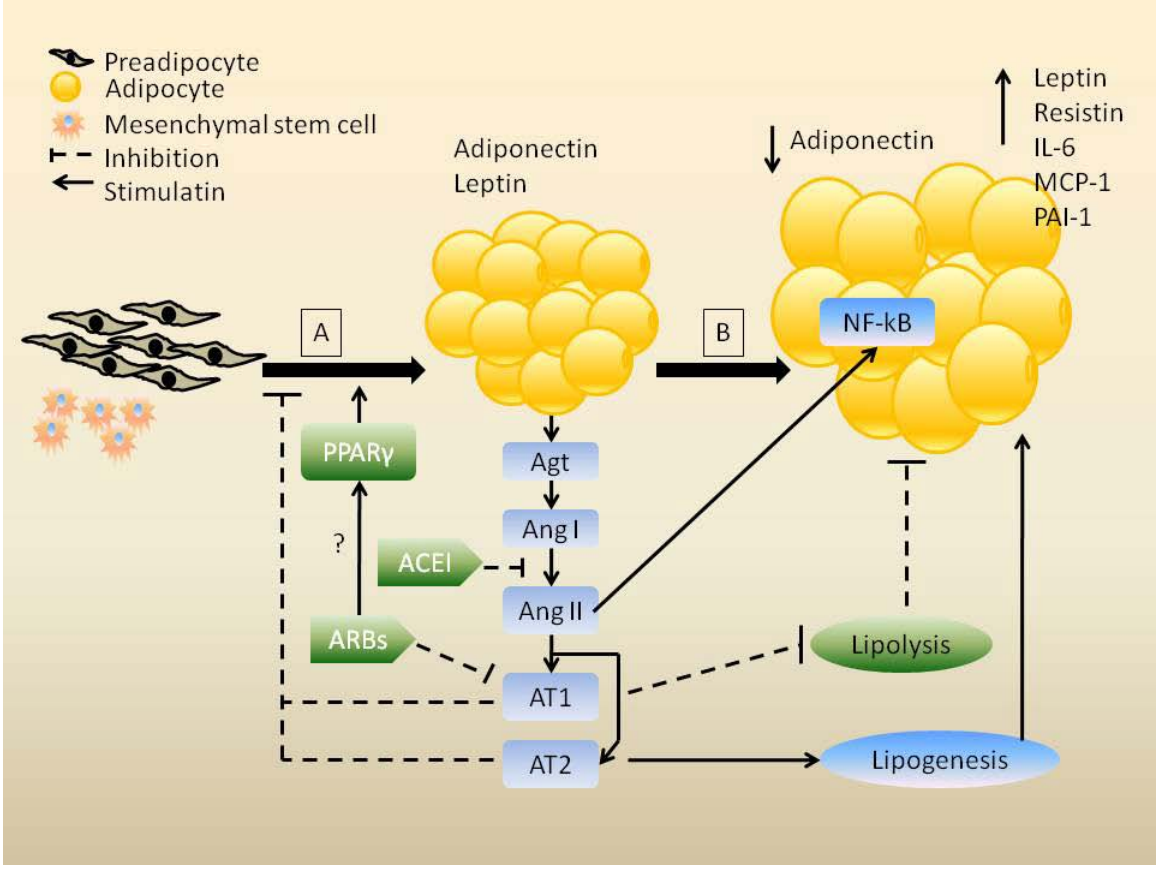

Figure 5. Effects of Ang II and RAS blockade on adipose tissue function

Adipose tissue expansion during positive energy balance involves a combination of adipocyte hypertrophy and hyperplasia. Adipogenesis via preadipocyte differentiation to adipocytes results in adipocyte hyperplasia (A). Mature adipocytes secrete Agt, which is converted to Ang II. Ang II acting on AT_1 and AT_2 inhibits preadipocyte differentiation. Stimulation of AT_2 also promotes lipogenesis, while activation of AT_1 inhibits lipolysis. Both processes promote adipocyte hypertrophy (B) which is associated with a pro-inflammatory adipokine profile.

overexpression in human obesity, some have reported no change or a negative association (Table 2). Since adipose Agt expression is acutely regulated by hormonal and nutritional signals, this could be a confounding factor when studying the chronic effect of obesity on adipose Agt expression. It is also possible that genetic factors, such as polymorphisms in RAS genes also play a role in this discrepancy of results [128]. This is supported by the fact that while adipose RAS is overactivated in most animal models of diet-induced obesity, it is downregulated in some models with genetic forms of obesity (Table 2). Given that not all obese individuals develop metabolic derangements, it is possible that adipose RAS is also overexpressed in some, but not all, obese individuals. In this context, it is important to study the effects of adipose RAS overactivation on adipose tissue function and systemic insulin sensitivity, to elucidate its role in the pathogenesis of metabolic derangements in obesity.

Since a reduced adipogenic capacity is linked to adipose tissue inflammation and systemic insulin resistance in obesity [217], it is important to investigate the effects of adipose RAS on both adipogenesis and lipogenesis, and conversely on both adipose hyperplasia and hypertrophy. It is important to recognize that lipogenesis and adipogenesis are distinct processes. The former refers to storage of lipids in adipocytes, which is positively regulated by Ang II as described above. Adipogeneis refers to formation of new adipocytes, either from preadipocytes or other precursors such as mesenchymal stem cells. In a state of positive energy balance, adipose tissue expansion occurs as a result of both these processes. Adipogenesis leads to adipocyte hyperplasia, while lipogenesis leads to adipocyte hypertrophy. An inadequate adipogenic capacity,

which is postulated to be linked to systemic insulin resistance, is characterized by adipocyte hypertrophy and a lower adipocyte number.

RAS blockade via either ACEI or ARB results in smaller adipocyte size in numerous rodent models of obesity [218-220]. Furthermore, ARB also increase the number of small differentiated adipocytes in diabetic rats [161]. Both these findings support the assertion that while RAS blockade inhibits lipogenesis, it also promotes adipogenesis *in vivo*. However, these findings are confounded by the fact that some ARB such as Losartan, activate PPARγ, an adipogenic transcription factor [221, 222]. Thus, the effects of ARB on adipogenesis could be attributed to indirect effects.

Rodents with genetic deletion of renin, Agt, AT_1 or AT_2 also exhibit smaller adipocytes (Table 3). Of these, mice lacking AT_2 have a relative increase in adipocyte number [178], suggesting an inhibitory effect of AT_2 on adipogenesis.

The only animal model available to study the paracrine effects of RAS overactivation on adipose tissue is the transgenic mouse model overexpressing Agt in the adipose tissue (aP2-Agt mice). These mice become moderately obese and develop large adipocytes [157]. Their adipocyte number is also reduced compared to wild-type mice [179]. This suggests that increased local levels of Ang II inhibit adipogenesis. When these mice are crossed with mice lacking the AT_2 gene, their adipocyte number and size becomes comparable to wild-type mice [179], demonstrating a critical role of AT_2 in mediating Ang II's inhibitory effects on adipogenesis. However, since their adipocyte number is still lower than AT_2 knockouts (with normal adipose Agt expression), AT_1 also seems to be, at least in part, involved in mediating Ang II's anti-adipogenic effects.

In vitro studies on Ang II's effects on adipogenesis are inconsistent; practical difficulties in dissociating adipogenesis from lipogenesis likely being the reason. Some early studies show that Ang II *increases* murine preadipocyte differentiation via prostacyclin and AT_2-dependent manner [223]. However, it was later shown that Ang II inhibits human mesenchymal stem cell differentiation into adipocytes in an AT_2-dependent manner [224]. There is also evidence that Ang II inhibits differentiation of human [225-227] and 3T3-L1 [228] preadipocytes in an AT_1-dependent manner *in vitro*. While there is some evidence to suggest that Ang II exerts these effects via mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (Erk) pathways [227], further studies are certainly warranted. Overall, this evidence suggests that Ang II promotes lipogenesis and inhibits adipogenesis leading to an adipose tissue phenotype characterized by large adipocytes (Figure 5). Whether this could be an important mechanism for insulin resistance in conditions of adipose RAS overactivation, remains to be tested.

Ang II promotes inflammation in several tissues [229]. Similarly, aP2-Agt mice express higher levels of inflammatory genes in the adipose tissue [179]; however, it is unclear whether this is a direct effect of Ang II. *In vitro* studies show that Ang II increases pro-inflammatory cytokines IL-6 and IL-8 secretion from human adipocytes [230] and MCP-1 from preadipocytes [231] in an NF-kB dependent manner. Conversely, RAS blockade reduces MCP-1 expression and macrophage infiltration in HF diet-induced obese mice [161]. Taken together this suggests that Ang II promotes adipose tissue inflammation in an NF-kB-dependent manner (Figure 5). Further studies on the

mechanisms of Ang II mediated stimulation of NF-kB in adipose tissue are thus warranted.

Dietary approaches to ameliorate metabolic derangements in obesity

Obesity is causally linked to insulin resistance and metabolic syndrome (discussed previously). Individuals with metabolic syndrome are at a higher risk of developing type-2 diabetes and other chronic and inflammatory diseases. Dietary interventions are useful in reducing the severity of these chronic diseases and in preventing them. Caloric restriction is the primary dietary approach employed for weight loss purposes [232]. Furthermore, in recent years, bioactive components of foods, such as omega-3 fatty acids, and various food-derived phytochemicals have gained popularity and are commonly used as natural preventive therapies for chronic diseases [233]. In this section, we will review the state of research interventions for metabolic disorders, using caloric restriction and those using a bioactive compound, omega-3 polyunsaturated fatty acids.

Caloric restriction for improvement of insulin resistance and metabolic derangements in obesity

Lifestyle modification, including restriction of energy intake, is as or more effective than pharmacotherapy in preventing the progression of metabolic syndrome to type 2 diabetes [234]. Caloric restriction leading to 5-10% loss of body weight, regardless of the macronutrient composition of the diet, significantly improves glycemic control in

obese individuals [235]. Even in patients with type-2 diabetes, caloric restriction and weight loss improves insulin sensitivity [236]. Since dysfunctions in adipose tissue, skeletal muscle, liver and endocrine pancreas alter glucose homeostasis in obesity, it is important to investigate whether improved insulin resistance following caloric restriction is accompanied with reversal of these tissue dysfunctions.

Enhanced insulin sensitivity following caloric restriction is at least in part due to loss of body weight and adipose tissue mass. Indeed, some studies show that weight loss consistently improves insulin sensitivity, regardless of whether it is induced by increased energy expenditure or reduced energy intake [237, 238]. However, other weight-independent mechanisms might also operate. For example, obese individuals subjected to short-term caloric restriction (1-week) exhibit improvements in insulin sensitivity despite minimal weight loss [239]. This is also postulated to be a mechanism responsible for rapid improvement of insulin sensitivity following bariatric surgery [239]. Moreover, caloric restriction in non-obese animals increases longevity and delays the onset of several chronic illnesses including type-2 diabetes [240]. In this context it is important to understand the mechanisms of improvements in insulin sensitivity following caloric restriction in obese individuals.

Caloric restriction and adipose tissue function

Caloric restriction leads to loss of adipose tissue mass in both subcutaneous and visceral fat depots [241]. It also favorably alters plasma adipokine profile (Table 4). Caloric restriction also reduces plasma leptin consistently across studies (Table 4) and

Table 4 . Change in plasma adipokines following caloric restriction in obese humans

Adipokine	Increased	No Change	Decreased
Leptin			[242-246]
Adiponectin	[244, 247-250]	[245, 246, 251-254]	
IL-6		[246]	[242, 249, 255-257]
RBP-4			[242, 258]
Visfatin	[259, 260]		[244]
TNF-α		[246, 257]	[255, 261]
PAI-1			[246, 262]
MCP-1			[248]

IL-6 – interleukin-6, RBP-4 – retinol binding protein-4, TNF – tumor necrosis factor, PAI-1 – plasminogen activator inhibitor-1, MCP-1 – monocyte chemotactic protein-1

increases sensitivity to leptin [263]. The effect of caloric restriction on plasma adiponectin level is variable, with some studies showing an increase while others showing no effects of caloric restriction on this anti-inflammatory adipokine (Table 4). Several reasons might account for these discrepancies. Fist, the restoration of plasma adiponectin to non-obese levels could be age-dependent as evident by studies showing that caloric restriction improves plasma adiponectin level in young, but not old rodents [259]. Second, there could be a temporal change in adiponectin response to caloric restriction [260]. Further, the magnitude of weight loss might influence adiponectin response. Finally, given that fatty acids can modulate adipose tissue adipokine secretion, the macronutrient composition of a caloric-restricted diet could also affect plasma adiponectin levels. However, this latter possibility remains to be tested. When the effect of caloric restriction on other adipokines are considered, it favorably changes plasma RBP4, IL-6, visfatin, TNF-α, PAI-1 and MCP-1 levels (Table 4).

Caloric restriction reduces adipocyte size in humans [264, 265], probably due to increased rates of lipolysis and a resistance to the insulin's anti-lipolytic effects [266]. The anti-lipolytic α-2 adrenergic receptor expression is also reduced following caloric restriction in obese individuals [267]. Caloric restriction also regulates expression of genes involved in lipogenesis, fatty acid oxidation [268, 269] and fatty acid metabolism in human adipose tissue. Examples include stearoyl-coenzyme A desaturase (SCD) and diacylglycerol acyl transferase 2 (DGAT2) [270], both of which are downregulated by caloric restriction.

It is possible that caloric restriction also restores the adipogenic capacity affected by obesity, by increasing the expression of several adipogenic factors [271] and the number of small insulin-sensitive adipocytes in rats [272] suggesting an improvement in adipogenic capacity.

Changes in expression of inflammatory genes in adipose tissue during caloric restriction is variable, with some studies showing increased expression of IL-6 and TNF-α [256] and others showing downregulation of inflammatory genes [273-275]. Moreover, adipose tissue gene expression pattern does not always follow the direction of change in plasma adipokines [246]. Therefore, it is possible that changes in adipose tissue inflammation following caloric restriction are of a temporal nature. Recent evidence confirms this as weight loss during caloric restriction is characterized by a dynamic immune response. In obese mice on a high-fat diet, caloric restriction initially induces macrophage recruitment into adipose tissue [276]. The macrophage number peaks at the time of maximal lipolysis, suggesting a role of fatty acid flux in their recruitment. With continued weight loss, the macrophage number decreases [276]. Similarly, in human adipose tissue, macrophage markers increase during caloric restriction and decrease during weight stabilization and maintenance [277], suggesting a dynamic immune response.

Endoplasmic reticulum stress, another triggering factor of adipose tissue inflammation, is alleviated by caloric restriction in obese mice [278]. Taken together, while the adipose tissue inflammation associated with obesity seems to be alleviated by caloric restriction, the exact nature of the dynamic immune response occurring during

caloric restriction is yet to be fully characterized. Further, the effect of macronutrient composition on adipose tissue inflammation also needs characterization.

Effects of caloric restriction on skeletal muscle and hepatic insulin sensitivity

Caloric restriction increases insulin-stimulated glucose disposal by the skeletal muscle [279]. This is primarily due to enhancement of insulin signaling [236, 280] in an Akt2-dependent fashion [281, 282]. Caloric restriction also results in increased glycogen synthesis as well as fatty acid oxidation by the skeletal muscle [283]. Further, an increase in skeletal muscle mitochondria content due to caloric restriction potentially leads to increased glucose utilization and fatty acid oxidation by the muscle as well [284]. While the exact mechanisms responsible for these changes are still unknown, but may be attributed to reduced intramyocellular triglyceride (IMTG) content during caloric restriction [236, 285]. Moreover, caloric restriction reduces the proportion of saturated fatty acids in IMTG and muscle cell membranes [286, 287]. Other potential mediators of caloric restriction-induced improvement in skeletal muscle insulin signaling include growth factor receptor-bound protein 2 (Grb2). Expression of this protein is reduced in caloric restriction. Further, experimental reduction of Grb2 enhances insulin signaling and increases insulin-stimulated glucose uptake by myoblasts *in vitro* and protects mice from high-fat diet induced insulin resistance [288].

Caloric restriction improves hepatic insulin sensitivity and restores the insulin mediated suppression of hepatic glucose production [289]. These improvements are primarily related to reductions in hepatic lipid [264, 290, 291] and visceral fat content

[292]. Although the effects of caloric restriction on all components of hepatic insulin signaling cascade have not been characterized, the obesity-induced increase in serine phosphorylation of IRS is reported to be reversed by caloric restriction [293].

Caloric restriction also attenuates the glucose-stimulated insulin response [294, 295] suggesting a beneficial effect on the endocrine pancreas.

Caloric restriction and longevity

Caloric restriction, defined as dietary restriction without induction of malnutrition, slows aging and extends longevity in different species including yeast, flies, nematodes, rodents and non-human primates [296]. The effect of caloric restriction on longevity of humans is still not clear [297], however studies are currently underway to study this effect [298]. Several mechanisms have been proposed for mediating the beneficial effects of caloric restriction on longevity. Reduced activation of nutrient-sensing pathways is proposed to be one such mechanism. Indeed, animals with mutations of these pathways, including insulin-like growth factor / insulin and mammalian target of rapamycin (mTOR) pathways, have increased lifespan. For example, mice with deletion of ribosomal S6 protein kinase 1 have extended lifespan and have gene expression patterns similar to that of caloric-restricted animals [299]. Adipose-specific insulin receptor knockout mice also have lower adiposity and extended lifespan [300].

Prevention of age-related deterioration of insulin sensitivity is another mechanism proposed to mediate effects of caloric-restriction on longevity. Growth hormone activity is known to be associated with insulin resistance, and growth-hormone receptor knockout

(GHRKO) mice are insulin sensitive and have a prolonged lifespan. Because caloric-restriction does not have additional effects on longevity on these mice, it is likely that GH also mediates effects of caloric-restriction on longevity.

Finally, the beneficial effects of caloric restriction in animals are at least in part mediated via the histone deacetylases sirtuins [301]. Mammalian sirtuins include SIRT1-7. SIRT1 is upregulated in caloric restriction and promotes fatty acid oxidation in the liver and skeletal muscle and improves glucose homeostasis [302]. Further, SIRT1 activators, such as resveratrol extend lifespan in organisms such as yeast [303]. SIRT2 is also upregulated in adipose tissue following caloric restriction [304], while SIRT3 is mainly expressed in mitochondria and regulates fatty acid metabolism [305].

While the aforementioned mechanisms are responsible for the beneficial effects of caloric restriction on longevity, it is not evident whether similar mechanisms operate in obese animals as well. Limited evidence suggests that SIRT1 activation protects from high-fat diet-induced metabolic derangements. For example, resveratrol prevents the development of insulin resistance in high-fat fed mice [306]. Although these sirtuins are implicated in mediating the beneficial roles of caloric restriction, whether these mechanisms operate in obese humans subjected to caloric restriction remains to be elucidated.

Macronutrient composition of a caloric restricted diet

Most health agencies recommend a low-fat diet for weight loss and improvement of metabolic health. However, low-carbohydrate, high-fat reduced-energy diets are also

effective in weight loss [307]. While energy restriction, rather than macronutrient composition, seems to be more important for improving insulin sensitivity [308], the effects of these high-fat reduced-energy diets on other cardiovascular risk factors are not well known. In contrast, low-fat diets are known to reverse coronary artery disease [309] and reduce cardiovascular disease risk factors such as low-density lipoprotein cholesterol levels [310]. Therefore, it is important to study the effects of energy-restricted diets with varying macronutrient compositions on metabolic markers. It is especially crucial to dissect the role of dietary fat in health and disease, due to its higher energy density.

Effect of macronutrient composition on degree of weight loss and insulin sensitivity

Caloric restriction, rather than macronutrient composition, appears to be more important for weight loss. Low-fat, low-carbohydrate, very-low carbohydrate and high-protein diets induce similar degrees of weight loss [311]. Although very low-carbohydrate ketogenic diets induce greater weight loss short-term, this difference is not significant after 1 year [310].

Caloric restriction leading to weight loss also seems to be more important for improvements in insulin sensitivity, than the macronutrient composition of the diet. In a large randomized controlled trial, lower intakes of dietary fat reduced the risk of type-2 diabetes only in the presence of weight loss [312]. However, animal studies show that very low-carbohydrate diets induce hepatic lipid accumulation and might not improve HF diet-induced insulin resistance to the same extent as high-carbohydrate low-fat diets [313, 314].

Effect of macronutrient composition on serum cholesterol levels

In addition to weight loss and improvement in insulin sensitivity, low-fat diets also reduce circulating total cholesterol and low-density lipoprotein (LDL) cholesterol levels. However, they have an undesirable effect of lowering high-density lipoprotein (HDL) cholesterol level as well. In contrast, low-carbohydrate diets reduce triglyceride and very low-density lipoprotein (VLDL) cholesterol levels, with a slight increase in LDL cholesterol levels [310, 315]. These low-carbohydrate diets also induce a greater degree of reduction in the total/HDL cholesterol ratio [235].

Effect of macronutrient composition on adipose tissue function

Caloric restriction alters lipolytic rates and lipogenesis in adipose tissue (discussed above). However, the fat/carbohydrate content of a caloric restricted diet does not change insulin-stimulated glucose transport or lipolysis in human subcutaneous adipose tissue [316].

Only a few studies have investigated the effect of macronutrient composition of a caloric-restricted diet on adipose tissue gene expression. In one study, dietary energy intake was restricted by 600 kcal/day in obese women by either a low-fat (20-25% of total energy by fat) or a moderate-fat (40-45% of total energy by fat) diet [246]. During 10 weeks of the intervention, body weight of women in both groups decreased an average of 7.5%. In the subcutaneous adipose tissue, mRNA expression of leptin and IL-6 were reduced by both diets, with no changes in TNF-α, PAI-1, IL-8 and adiponectin expression. When the protein secretion was considered, leptin, IL-6 and IL-8 were

reduced by both diets, while TNF-α reduced only by the low-fat diet. This shows that during caloric restriction, the effect of macronutrient composition is minimal. In another study, microarray analysis of adipose tissue of obese women showed that a majority of genes were differentially expressed in response to caloric restriction, but only a few genes responded to the fat / carbohydrate content of the diet [315]. These latter genes included *FABP4, SIRT3, NR3C1, FNTA, and GABARAPL2,* which were expressed at higher levels in the low-carbohydrate group.

In conclusion, caloric restriction, regardless of the macronutrient composition of the diet, induces weight loss and improves systemic insulin sensitivity. However, the effect of macronutrient composition in reduced-energy diets on adipose tissue function and other cardiovascular risk factors need further characterization.

Long-chain (n-3) polyunsaturated fatty acids for improvement of insulin resistance and metabolic derangements in obesity

Disclosure: The work described in this section has been submitted for the following publication, with minor modifications in the numbering of tables and figures:
"Kalupahana NS, Claycombe KJ and Moustaid-Moussa N. (n-3) fatty acids alleviate adipose tissue inflammation and insulin resistance: mechanistic insights. *Advances in Nutrition* in press, 2011"

While caloric restriction leading to weight loss is a successful dietary intervention for improving obesity-associated metabolic disorders, other dietary interventions such as

ones targeted at reducing adipose tissue inflammation, regardless of weight loss, have not been explored in detail. Long-chain (n-3) polyunsaturated fatty acids of marine origin [(n-3) LC-PUFA] namely eicosapentaenoic acid [20:5 (n-3), EPA] and docosahexaenoic acid [22:6 (n-3), DHA] have known anti-inflammatory properties [317]. Moreover, they reduce plasma triglycerides, reduce cardiac events and delay the progression of atherosclerosis [318, 319]. They also have anti-obesity effects on humans [320] and rodents [321].

In contrast, the effect of EPA/DHA on insulin sensitivity is not well characterized. While EPA/DHA consistently prevent the development of insulin resistance associated with high-fat [322, 323] or high-sucrose [324] feeding in rodents, they do not improve insulin sensitivity in individuals with type-2 diabetes [325, 326]. However, preliminary evidence suggests that EPA/DHA might help delay the progression of metabolic syndrome to type-2 diabetes [327]. In this context, elucidating the mechanisms responsible for improvement of insulin sensitivity due to EPA/DHA might enhance the understanding of the pathophysiology of obesity-associated insulin resistance and could potentially lead to discovery of novel therapeutic targets for the metabolic syndrome.

(n-3) and (n-6) polyunsaturated fatty acids

(n-3) and (n-6) polyunsaturated fatty acids are the two main classes of essential fatty acids. In (n-3) fatty acids, the first double bond is located between the 3rd and 4th carbons counting from the methyl end (Figures 6 and 7), while in (n-6) fatty acids this is

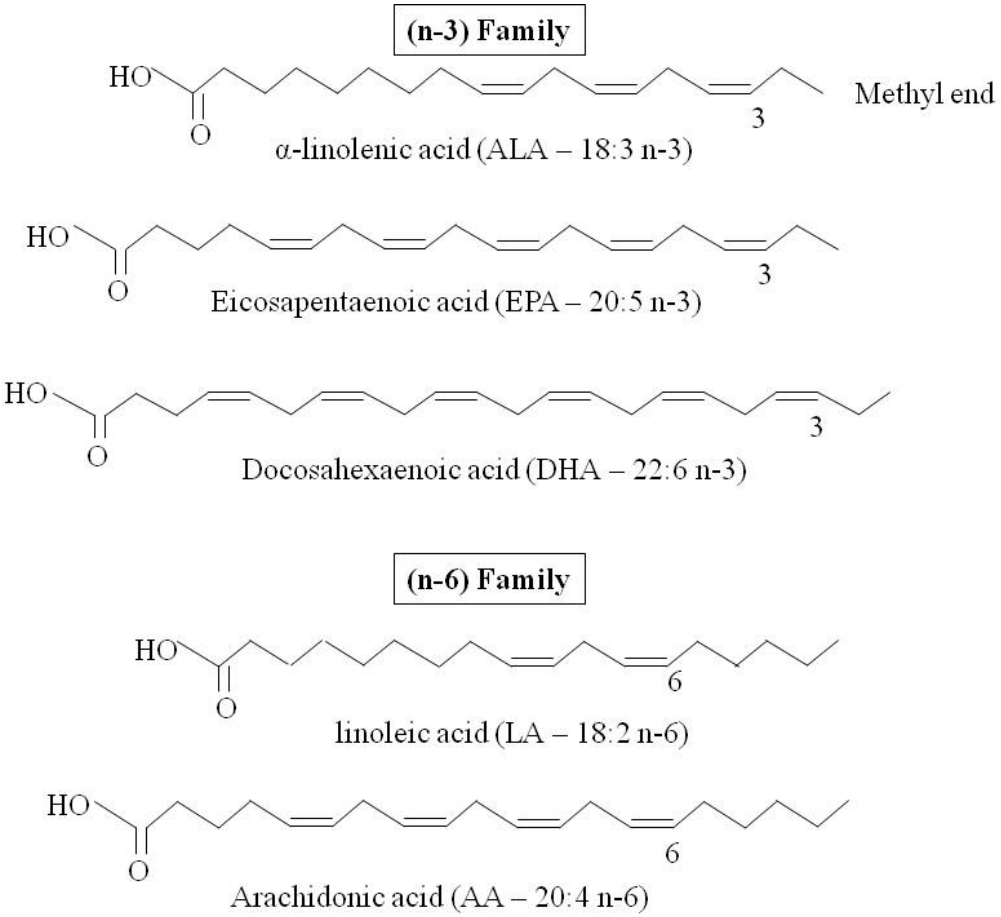

Figure 6. Structures of main (n-3) and (n-6) polyunsaturated fatty acids

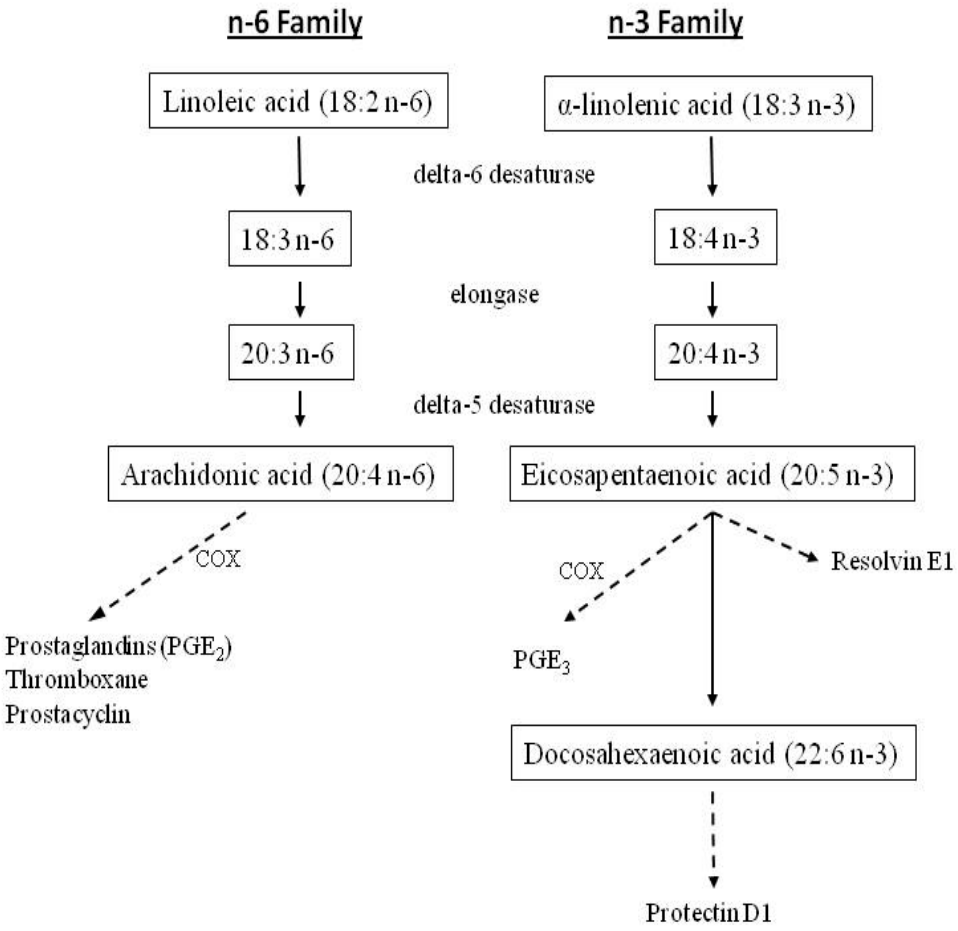

Figure 7. Metabolism of (n-3) and (n-6) fatty acids

Linoleic (LA) and α-linolenic acid (ALA) are the parent (n-6) and (n-3) long-chain polyunsaturated fatty acids. LA is converted to arachidonic acid (AA), while ALA is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AA-derived eicosanoids are pro-inflammatory, while EPA-derived ones are less so. EPA and DHA metabolites such as resolvins and protectins have important roles in resolution of inflammation.

COX – cyclooxygenase

located between the 6^{th} and 7^{th} carbons from that end. These are considered essential fatty acids because humans cannot synthesize them due to absence of delta-12 and 15 desaturase enzymes. Linoleic acid (LA) is the parent long-chain (n-6) fatty acid, which can be converted into arachidonic acid (AA) (Figure 7). LA is found mainly in vegetable oils such as corn, sunflower and soybean oil, while AA is found in foods of animal origin such as meat and egg-yolk [328]. α-linolenic acid (ALA), is the parent (n-3) fatty acid, which can be converted to EPA and DHA. ALA is found in nuts such as walnuts and flax seed, while EPA and DHA are found primarily in foods of marine origin such as oily fish. AA-derived eicosanoids such as PGE_2 and thromboxane A_2 are pro-inflammatory, while EPA-derived ones such as PGE_3 are less inflammatory. EPA and DHA are also metabolized into resolvins and protectins, which have important roles in resolution of inflammation (Figure 7).

The ratio of (n-6) /(n-3) polyunsaturated fatty acids in the western diet ranges from about 10/1 to 20/1 [329]. In countries with a relatively higher fish consumption such as Japan, this ratio is 4/1 [330]. Dietary intake of these fatty acids affects the proportion of AA/EPA ratio in phospholipids, which affects cardiovascular disease risk [331]. The (n-3) index is a measure of erythrocyte EPA+DHA to total fatty acid ratio, which has been proposed to be used as a cardiovascular disease risk factor. Individuals with a low (n-3) index have a higher risk of cardiac events [332]

EPA/DHA and hepatic insulin sensitivity

The effect of (n-3) LC-PUFA, mainly EPA, on lowering plasma triglycerides is well established. This effect is at least in part due to their ability to inhibit hepatic

enzyme diacylglycerol acyltransferase (DGAT) [333], which catalyses the final reaction of triglyceride synthesis. In addition to this triglyceride lowering effect, EPA/DHA also prevents the development of hepatic steatosis [334] and insulin resistance [323] associated with high saturated fat (HF) feeding in rodents.

Lipid accumulation in the liver depends on non-esterified fatty acid delivery to the liver, *de novo* lipogenesis and the rate of fatty acid oxidation. In obesity, there is a net increase in fatty acid availability, promoting lipid deposition in the liver. Moreover, lipogenic gene transcription factors such as sterol regulatory element-binding protein (SREBP)-1c, are expressed at a higher level in obesity [335]. This leads to increased expression of hepatic lipogenic genes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [336]. Further, obesity is also associated with suppression of peroxisome proliferator-activated receptor (PPAR)-α [335] leading to reduced fatty acid oxidation [336]. All these processes are linked to the development of hepatic steatosis. Excessive lipid accumulation in the liver leads to hepatic insulin resistance and blunting of insulin-mediated suppression of hepatic glucose production.

EPA reduces lipogenesis and increases fatty acid oxidation [337], preventing lipid accumulation in the liver, leading to improvements in hepatic insulin resistance (Figure 8). Moreover, EPA reduces lipogenesis via inhibition of lipogenic transcription factors such as SREBP-1c, nuclear factor-Y [338] and carbohydrate-responsive element-binding protein (ChREBP) [339]. EPA stimulates fatty acid oxidation via activation of PPARα

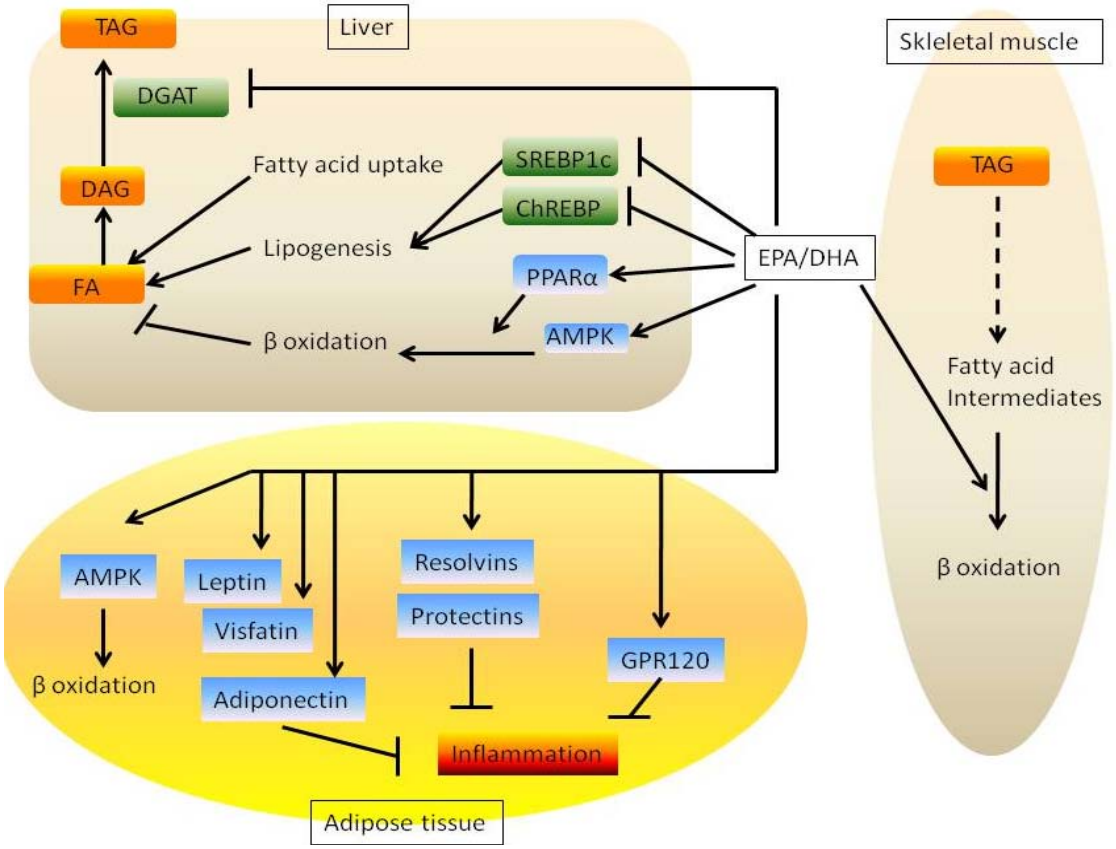

Figure 8. Effect of EPA/DHA on liver, skeletal muscle and adipose tissue metabolism

EPA/ DHA promote hepatic fatty acid oxidation and suppress lipogenesis. This leads to reduced accumulation of TAG in the liver. These fatty acids also increase adipose tissue fatty acid oxidation and increase secretion of adiponectin, leptin and visfatin. EPA/DHA also alleviate adipose tissue inflammation via GPR120 and resolvins / protectins. In the skeletal muscle, EPA/DHA promote fatty acid oxidation, thereby preventing accumulation of fatty acid intermediates. All these mechanisms are responsible for the EPA/DHA- mediated improvement in insulin sensitivity. FA – fatty acids, DAG – diacylglycerol, TAG – triacylglycerol, DGAT – diacylglycerol acyl transferase.

[336, 340-342] and AMP-activated protein kinase (AMPK) [343]. PPARα is required for EPA's beneficial effects on hepatic insulin sensitivity, as evident by a lack of EPA effect in restoring hepatic insulin sensitivity in PPARα null mice fed a HF diet [344]. Interestingly, these mice continue to exhibit low plasma triglyceride levels, concomitant with diacyl glycerol accumulation in the liver, suggesting that EPA exerts a PPARα-independent effect on hepatic DGAT. AMPKα2 is another signaling enzyme,coordinately regulated with PPARα during fat oxidation. As expected, and in line with the PPARα null mice phenotype, AMPKα2 null mice do not exhibit EPA's beneficial effects on improvement in hepatic insulin sensitivity [345].

Effects of EPA/ DHA on adipose tissue function

EPA/DHA reduce adiposity in humans [346] especially when combined with caloric restriction [320]. These fatty acids also prevent the development of high-fat diet induced adiposity and adipocyte hypertrophy in rodents [347]. There are two possible mechanisms for these anti-obesity effects of EPA/DHA. First, EPA/DHA is known to increase fatty acid oxidation in liver, adipose tissue [348] and small intestine [349] *in vivo* and adipocytes [350] and myotubules [351] *in vitro*. Second, they are known to inhibit hepatic lipogenesis (Figure 8). Both these processes shift the balance of fatty acid metabolism toward oxidation, rather than storage. EPA/DHA activate AMPK in adipose tissue and cultured adipocytes, which could be a mechanism for their effect on fatty acid oxidation [352, 353]. Further, these PUFAs are also known to induce mitochondrial biogenesis [318].

While it is possible that improvements of systemic insulin resistance due to EPA/DHA are secondary to reduction in adipose mass, this could also be due to direct actions of these fatty acids in improving adipose tissue function. Indeed, some studies have shown that these (n-3) LC-PUFA-mediated insulin sensitivity is preserved even in the presence of increased adipose mass [354].

EPA/DHA modulate adipokine secretion from adipose tissue (Figure 8). They increase plasma adiponectin levels in obese humans [355, 356] and rodents [357], which could be a potential mechanism by which EPA/DHA improve insulin sensitivity. This effect of EPA/DHA on adiponectin is PPARγ-dependent, because adiponectin is not elevated in response to fish oil in mice lacking PPARγ [358]. They also induce leptin and visfatin secretion and reduce the expression of several proinflammatory cytokines from the adipose tissue including TNFα and IL-6 [359-361]. Current evidence suggests that these anti-inflammatory actions of EPA/DHA play a major role in their insulin-sensitizing effects.

Adipose tissue macrophage infiltration and phenotypic switch is causally linked to insulin resistance in obesity (discussed previously). EPA/DHA prevent HF diet-induced adipose tissue macrophage infiltration in mice [362]. Production of pro-inflammatory cytokines by macrophages is dependent on activation of the NF-kB and JNK pathways. EPA/DHA bind to G protein-coupled receptor 120 (GPR120) and inhibit NF-kB and JNK, attenuating this response [363]. The importance of this receptor is highlighted by the finding that the EPA-mediated improvement in insulin sensitivity is absent in mice lacking GPR120. Another G protein-coupled receptor, GPR40 is also known to be

activated by long-chain PUFA [364]. Recent evidence has highlighted the role of EPA/DHA in resolving inflammation, through mechanisms involving EPA-derived resolvin E1 and DHA-derived protectin D1 [365]. In HF-fed mice, protectin D1 is lacking in the adipose tissue and skeletal muscle. Moreover, transgenic restoration of (n-3) PUFA and protectin D1 prevents the HF diet-induced insulin resistance, highlighting the important role of this DHA-derivative [366].

EPA/DHA and skeletal muscle metabolism

Triglyceride accumulation in skeletal muscle fibers has been linked to insulin resistance (discussed previously). Proposed mediators include increased fatty acid availability and impaired fatty acid oxidation in the skeletal muscle. The latter is also associated with accumulation of fatty acid intermediates such as diacylglycerol and ceramides. Exposure of myotubules to EPA enhances glucose uptake [367], indicating increased insulin sensitivity. EPA also protects from the development of HF-induced skeletal muscle insulin resistance *in vivo* [368]. Interestingly, EPA increases both TAG accumulation and fatty acid β oxidation (Figure 8) while improving skeletal muscle insulin sensitivity both *in vitro* [351] and *in vivo* [369].

Because EPA/DHA also reduce skeletal muscle ceramide content [357], it is possible that their effect on maintaining skeletal muscle insulin sensitivity is related to their ability to normalize fatty acid oxidation with lower accumulation of fatty acid intermediates. Saturated fatty acids induce skeletal muscle insulin resistance via activation of the NF-kB pathway [370]. Since EPA/DHA inhibit this pathway in other

tissues, it will be interesting to determine whether EPA/DHA inhibit this pathway in the skeletal muscle, and subsequently prevent the saturated fatty acid-mediated insulin resistance.

In summary, EPA/DHA prevent excessive adiposity and insulin resistance in rodents. Mechanistically, this is related to the ability of these fatty acids to increase hepatic, skeletal muscle and adipose tissue fatty acid oxidation and their ability to reduce lipogenesis. EPA/DHA also have important anti-inflammatory properties which modulate adipose tissue inflammation via GPR120-mediated suppression of macrophage pro-inflammatory cytokine secretion, resolvin and protectin-mediated resolution of inflammation. Through modulation of adipokine secretion, these fatty acids also favor insulin sensitivity. Most studies have addressed preventive effects of these PUFA, however whether these macronutrients are also able to reverse insulin resistance in obesity or their mechanisms for modulating adipocyte secretory function needs further characterization.

Goal and specific aims

The overarching goal of this dissertation research is elucidating adipose tissue function in metabolic syndrome using genetic and nutritional manipulations/approaches, with specific emphasis on the roles of inflammation and angiotensinogen.

Specific Aims

I. Determine the role of adipose tissue angiotensinogen (Agt) overexpression in the pathogenesis of insulin resistance in obesity

In this specific aim, we test the hypothesis that overexpression of Agt from adipose tissue induces systemic insulin resistance via NADPH oxidase and NF-kB-dependent increases in adipose tissue inflammation (Figure 9). We have the following objectives.

A. Determine the metabolic and adipose tissue gene expression changes resulting from overexpression of Agt in adipose tissue.
B. Determine the metabolic changes resulting from high-fat feeding in mice with overexpression of Agt in adipose tissue in the presence and absence of ACE inhibitor captopril.
C. Determine whether the effects of adipose Agt overproduction on adipokine secretory patterns in murine adipocytes are mediated via NADPH oxidase and NF-kB

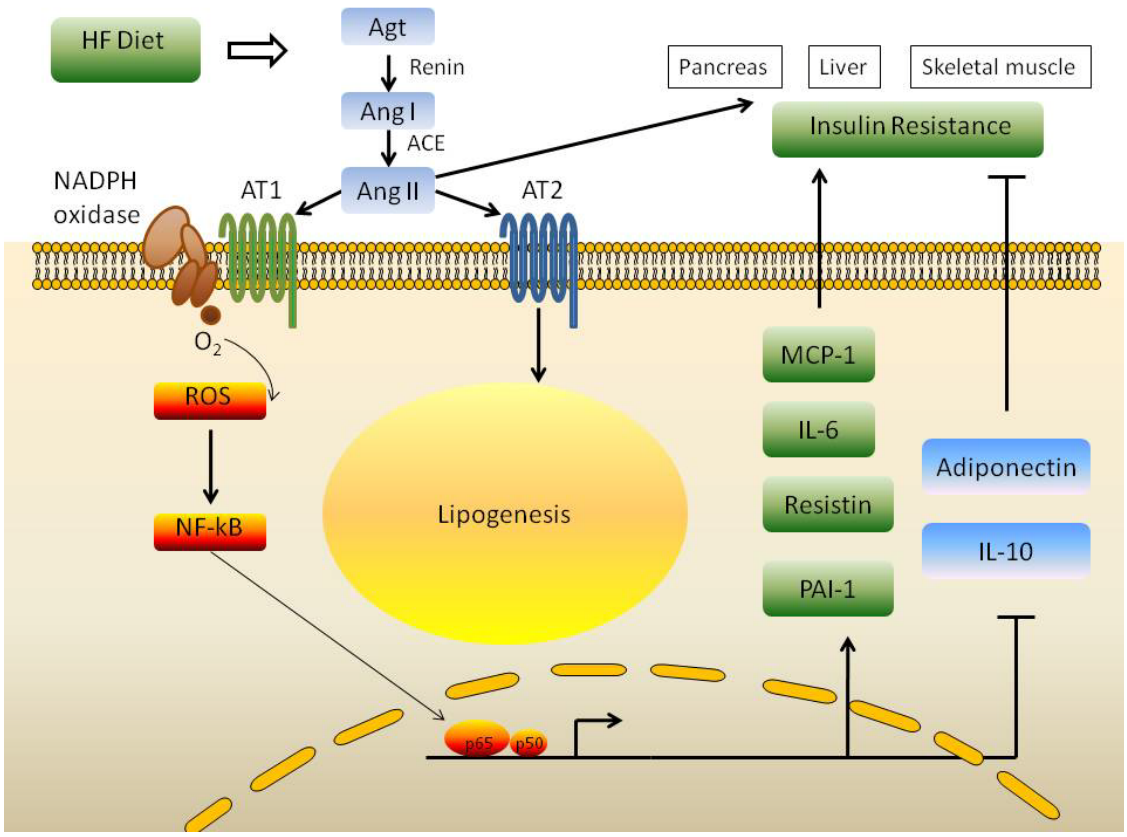

Figure 9. Hypothesis for specific aim I

We propose that overproduction of Agt from adipose tissue leads to increased lipogenesis and activation of NADPH oxidase, which in turn lead to activation of the NF-kB pathway in adipocytes. This will increase transcription of pro-inflammatory adipokines and suppress the anti-inflammatory adipokines leading to systemic insulin resistance.

II. Determine the role of energy-restricted high-fat diets in reversing metabolic derangements of obesity

In this specific aim, we test the hypothesis that weight loss due to an energy-restricted high-fat diet is accompanied by improvements in insulin sensitivity, adipose tissue inflammation and metabolic markers (Figure 10). To test this hypothesis, we have the following objective.

A. Determine the changes in metabolic markers and adipose tissue gene expression patterns in mice fed a low-fat, high-fat or a high-fat energy-restricted diet

III. Elucidate mechanisms of (n-3) polyunsaturated fatty acid-mediated prevention and reversal of insulin resistance in high-fat diet-induced obesity

In this specific aim we test the hypothesis that eicosapentaenoic acid (EPA) would prevent and reverse the metabolic derangements occurring in diet-induced obesity (DIO) via modulation of adipose tissue inflammation (Figure 10). We have the following objectives.

A. Determine metabolic alterations in adipose tissue and changes in glucose tolerance, insulin sensitivity and circulating metabolic biomarkers in mice fed a low-fat, high-fat or a high-fat EPA diet
B. Determine mechanisms by which EPA modulates HF diet-induced adipose tissue inflammation

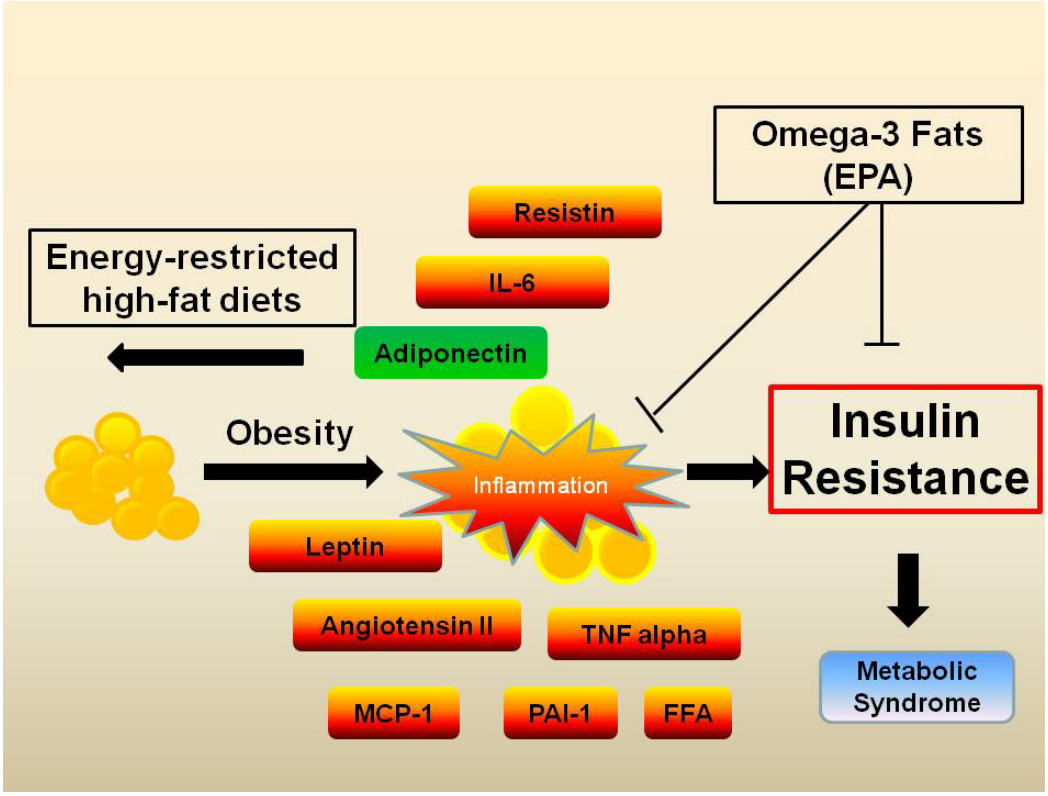

Figure 10. Hypotheses for specific aims II and III

Obesity leads to adipocyte hypertrophy and a dysregulation of adipokine secretion favoring inflammation. We propose that loss of adiposity due to energy-restricted high-fat diets will also lead to improvements in adipokine secretory patterns and metabolic markers. We also propose that EPA would modulate adipose tissue inflammation and thereby improve systemic insulin resistance induced by high-fat feeding.

CHAPTER III

ROLE OF ADIPOSE TISSUE ANGIOTENSINOGEN (AGT) OVEREXPRESSION IN THE PATHOGENESIS OF INSULIN RESISTANCE IN OBESITY

Disclosure: The work described in this chapter in its entirety has been submitted for the following publication, with minor modifications in the numbering of tables and figures: "Kalupahana NS, Massiera F, Quignard-Boulange A, Ailhaud G, Voy BH, Wasserman DH and Moustaid-Moussa N. Overproduction of angiotensinogen from adipose tissue induces adipose tissue inflammation, glucose intolerance and insulin resistance. Submitted to *Endocrinology"*

Introduction

The renin-angiotensin system (RAS) is classically known for its role in the regulation of blood pressure and fluid balance [371]. Angiotensinogen (Agt) is cleaved by the enzymes renin and angiotensin-converting enzyme (ACE) successively, to form angiotensin II (Ang II), the main bioactive peptide of this system. In addition to Ang II, several other angiotensin peptides such as Ang 1-7 are also generated by the RAS [372]. It is also well documented that other non classical enzymatic and non enzymatic pathways can generate many of the RAS intermediate peptides [371]. Ang II exerts its physiological actions, primarily via two G-protein coupled receptors, Ang II Type 1 (AT_1) and Type 2 (AT_2) receptors [371]. Hence, ACE inhibitors and angiotensin receptor blockers (ARBs) are common drug targets for anti-hypertensive therapy [371]. Interestingly, several clinical trials have shown that the risk for type-2 diabetes mellitus is

lower in hypertensive individuals treated with ACE inhibitors or ARBs compared to those treated with other anti-hypertensive medications [373]. Subsequent randomized controlled trials have also shown that RAS blockade improves glucose tolerance and insulin sensitivity in previously insulin resistant individuals [374]. Further, RAS blockade also ameliorates insulin resistance and glucose intolerance in several rodent models of obesity [188, 375]. However, the exact mechanism of modulation of insulin sensitivity via RAS blockade is not fully understood.

Adipose tissue is well recognized now as an important endocrine organ which secretes a number of bioactive peptides collectively known as adipokines. These include leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1(PAI-1), monocyte chemotactic protein-1(MCP-1) and Ang II [23]. Obesity leads to a chronic low-grade inflammatory state in the adipose tissue and a dysregulation of adipokine secretory patterns, which is causally linked to the pathogenesis of metabolic syndrome and Type-2 diabetes [376]. Several lines of evidence point to the adipose RAS as a potential link between obesity and insulin resistance. Indeed, adipose tissue synthesizes and secretes the major components of RAS [377]. There is also evidence for overactivation of adipose tissue RAS in obesity in rodents [143, 152], and for a positive correlation between adipose tissue Agt levels and body mass index in humans [52]. Moreover, circulating levels of Agt correlate with BMI and estimated total adipose tissue-derived Agt in humans [123], suggesting an endocrine role for adipose Agt. Further, Ang II secretion from adipose tissue is increased following sympathetic stimulation in obese, but not lean, individuals [129]. Conversely, plasma and adipose Agt

levels are decreased following weight-loss [130]. Despite this strong evidence for an association between adipose RAS overactivation and insulin resistance, it is hitherto unknown whether the former is causally linked to the latter.

More recent studies targeted manipulation of RAS (overexpression or deletion) and subsequent effects on obesity and insulin sensitivity. Loss of function in any single component of the RAS tested so far, provides protection from diet-induced obesity and insulin resistance; i.e. Agt, Renin, ACE, AT_1 or AT_2 knockout rodents are lean and insulin sensitive [173-175, 178, 378]. Systemic RAS overactivation via gene overexpression or chronic Ang II infusion also induces insulin resistance, but not necessarily obesity [159, 197, 202]. Considering the relationship of adipose tissue inflammation to insulin resistance, it is possible that overactivation of adipose RAS specifically, leads to obesity and associated insulin resistance and inflammation. The hypothesis that adipose RAS overactivation will induce insulin resistance via increasing adipose tissue inflammation was tested in the present studies using a mouse model which overexpresses Agt in adipose tissue via the adipose aP2 promoter (aP2-Agt mice). These mice have elevated plasma Agt and develop hypertension [157]. As previously reported, male aP2-Agt mice exhibit higher fat pad weights compared to wild type littermates. Here, we further demonstrate that these mice also develop adipose tissue inflammation, glucose intolerance and insulin resistance even on a low-fat diet. Moreover, the glucose intolerance was significantly improved when aP2-Agt mice were treated with ACE inhibitor, captopril. Our studies thus demonstrate that adipose tissue RAS overactivation causes systemic insulin resistance in an Ang II-dependent manner.

Research Design and Methods

Animals

Generation of transgenic mice overexpressing Agt in adipose tissue using the adipose aP2 promoter (aP2-Agt mice) has been described previously [157]. Mice used in the current study were bred on a C57BL/6J background and maintained in our animal facility at the University of Tennessee. Genotyping of the offspring was performed using PCR of genomic DNA using transgene-specific (5'-CTTTGCCTTTCTCTCCACAG-3') and intron-specific (5'-TTATCTCGCAGGGTCTTCTC-3') oligonucleotides. All mice were housed under 12-hour light/dark cycles with free access to food and water. For the first study, mice were fed a regular low-fat (LF) diet from weaning. For the second study, a separate cohort of male mice were fed a high-fat (HF) diet (45, 20, and 35% of energy from fat, protein, and carbohydrate, respectively; D12451 Research Diets, New Brunswick, NJ)) [379] for 12 weeks from the time of weaning. The mice on the HF diet were housed individually. Half the number of HF-fed mice was given the ACE inhibitor captopril with drinking water (30mg/L). At the end of each study, mice were feed-deprived for 6 hours and then killed using the CO_2 inhalation method. Blood was collected into tubes with EDTA, kept on ice for 10 min, centrifuged at 3000 X g for 20 min, and plasma samples were collected and stored at -80^{0}C for subsequent analyses. Gonadal (epididymal or periovarian), inguinal, retroperitoneal and subscapular fat pads were dissected, snap-frozen in liquid N_2, and stored at -80^{0}C for subsequent analyses. These protocols were all approved by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville.

Glucose tolerance test

Mice were feed-deprived for 6 hours with free access to water. A drop of tail blood was used to measure the blood glucose levels using the One Touch Ultra glucometer. Next, 1 g/kg body weight of 20% D-glucose was injected intraperitoneally. Serial blood glucose measures were taken at 15, 30, 60, and 120 min after the injection.

Hyperinsulinemic, euglycemic clamp

Detailed procedure has been previously reported [380]. Catheters were chronically implanted in the jugular vein (for infusions) and carotid artery (for sampling) 5 to 7 days prior to clamps (n=8-10). Insulin was continuously administered at 4 mU/kg/min. Arterial glucose levels were measured every 5-10 min and glucose infusion rates were adjusted to maintain fasting glucose. Mice were infused with [3-^{3}H]glucose at a rate of 0.4 μCi/min. Endogenous glucose appearance (Ra) and disappearance (Rd) rates were calculated as described previously [380]. Glucose clearance was calculated by dividing the Rd by the arterial glucose concentration. To measure a tissue-specific index of glucose metabolism (Rg), mice were injected with 12 μCi of [^{3}H]-labeled 2-deoxyglucose ([2-^{3}H]DG). Arterial plasma samples were collected in intervals for 40 minutes before mice were anesthetized and tissues were extracted and frozen in liquid nitrogen until further analysis.

Plasma analyte measurements

Commercially available ELISA kits were used to measure plasma Agt, leptin and total adiponectin (Linco Research, Billerica, MA) concentrations, whereas a colorimetric

assay was used to measure plasma nonesterified fatty acids (Wako Chemicals USA, Richmond, VA). Plasma insulin, monocyte chemotactic protein-1 (MCP-1) and resistin concentrations were measured using a commercially available microsphere-based multiplexing system (Luminex xMAP, Millipore, Billerica, MA).

Adipose tissue adipokine measurements

Because the epididymal (gonadal) fat depot is known to show a pronounced dysregulation of adipo/cytokine secretory patterns in response to high-fat diets and other metabolic challenges [381], we used it to study the adipo/cytokine changes in our mice. Epididymal adipose tissue was homogenized in modified radio-immunoprecipitation assay buffer containing a cocktail of protease inhibitors for total protein extraction. The protein concentration was determined by the Bradford assay (18). Luminex xMAP was used to measure adipokines. Analyte values in the adipose protein extracts were normalized to total protein concentration.

Proteomics

Proteins were extracted from epididymal adipose tissue of aP2-Agt and control littermates matched for adipose mass, and labeled with cyanine 3 or 5 (green or red), respectively. Both samples were run on a 2-dimensional gel electrophoresis (Applied Biomics). A complete analysis of all differentially expressed proteins was obtained using Decyder software from which quantitative data were derived. Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and obtained using the

spot picker robot, and proteins within each spot were enzymatically digested and analyzed by Mass Spectrometry. Proteins identified from this analysis were then uploaded into DAVID Bioinformatics Resources, where the functional annotation chart was used to search for significantly enriched gene ontology categories. Differential expression of these proteins was further confirmed by western blotting.

Cell culture experiments

Adipocytes were cultured as described previously [382]. Briefly, 3T3-L1 preadipocytes were cultured in 6-well plates in regular growth media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S). At confluence, the cells were differentiated by the addition of growth media supplemented with 250 nmol/L dexamethasone and 0.5 mmol/L methyl isobutylxanthine for 72 h, after which regular media were added for 2 additional days. Subsequently, cells were placed in serum-free media [DMEM, P/S, and 1% bovine serum albumin (BSA)] for 18 hours prior to applying various treatments for 24 hours, as described in the results and figure legends. Secreted adipokine levels were assayed in culture media, using Luminex Xmap.

Western immunoblotting

Total proteins were isolated from epididymal adipose tissue, homogenized in modified RIPA buffer containing a cocktail of protease inhibitors. Twenty micrograms of total protein was loaded into each lane and separated by electrophoresis in an 8–10%

polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane in transfer buffer, blocked overnight with 5% non-fat dry milk in Tris-buffered saline, and 0.1% Tween, incubated with polyclonal antibodies against monoglyceride lipase (MGL) and actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary antibodies containing horseradish peroxidase and finally detected by chemiluminescence.

Statistical analysis

Body and fat-pad weight, area under the glucose curve (AUC) and plasma biomarkers were analyzed by ANOVA using the general linear models procedure, taking the effect of the genotype, sex, and interactions between these factors into account. For the diet study, a similar analysis was performed taking the effect of genotype, treatment and interactions between these factors into account. Glucose infusion rate and tissue glucose uptake in the clamp studies, adipose tissue cytokine levels and MGLL expression was compared using the student t test. In the cell culture studies, one way-ANOVA was used. If the F test was significant, group means were compared using the Tukey's post hoc test for multiple comparisons. The fold difference in protein expression between replicate gels in proteomic studies was compared using the 1-sample t test. The level of significance for all tests was $P < 0.05$. All data are expressed as mean ± SD.

Results

Adipose Agt overexpression induces higher adiposity, glucose intolerance and insulin resistance

As expected, the aP2-Agt transgenic mice had higher adipose Agt levels (5.2 ± 3.9, 16.9 ± 8.6, 15.9 ± 7.7 and 20.4 ± 10.8 μg/g protein in female wild-type (Wt), female transgenic, male Wt and male transgenic respectively; $p < 0.05$ for genotype effect) at the age of 24-weeks. Similar to previous reports [157], the transgenics also had approximately 20% higher plasma Agt levels (Table 5) than the Wt mice. These mice also exhibited higher fat pad weights, while there was no genotype effect on body weight (Figure 11A and B). An intra-peritoneal glucose tolerance test performed at 22-weeks of age showed that male transgenics were glucose intolerant as compared to Wt males, indicated by a higher area under the glucose curve (AUC -Figure 11C and D). However, this difference in glucose tolerance was not seen in female mice.

While the transgenics also had higher plasma leptin levels compared to their Wt counterparts, as expected from fat pad weight differences, there was no significant genotype effect on plasma levels of several other adipocytokines (Table 5). Next, to determine if overexpression of Agt in adipose tissue causes insulin resistance, we performed hyperinsulinemic, euglycemic clamps on male aP2-Agt mice. The transgenics had a lower steady-state glucose infusion rate compared to Wt mice, indicating lower insulin sensitivity (Figure 12B, C). The difference in glucose clearance between basal and clamp was also lower in the transgenics (Figure 12D). Isotope studies for tissue glucose uptake showed that Rg was significantly lower in the gastrocnemius and vastus muscles

Table 5. Plasma biomarkers in low-fat fed aP2-agt mice[#]

	Females		Males		P Value		
	Wt	Tg	Wt	Tg	Sex effect	Genotype Effect	Sex X Genotype Effect
Insulin (pmol/L)	131 ± 93	164 ± 67	191 ± 73	312 ± 133	**0.019***	0.157	0.725
Glucose (mg/dL)	204 ± 16	183 ± 9	199 ± 15	221 ± 22	0.352	0.753	0.166
Adiponectin (mg/L)	18.2 ± 7.0	19.7 ± 6.1	9.8 ± 3.1	9.9 ± 2.9	**0.001***	0.743	0.769
Leptin (μg/L)	1.6 ± 1.0	4.1 ± 2.5	2.9 ± 1.7	6.0 ± 4.6	0.204	**0.046***	0.508
Agt (mg/L)	2.8 ± 0.8	3.4 ± 0.8	3.4 ± 0.5	4.4 ± 0.4	**0.021***	**0.022***	0.617
NEFA (mmol/L)	0.55 ± 0.19	0.70 ± 0.19	0.62 ± 0.22	0.71 ± 0.19	0.649	0.200	0.727
MCP-1 (ng/L)	18.9 ± 7.0	25.4 ± 15.0	22.6 ± 14.7	24.0 ± 9.2	0.894	0.638	0.761
Resistin (ng/L)	1400 ± 211	1748 ± 566	1184 ± 279	1032 ± 99	**0.034***	0.777	0.267

[#]Data is presented as mean ± SD (n=4-9 per group); Wt – wild-type, Tg - transgenic
* $p < 0.05$

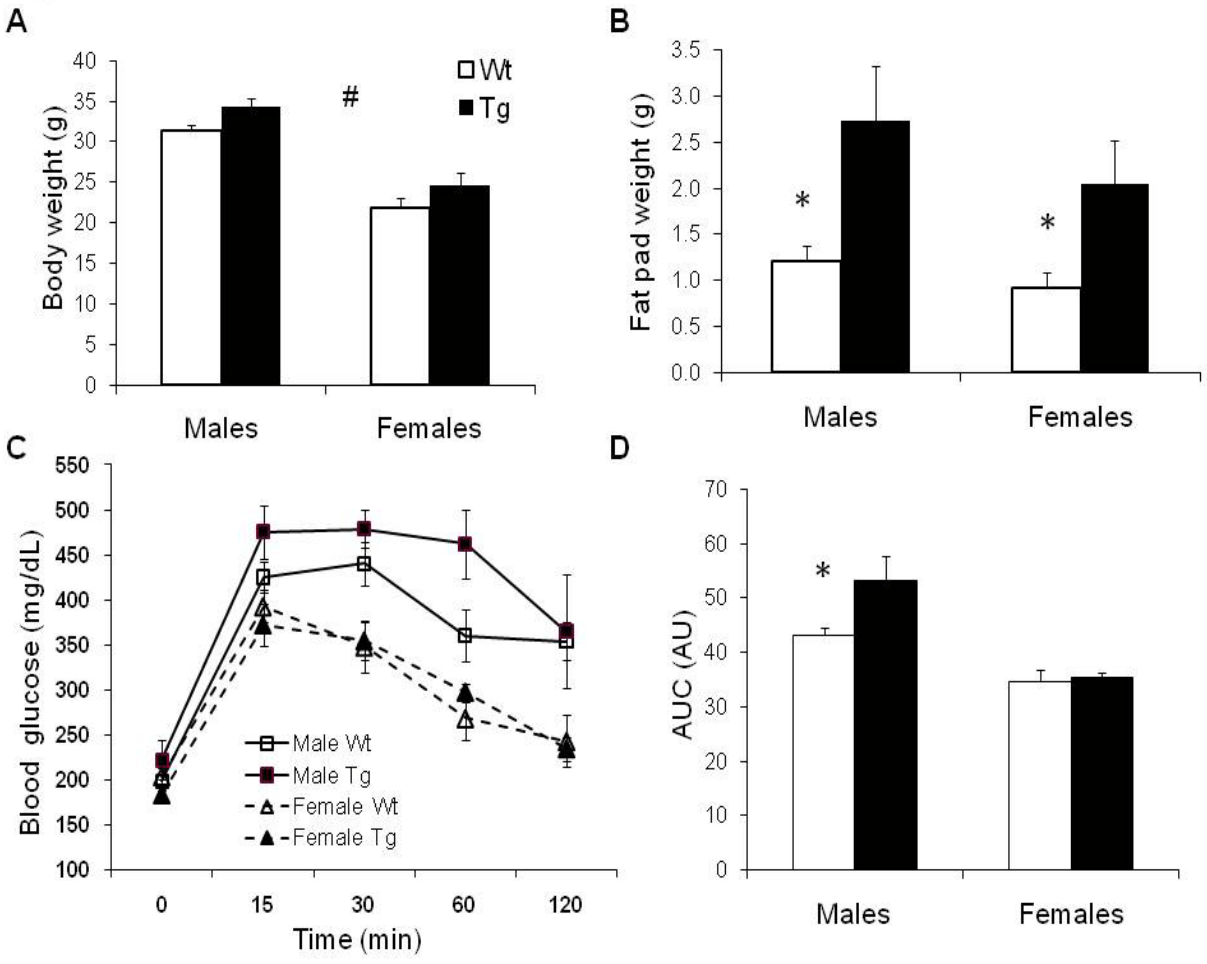

Figure 11. Adipose Agt overexpression induces adiposity and glucose intolerance

Wild-type (Wt) and transgenic (Tg) aP2-Agt mice were fed a low-fat diet from weaning until 24-weeks of age. Body weight (A) and fat pad weight (B) are shown. At 22-weeks of age, an intraperitoneal glucose tolerance test was performed. Change in blood glucose over time (C) and area under the glucose curve (AUC) (D) are shown. Data are presented as mean ± SEM (n=4-9 per each group). # $p < 0.05$ for sex effect; * $p < 0.05$ for genotype effect.

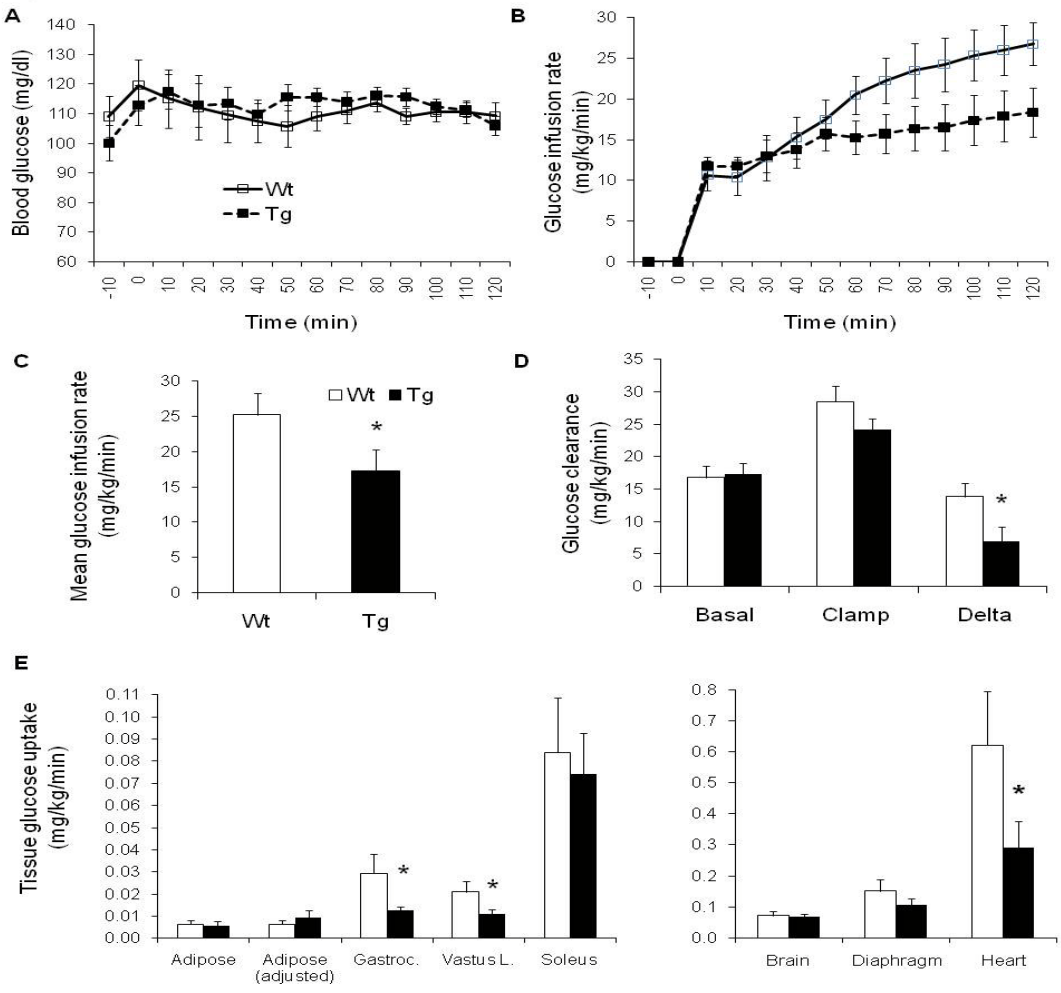

Figure 12. Adipose Agt overproduction induces insulin resistance

Low-fat diet fed, male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were subjected to a hyperinsulinemic, euglycemic clamp. Change in blood glucose (A) and glucose infusion rate (B) over time, mean steady-state glucose infusion rate (C) and glucose clearance rate (D) are shown. Using [^{3}H]-labeled 2-deoxyglucose, tissue-specific index of glucose metabolism (Rg) was measured (E). Data are presented as mean ± SEM (n=8-10 in each group). * $p < 0.05$

in the transgenic mice compared to the Wt mice (Figure 12E). Interestingly, the transgenics also had lower cardiac Rg. While there was a trend for an increase in adipose tissue Rg (adjusted for adipocyte number) of transgenics, this was not significant.

Captopril attenuates glucose intolerance in HF-fed aP2-Agt and Wt mice

HF feeding is known to induce glucose intolerance and insulin resistance in C57BL/6J mice [77]. To test that adipose RAS overactivation exacerbates HF diet-induced glucose intolerance, we fed transgenic and Wt litermates a HF diet for 12 weeks. To determine if the glucose intolerance was Ang II-dependent, we also treated a cohort of HF-fed mice with the ACE inhibitor, captopril (30mg/L in drinking water). In contrast to males on the LF diet (figure 11D), the glucose tolerance was similar in both transgenics and Wt mice on the HF diet, as indicated by similar AUCs (Figure 13C and D). Captopril prevented the HF-diet induced increase in adiposity (Figure 13A and B) and glucose intolerance in both transgenic and Wt mice (Figure 13C and D). Thus, while aP2-Agt mice exhibited glucose intolerance compared to control littermates when fed low-fat diets, HF feeding normalized these difference in glucose tolerance between genotypes. Further, ACE inhibition improved HF diet-induced glucose intolerance in both genotypes.

Adipose Agt overexpression increases markers of adipose inflammation

Chronic low-grade inflammation in adipose tissue is causally linked to the pathogenesis of insulin resistance in obesity. To determine if adipose inflammation is a potential mechanism for the insulin resistance in aP2-Agt mice, we measured selected

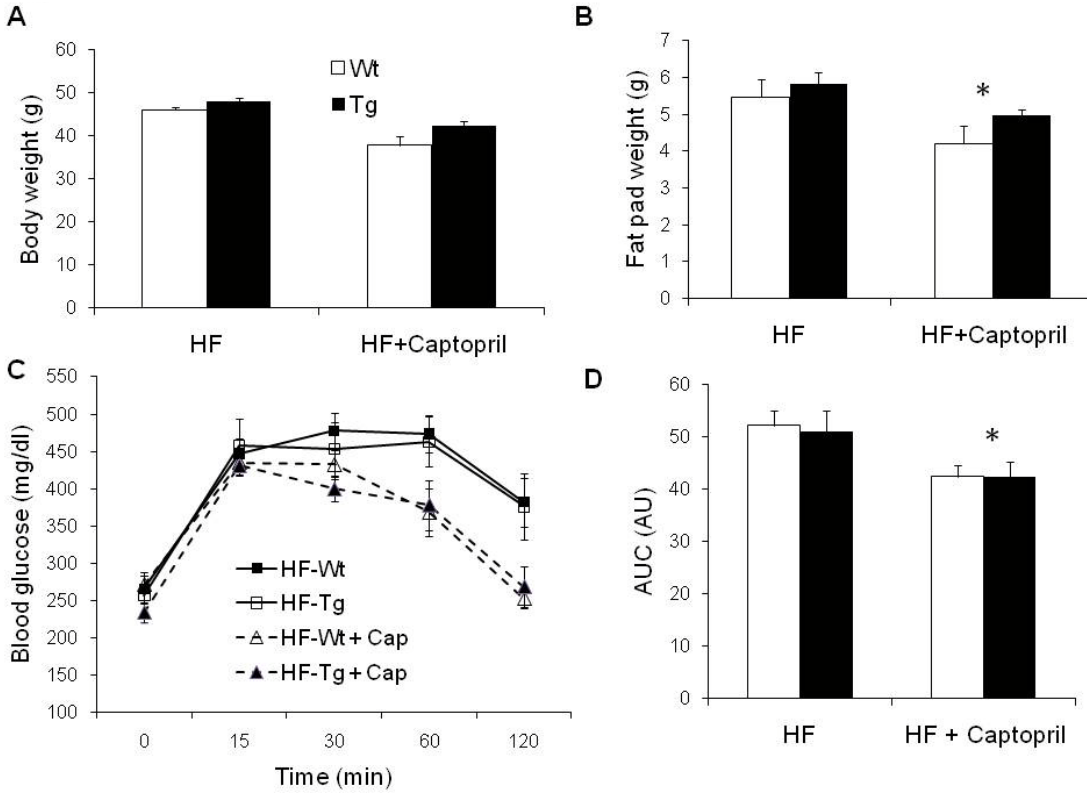

Figure 13. Captopril attenuates high-fat diet induced adiposity and glucose intolerance Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a high-fat diet (45, 20, and 35% of energy from fat, protein, and carbohydrate, respectively) for 11 weeks. Half of each genotype was given captopril (cap - 30mg/L) in drinking water. Body weight (A) and fat pad weight (B) are shown. At 10 weeks on the high-fat diet, an intra-peritoneal glucose tolerance test was performed. Change in blood glucose concentration over time (C) and area under the glucose curve (AUC) (D) are shown. Data are presented as mean ± SEM (n=8-12 in each group). * $p < 0.05$ treatment effect.

markers of adipose inflammation. Pro-inflammatory cytokine MCP-1 was nearly two-fold higher in the epididymal adipose tissue of the transgenics compared to Wt litermates (Figure 14). Further, expression of interleukin-10 (IL-10), an anti-inflammatory cytokine was significantly lower in the transgenics. The hematopoietic cytokine IL-7 and leukocytotrophic cytokine IL-2 were expressed lower in the transgenics. These findings indicate that adipose Agt overexpression induces adipose tissue inflammation.

Angiotensin II increases secretion of MCP-1 and resistin in adipocytes

Previous studies have reported that aP2-Agt mice exhibit adipocyte hypertrophy [157]. Because adipocyte hypertrophy is associated with a pro-inflammatory adipokine secretory profile, increased MCP-1 levels in adipose tissue of transgenics could be due to either a direct action of Ang II on adipocytes, or an indirect effect due to adipocyte hypertrophy. To address this issue, we subjected 3T3-L1 murine adipocytes to a short-term (24-hour) treatment of Ang II (10 nM). As shown in figure 15, Ang II increased the secretion of MCP-1 and resistin from adipocytes. Because Ang II is known to activate the nuclear factor kappa B (NF-kB) pathway, we treated the adipocytes with Ang II in the presence of an NF-kB inhibitor (Bay 11-7082). As expected, Bay 11-7082 suppressed the Ang II-induced increases in MCP-1 and resistin secretion from adipocytes. This indicates that Ang II induces MCP-1 and resistin secretion from adipocytes in an NF-kB pathway-dependent manner. To further investigate the mechanism of Ang II-mediated stimulation of the NF-kB pathway, we treated the adipocytes with Ang II in the presence of NADPH

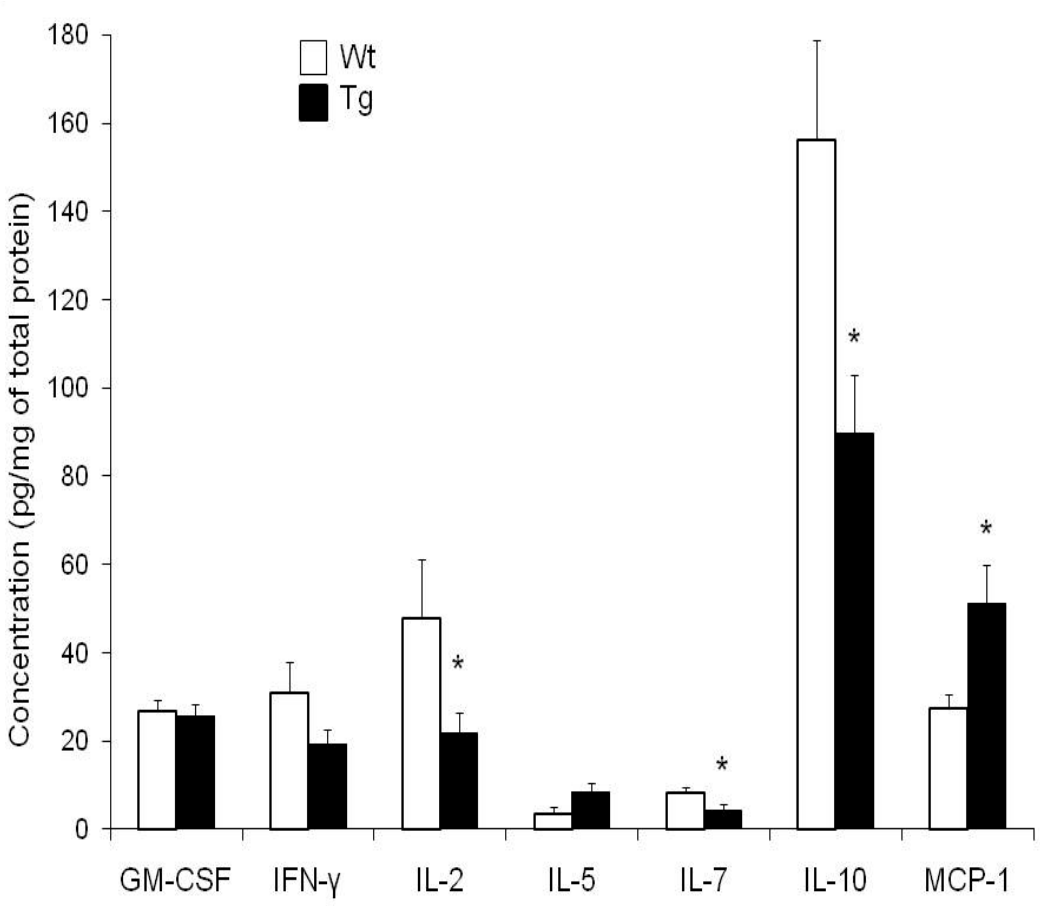

Figure 14. Adipose Agt overexpression induces inflammatory markers in adipose tissue Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a low-fat diet from weaning until 24-weeks of age. Epididymal adipose tissue cytokine levels normalized to total protein content are shown. Data are presented as mean ± SEM (n=5 per group). * $p < 0.05$

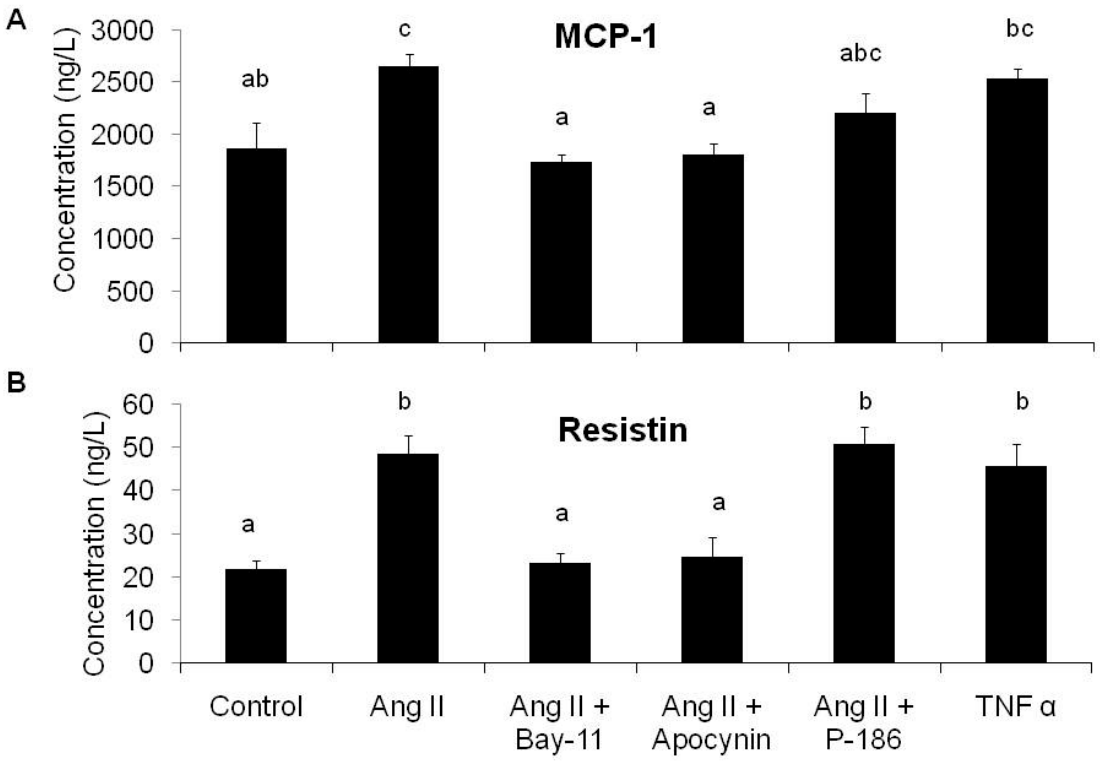

Figure 15. Ang II induces MCP-1 and resistin secretion from 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were treated with control (DMSO), Ang II (10nM), Ang II plus NFkB inhibitor Bay 11-7082 (5μM), Ang II plus NADPH oxidase inhibitor apocynin (200μM), Ang II plus AT_2R antagonist P-186 (100nM) or TNF α (100pM) for 24 hours. Culture media MCP-1 (A) and resistin (B) levels are shown. Data are presented as mean ± SEM (n=3 per each group). Different letters indicate a significant difference ($p < 0.05$)

oxidase inhibitor, apocynin. As shown in Figure 15, apocynin also completely prevented the Ang II-induced increases in MCP-1 and resistin secretion. We have previously shown that Ang II effects in 3T3-L1 adipocytes are mediated by AT_2 [216]. To test whether these changes were mediated via AT_2, we treated the adipocytes with Ang II in the presence of an AT_2 antagonist. We found that this antagonist was able to partially prevent the Ang II-induced MCP-1, but not resistin secretion.

Adipose angiotensinogen overexpression induces MGL expression

Proteomic studies of epididymal adipose tissue were used to identify novel proteins induced by adipose angiotensinogen overexpression of adipose weight-matched transgenic and Wt litermates, using 2DIGE. Several spots differentially expressed between the Wt and transgenic mice were identified (Figure 16A and B), quantified (Figure 6C), and proteins were identified by mass spectrometry. The detailed list of proteins is given in Table 6. Transgenics exhibited higher expression of glycerol-3-phosphate dehydrogenase, a major lipogenic enzyme and MGL, a lipolytic enzyme. The differential expression of MGL was confirmed by western blotting as shown in Figure 16D and E. Proteins expressed at low levels in the transgenic mice include catalase, an antioxidant enzyme.

Discussion

These studies provide the first evidence that adipose RAS components directly link to insulin resistance. Male aP2-Agt mice develop glucose intolerance and systemic

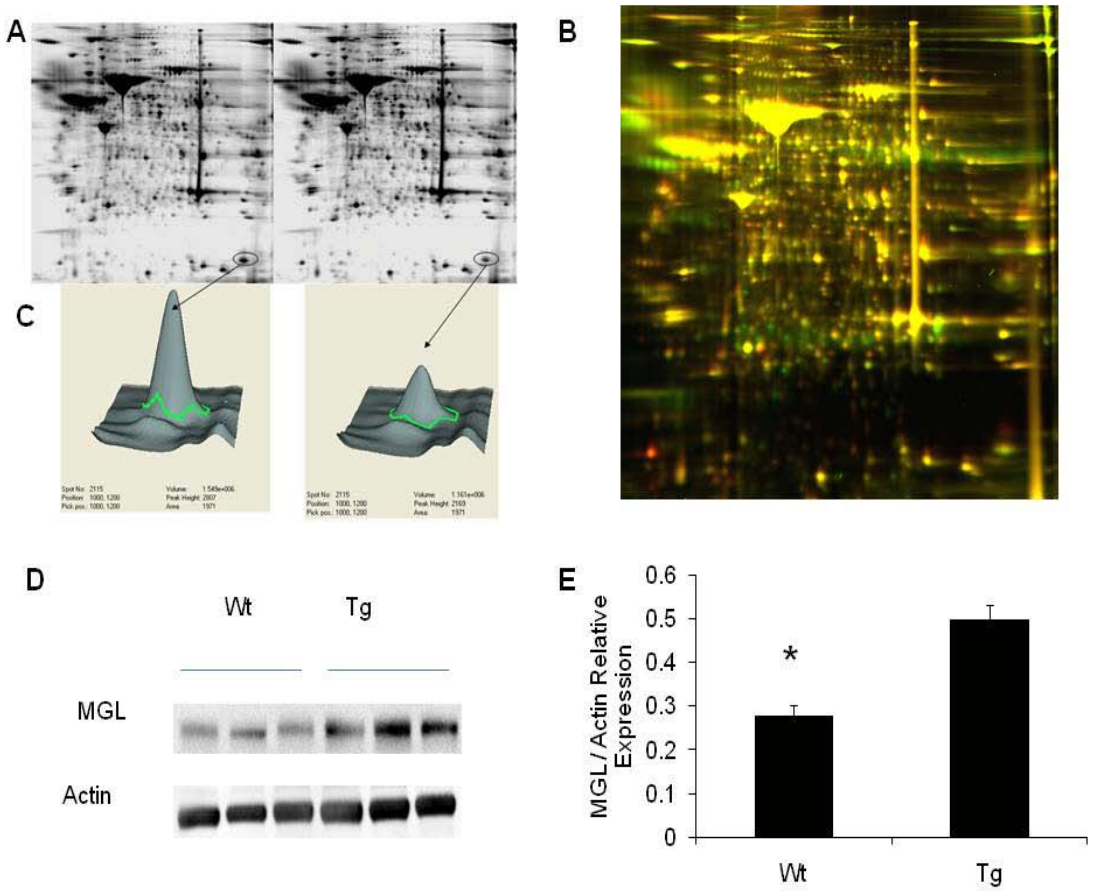

Figure 16. Adipose agt overexpression induces monoglyceride lipase in adipose tissue

Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a low-fat diet from weaning until 24-weeks of age. Epididymal adipose tissue proteomic studies were done in adipose weight-matched mice. Differentially expressed spots between Wt and Tg are shown (A and B). Spots were quantified (C), picked and identified using mass spectrometry. Detailed list of proteins are given in Table 6. Monoglyceride lipase expression between Wt and Tg was confirmed by western blot (D and E). Data are expressed as mean ± SEM (n=4-5 per group). * $p < 0.05$

Table 6. Proteins differentially expressed in adipose tissue in low-fat fed aP2-Agt mice

Protein Name	GI Accession No	Tg/Wt spot volume ratio[#]
Tg higher Expression than Wt		
Triosephosphate isomerase 1	6678413	1.67
Gpd1 protein (glycerol-3-phosphate dehydrogenase)	13543176	1.36
Vinculin	31543942	1.46
Isocitrate dehydrogenase 3 (NAD+) alpha	18250284	1.49
Eukaryotic translation initiation factor 5A, isoform CRA_a	148680528	2.42
Vitamin D-binding protein	193446	1.40
Ehd2 protein	20072042	1.43
Medium-chain acyl-CoA dehydrogenase	6680618	1.52
Polymerase I and transcript release factor	6679567	1.59
Polymerase I and transcript release factor	6679567	1.66
Glutathione S-transferase, alpha 4	15215030	1.65
Fumarate hydratase 1	33859554	1.79
Mgll protein (monoglyceride lipase)	34786023	2.40
Anxa1 protein	12805619	1.92
Tg lower expression than Wt		
Apolipoprotein A-I	6753096	0.17
Immunoglobulin gamma-1 heavy chain	26665404	0.25
Mannose binding lectin, serum (C)	6754656	0.20
Arhgdib protein	21618829	0.26
Galactokinase 1, isoform CRA_a	148702595	0.35
Medium-chain acyl-CoA dehydrogenase	6680618	0.35
Apolipoprotein A-I precursor - mouse	109571	0.35
Dnm1l protein	51259985	0.21
Villin 2	37573976	0.41
Major urinary protein 2	47059037	0.35
Transthyretin	56541070	0.39
Proteasome beta 3 subunit	6755202	0.50
DOM1	21322147	0.50
Carbonic anhydrase 3	31982861	0.20
Similar to Sly protein	38090288	0.29
Hemopexin	160358829	0.64
Catalase	157951741	0.64
Glutathione S-transferase M2	6680121	0.68
G protein beta subuit like	475012	0.68
Anti-human seminoprotein monoclonal antibody	27227449	0.25
65-kDa macrophage protein	984636	0.34

[#]Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and analyzed by mass spectrometry. Tg – transgenics, Wt- wild-type

insulin resistance, which is at least in part due to reduced skeletal muscle glucose uptake. Moreover, the inflammatory profile of these transgenic mice is characterized by elevated adipose MCP-1 levels. We further confirmed this *in vitro*, demonstrating that Ang II increases MCP-1 secretion from murine adipocytes, in an NF-kB and NADPH oxidase-dependent manner.

RAS overactivation, adiposity and insulin resistance

In agreement with previous reports, our aP2-Agt mice developed increased adiposity even on a low-fat diet [157]. We have previously shown that Ang II increases triglyceride content and lipogenic enzyme levels in murine adipocytes *in vitro* [216]. Similarly, we found that the expression of glycerol-3-phosphate dehydrogenase, a key lipogenic enzyme, was expressed at higher levels in the adipose tissue of aP2-Agt transgenics compared to non-transgenic controls. Interestingly, other models of systemic RAS overactivation such as chronic Ang II infusion [159] and overexpression of human AGT in liver [152], do not exhibit higher adiposity. The increased adiposity seen in aP2-Agt mice strongly suggests that it is due to autocrine / paracrine actions of Ang II on adipose tissue.

In addition to being moderately obese, male aP2-Agt mice are also glucose intolerant and insulin resistant. Previous rodent models of RAS overexpression have shown that chronic systemic RAS overactivation leads to insulin resistance. These include the renin overexpressing hypertensive transgenic TG(mRen2)27 rat [191], and chronic Ang II infusion models [202]. In this study we show that specific adipose Agt

overexpression is sufficient to produce a level of glucose intolerance that is comparable to Wt mice on a HF diet. An important finding of these studies is that HF feeding did not further exacerbate glucose intolerance in aP2-Agt mice. This suggests that similar mechanisms operate in both instances. It is interesting that female aP2-Agt transgenics remained glucose tolerant, which is likely due to the effects of sex hormones as reported previously [383]; however further studies beyond the scope of this work, are warranted.

Since systemic RAS blockade improves insulin resistance and glucose tolerance in numerous human and rodent studies [191, 374, 384], we tested whether blocking RAS would improve metabolic alterations in the aP2-Agt mice. As expected, treatment with ACE inhibitor captopril prevented the HF diet-induced glucose intolerance in both transgenic and Wt mice. Previous studies in rodents with ACE knockout [175], renin inhibition [191], renin knockout [173], AT_1 blockade [375] or AT_2 deletion [178] have all shown improvements in insulin sensitivity and glucose tolerance. ACE2 overactivation which lowers Ang II and increases Ang (1-7) has also been shown to promote insulin sensitivity and glucose tolerance [206, 372].

Mechanisms of insulin resistance induced by RAS overactivation

Most models of obesity and insulin resistance exhibit both muscle and adipose insulin resistance. Here, we found that while adipose tissue Rg was not impaired in the aP2-Agt mice, these mice exhibited reduced skeletal muscle and heart Rg. This could at least in part explain the whole body insulin resistance in transgenic mice. Several potential factors might have caused skeletal muscle insulin resistance. We found that the

level of pro-inflammatory cytokine MCP-1 was almost two-fold higher in the adipose tissue of transgenics compared to the non-transgenic control mice, while the level of anti-inflammatory cytokine IL-10 was lower. Together, these findings indicate increased inflammation in the adipose tissue of the aP2-Agt mice. Altered adipokine secretory patterns due to adipose tissue inflammation could be a cause of skeletal muscle insulin resistance [96]. It should however be noted that the plasma levels of several adipokines (with the exception of leptin) was comparable between the Wt and transgenic mice. Thus, a possible paracrine role of adipose tissue could be important in the reduced skeletal muscle and heart Rg in the aP2-Agt mice. Indeed, previous studies have linked adipose tissue distribution within skeletal muscle to insulin resistance [385]. Epicardial fat is also known to produce proinflammatory cytokines, which is implicated in the post-operative insulin resistance [386, 387]. *In vitro* studies have also shown that adipokine secretion can impair insulin signaling of myocytes, suggesting cross-talk between the two tissues [388].

The aP2-Agt transgenics also exhibited higher plasma Agt levels. Higher Agt and Ang II levels were previously reported to induce skeletal muscle and vascular smooth muscle insulin resistance by several mechanisms. First, the skeletal muscle blood flow can be reduced due to the pressor effect of Ang II, resulting in lower glucose delivery. Second, Ang II can also inhibit insulin signaling in muscle [389], resulting in reduced translocation of Glut4, leading to reduced glucose transport. Increased Ang II levels are also known to impair glucose-stimulated insulin release from the pancreatic islets [204]. Thus, the glucose intolerance in the aP2-Agt transgenics could be attributed to a

combination of reduced skeletal muscle glucose uptake as well as reduced insulin response in the pancreas, which could be exacerbated by their elevated blood pressure [157]. Additionally, previous studies have also reported that Ang II increases hepatic glucose production, which could also contribute to glucose intolerance [202]. We propose that these metabolic alterations are mediated by Ang II, as evidenced by an improvement of the glucose tolerance in the mice treated with ACE inhibitor captopril. While elevated plasma Agt levels could contribute to the systemic insulin resistance in aP2-Agt mice as outlined above, it is unlikely that it played a major role, because the difference in plasma Agt levels between the two genotypes was modest compared to circulating concentrations shown to be effective in causing insulin resistance in previous studies [202].

Ang II and adipose tissue inflammation

Adipose Agt overexpression increases markers of adipose tissue inflammation (increased MCP-1 and decreased IL-10 in this study and increased IL-6 and IL1β in previous reports [179]). aP2-Agt mice also exhibit higher adiposity and adipocyte hypertrophy [157], and because the latter is associated with a pro-inflammatory adipokine profile, the increased MCP-1 could be a result of adipocyte hypertrophy. However, we showed that Ang II can also directly increase MCP-1 and resistin secretion from adipocytes *in vitro*. Ang II's ability to promote MCP-1 production in other cell types such as preadipocytes [390] and pancreatic islets [391] has been reported previously. Further, RAS blockade improved plasma MCP-1 and resistin levels in humans [392, 393].

Ang II is also documented to simulate NADPH oxidase, leading to increased production of reactive oxygen species, which can activate the NF-kB pathway and production of proinflammatory cytokines in the skeletal muscle [196] and vascular smooth muscle [394]. Thus, we investigated whether similar mechanisms operate in the adipose tissue. We found that Ang II-induced MCP-1 and resistin secretion from adipocytes was completely abolished by treatment with NADPH oxidase or NF-kB inhibitors.

From our proteomic studies, we found that the expression of MGL, a lipolytic enzyme, was two-fold higher in the epididymal adipose tissue of transgenics. MGL cleaves 2-arachodonyl glycerol to form arachidonic acid (AA). Activation of MGL has been shown to increase AA levels in vascular smooth muscle [395]. Elevated AA levels induce secretion of pro-inflammatory cytokines [77] and PGE_2 [382] in adipocytes *in vitro,* thus MGL could be another mediator of Ang II-induced adipose inflammation. We have previously shown that Ang II dose-dependently increases PGE_2 secretion in murine adipocytes *in vitro* [396]. However, direct effects of Ang II on MCP-1 via prostaglandins remain to be tested.

Overall, the aP2-Agt transgenic mouse model is a valuable model for studies of human obesity associated with insulin resistance and hypertension. This study provides for the first time evidence of a causal link between adipose tissue Agt overproduction and the generation of glucose intolerance and systemic insulin resistance. The mechanisms are at least in part due to skeletal and cardiac muscle insulin resistance, resulting from increased adipose tissue inflammation and systemic levels of Ang II.

CHAPTER IV

ROLE OF ENERGY-RESTRICTED HIGH-FAT DIETS IN REVERSING METABOLIC DERANGEMENTS OF OBESITY

Disclosure: The work described in this chapter in its entirety has been published in the following reference with minor modifications in the numbering of tables and figures:

"Kalupahana NS, Voy BH, Saxton A and Moustaid-Moussa N. Energy-restricted high-fat diets only partially improve markers of systemic and adipose tissue inflammation. *Obesity (Silver Spring)* 2011 Feb;19(2):245-54"

Introduction

Obesity is associated with a number of co-morbidities including the metabolic syndrome, which is characterized by atherogenic dyslipidemia, hypertension, insulin resistance, and pro-inflammatory and pro-thrombotic states [397]. Individuals with the metabolic syndrome have a greater risk of developing type II diabetes mellitus and cardiovascular diseases [397]. Our previous work and that of others have shown that in addition to serving as an energy store, adipose tissue also functions as an endocrine organ by secreting adipokines such as leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1(PAI-1), monocyte chemotactic protein-1(MCP-1) and angiotensin II [23, 382]. There is evidence that with the increasing accumulation of fat inside adipocytes, the endocrine function of the adipose tissue becomes dysregulated resulting in a change in adipokine secretory patterns, with a predominant pro-inflammatory profile [90] . Further, there is evidence that with

increasing accumulation of triglycerides, there is an onset of adipocyte death, remodeling and inflammation [93, 381]. These changes of adipokine secretory patterns and release of inflammatory mediators contribute to the development of insulin resistance, cardio-vascular pathologies and the proinflammatory and prothrombotic state associated with obesity.

Energy restriction is a main form of dietary intervention to treat obesity and metabolic syndrome. It has been shown to not only reduce body weight and adipose tissue mass, but also reverse the metabolic derangements resulting from diet-induced obesity including an improvement of insulin sensitivity [398], and downregulate inflammatory genes in the adipose tissue [273, 399]. The role of macronutrient composition in a weight-loss diet, however, remains contentious [307]. Most health agencies advocate a low-fat diet, while there are claims that low-carbohydrate, high-fat, energy-restricted diets are more effective in bringing about weight loss [400]. Since the increasing portion sizes of meals over the years have been linked to the obesity epidemic [401], a reduction in portion sizes without changing the macronutrient composition seems a logical alternative to reverse obesity. However, it not clear whether weight loss by these high-fat, energy restricted diets is accompanied with improvements in the adipose tissue inflammation and metabolic markers. Thus we tested the hypothesis that weight loss due to a high-fat, energy restricted diet (analogous to a reduction in portion size without changing food items) is accompanied with improvements in adipose tissue inflammation and markers of the metabolic syndrome.

Methods and Procedures

Animals and Diets

Male C57BL/6J mice aged 7-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME). After a one-week period of acclimation, they were fed either a control low-fat (LF, n=15) diet (10, 70 (35% from sucrose) and 20% of total energy from fat, carbohydrates and proteins respectively; Research Diets: D12450B) or a high-fat (HF, n=28) diet (45, 35 (17% from sucrose) and 20% of energy from fat, carbohydrates and proteins respectively; Research Diets: D12451). Detailed diet composition can be found at researchdiets.com. The mice were individually housed with standard 12 hour light / dark cycles. After four months, half the mice on the high-fat diet were switched to an energy-restricted (HFR) diet with the same macronutrient composition as the previous high-fat diet. (Ad-libitum food intake of the HF group was measured daily and on the next day 70% of that amount was then given to the HFR mice; thus HFR mice were fed 70% of calories taken by the HF group), while the other half continued on the ad-libitum high-fat feeding. Mice were terminated two months later, using the CO_2 inhalation method, following an 8-hour food deprivation with water available. These protocols were all approved by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville

Plasma glucose, insulin, triglycerides and non-esterified fatty acids measurements

Following sacrifice, blood was collected into tubes with EDTA, kept on ice for 10min, centrifuged at 10,000 g for 10minutes, supernatant collected and stored at -80^{o}C

for subsequent analyses. Glucose and insulin concentrations were measured by glucose oxidase and a commercially available enzyme immuno assay (EIA) kit (Crystal Chem Inc., Downers Grove, IL) respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as glucose concentration (mmol/l) * insulin concentration (mU/l) / 22.5 [402]. A commercially available EIA kit was used to assay the plasma total adiponectin level (Linco Research, Billerica, MA), while colorimetric assays were used to measure plasma triglycerides and non-esterified fatty acids (Wako chemicals USA inc., Richmond, VA). All assays were performed according to the manufacturers' protocols.

RNA isolation and microarray

Total RNA was isolated from gonadal (epididymal) adipose tissue using Tri-reagent (Sigma Inc., St.Louis, MO). Five μg of total RNA was used to setup first strand cDNA synthesis reaction with an Affymetrix oligo (dT) primer and Superscript II reverse transcriptase (First strand cDNA synthesis kit, Invitrogen). Second strand synthesis reaction was setup by addition of E.coli DNA ligase, E.coli Pol I, and RNAse H to the first strand. cRNA labeling, hybridization to, washing and scanning of the GeneChip Mouse Genome 430 2.0 array were performed using standard protocols [403]. RNA samples from each of 3 animals in each group were used for microarray analysis.

Western blotting

Total proteins were isolated from gonadal (epididymal) adipose tissue by homogenizing with modified RIPA buffer containing a cocktail of protease inhibitors. Protein concentration was determined by the Bradford assay [404]. Twenty micrograms of total protein was loaded into each lane and separated by electrophoresis in an 8-10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane in transfer buffer, blocked overnight with 5% non-fat dry milk in tris-buffered-saline and 0.1% tween, incubated with polyclonal antibodies against PAI-1(Santa Cruz biotechnology) and tubulin (Millipore), incubated with secondary antibodies containing horse radish peroxidase (HRP) and finally detected by chemiluminiscence.

Plasma and adipose tissue adipo / cytokine measurements

Plasma leptin and PAI-1, and cytokine content in the gonadal adipose tissue homogenized in modified RIPA buffer were measured using a commercially available microsphere based multiplexing system (Luminex xMAP–Millipore, Billerica, MA). Adipose tissue cytokines were normalized to total protein content.

Liver triglycerides and oil-red-o staining

Livers were homogenized as described by Miao et al [405]. Briefly, they were homogenized in saline and solubilized in 1% deoxycholate. Triglycerides were assayed using a colorimetric method (Wako chemicals USA Inc.) and normalized to liver weight.

Frozen sections of livers were made, stained with oil red o and counterstained with DAPI as described previously [406].

Quantitative real-time PCR

Total RNA (5µg) was reverse transcribed and quantitative real time PCR was performed using the SYBR Green method with ABI 7300 Sequence Detection System. Levels of RNA were normalized to those of a housekeeping gene, acidic ribosomal phosphoprotein P0 (*36b4*). The primers were ordered from Sigma Genosys (Sigma Inc., St.Louis, MO) and the sequences are given in Table 7. Relative expression of RNA was compared to the HF group using the delta-delta ct method [407].

Statistical Analysis

Body weight and other parameters were compared for between-group differences by one way ANOVA using SPSS (ver15.0 SPSS Inc., Chicago, IL) software. If the F test was significant, group means were compared using the Bonferroni post-hoc test for multiple comparisons. Adipose cytokines and liver triglycerides were compared between HF and HFR groups using the independent samples t-test, while RT-PCR data were expressed relative to the HF group using the one-sample t-test to determine significance. Microarray data were uploaded and stored in the UTMD (University of Tennessee Microarray Database). UTMD is an implementation of the Stanford Microarray Database (SMD) package. Data was analyzed using Significance Analysis of Microarrays (SAM – ver 3.0) [408] and DAVID bioinformatics resources, 2008 [409]. Differentially expressed

Table 7. Primer sequences used for quantitative real-time PCR

Gene Symbol	Gene Name	Forward primer (5'-3')	Reverse primer (5’-3’)
Thbs1	Thrombospondin 1	GGGGAGATAACGGTGTGTTTG	CGGGGATCAGGTTGGCATT
Cd59a	CD59a antigen	GTTAGCCTCACATGCTACCAC	AGGAGAGCAAGTGCTGTTCATA
Adpn	Adiponutrin	TCACCTTCGTGTGCAGTCTC	CCTGGAGCCCGTCTCTGAT
Scd2	Stearoyl-Coenzyme A desaturase 2	GCATTTGGGAGCCTTGTACG	AGCCGTGCCTTGTATGTTCTG
Tlr1	Toll-like receptor 1	TGAGGGTCCTGATAATGTCCTAC	AGAGGTCCAAATGCTTGAGGC
Fbln5	Fibulin 5	GCTTGTCGTGGGGACATGAT	TGGGGTAGTTGGAAGCTGGTA
Fasn	Fatty acid synthase	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
36b4	Acidic ribosomal phosphoprotein P0	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC
Lep	Leptin	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
Sfrp5	Secreted frizzled-related sequence protein 5	CACTGCCACAAGTTCCCCC	TCTGTTCCATGAGGCCATCAG
An	Adiponectin	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT

genes were selected based on false discovery rate (FDR) less than 5% [410] for both SAM and GO enrichment analyses. All data are expressed as mean ± SD.

Results

Animal characteristics and metabolic markers

As expected, mice lost 7.6 ± 3.4g of weight during the two-month period of energy restriction (HFR). Their final body weights (33.6 ± 1.8g) were also significantly lower than the mice on low-fat (LF) diet (39.1 ± 3.4g) (Figure 17A). The net energy intake of the HFR group was 30% lower than the HF group and approximately 15% less than that of the LF group (based on energy density of the diets and estimates of average daily food intakes). Mice on LF and HFR groups had lower fat pad weights (Figure 17B) than the high fat (HF) group (1.3 ± 0.5 and 1.0 ± 0.2 vs. 2.9 ± 0.5g). Plasma leptin levels showed a pattern similar to the fat pad weights (Figure 17C) with the HF group having the highest level (13.1 ± 8.3 ng/ml) followed by the LF (7.0 ± 3.4 ng/ml) and HFR (2.7 ± 1.4 ng/ml) groups. The fad pad and plasma leptin levels were not significantly different between LF and HFR groups. The adiposity index (fat pad weight / “dead” weight) showed a pattern similar to that of the fat pad weight, indicating a lower body fat content in the HFR and LF mice.

The HFR group exhibited better insulin sensitivity than the other two groups as indicated by their lower fasting plasma glucose when compared with the LF group (10.6 ± 1.8 in HFR vs12.1 ± 1.5 mmol/l in LF), lower plasma insulin levels (40.5 ± 21 in HFR vs. 161.3 ± 125.3 in HF and 180.3 ± 169.3 pmol/l in LF) and HOMA-IR when compared

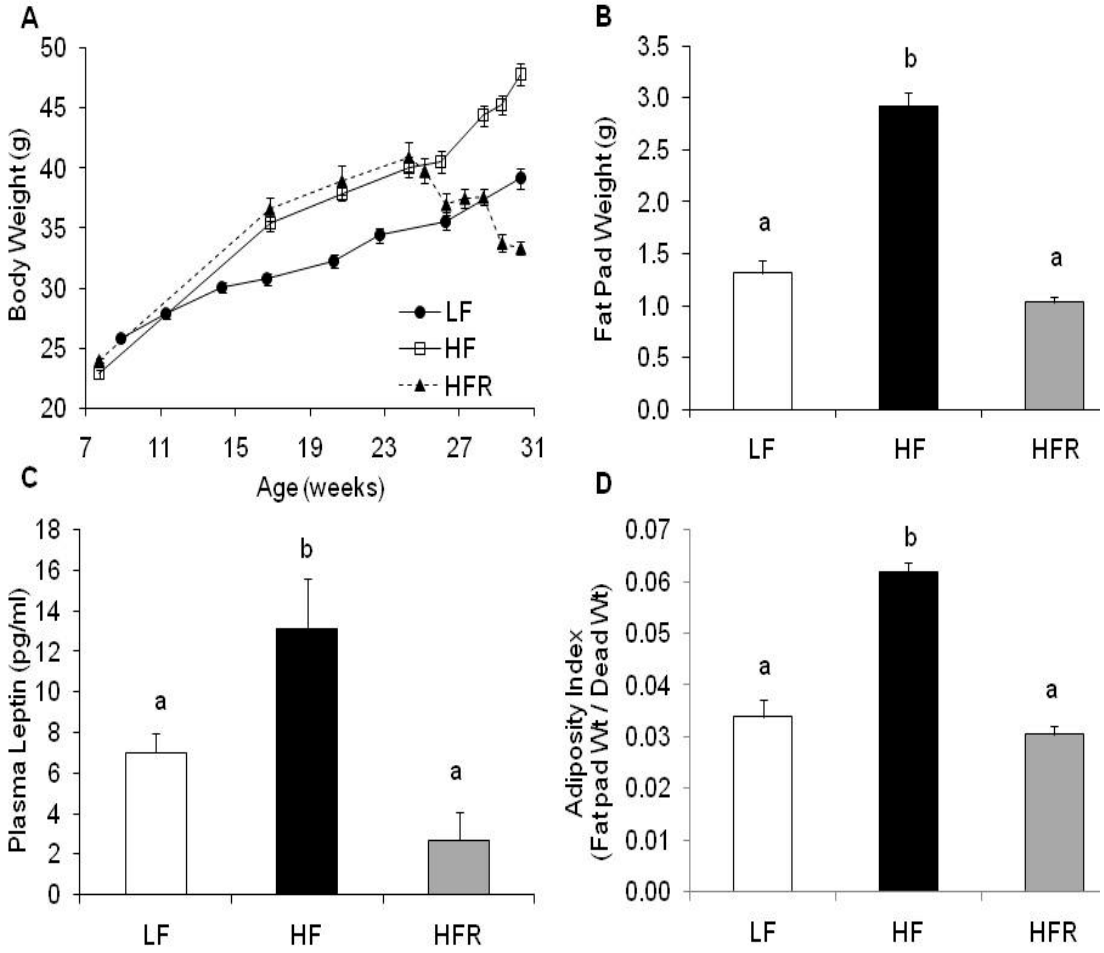

Figure 17. Body and fat pad weights of LF, HF and HFR groups

Weekly body weight (A) and gonadal fat pad weight (B), plasma leptin concentration (C) and adiposity index (gonadal fat pad / body weight ratio D) at sacrifice of C57BL/6J mice fed a high-fat (HF) low-fat (LF) or a high-fat reduced energy (HFR) diet (initially fed HF diet and later restricted to a total energy intake to 70% of initial intake). Different letters indicate a significant difference. (n=14-15 mice in each group)

to the other two groups (Figure 18A, B and C). Plasma non-esterified fatty acid concentration was lowest in the LF group (0.3 ± 0.1 mmol/l) followed by the HFR (0.56 ± 0.15mmol/l) and HF (0.77 ± 0.09mmol/l) groups (Figure 18D and E). Plasma triglycerides, in contrast, were significantly lower in the HFR group (43.7 ± 18.9mg/dl) than both LF (63.7 ± 20.7mg/dl) and HF (62.0 ± 15.3mg/dl) groups. Surprisingly, plasma total adiponectin level was significantly lower in the HFR (2.9 ± 0.4 μg/ml) group when compared to the HF (3.7 ± 0.7 μg/ml) group (Figure 18F). Plasma PAI-1 levels, however, were lower in the LF group (551 ± 89.9 pg/ml) than both HF (1557 ± 338 pg/ml) and HFR (1600 ± 182 pg/ml) groups (Figure 18G).

Gonadal adipose tissue cytokines, gene expression and hepatic steatosis

Since the HFR group had persistent elevation of plasma PAI-1 and reduced adiponectin levels, we next studied gonadal white adipose tissue (GWAT) cytokine levels to examine whether similar changes were seen there. This showed that in contrast to plasma levels, GWAT PAI-1 levels were comparable between LF and HFR groups, which were significantly lower than that of the HF group (Figure 19A). Next we compared levels of other cytokines between the HF and HFR groups to see whether caloric restriction has improved levels of these as well. This showed that while the HFR group had lower MCP-1 and IL-2 levels, the levels of GM-CSF, IFN-γ, IL-1β, IL-6 and IL-10 were comparable between the HF and HFR groups (Figure 19B).

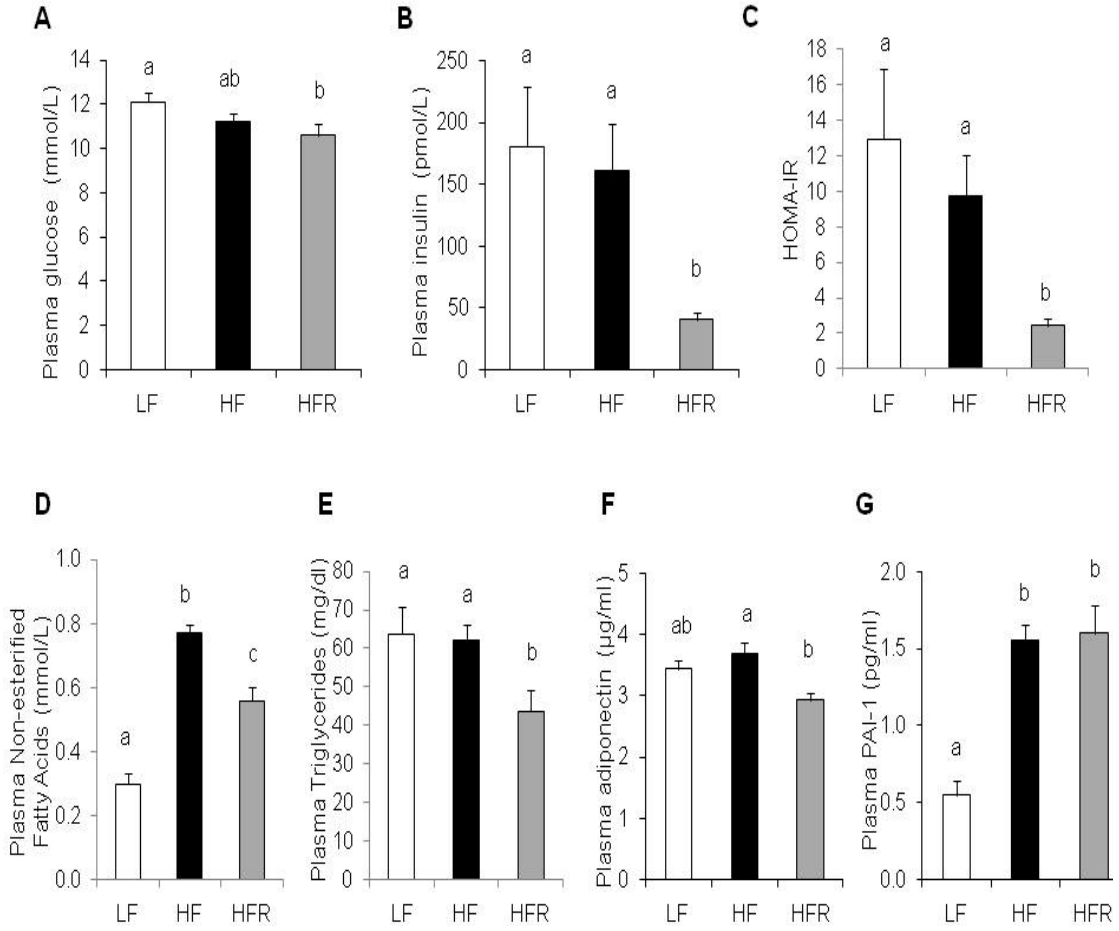

Figure 18. Plasma metabolic markers of LF, HF and HFR groups

Fasting plasma glucose (A), insulin (B), homeostasis model assessment of insulin resistance HOMA-IR (C), non-esterified fatty acids (D), triglycerides (E), adiponectin (F) and plasminogen activator inhibitor-1 (PAI-1) (G) concentrations at sacrifice of C57BL/6J mice fed a high-fat (HF) low-fat (LF) or a high-fat reduced energy (HFR) diet (initially fed HF diet and later restricted to a total energy intake to 70% of initial intake). Different letters indicate a significant difference. (n=14-15 mice in each group).

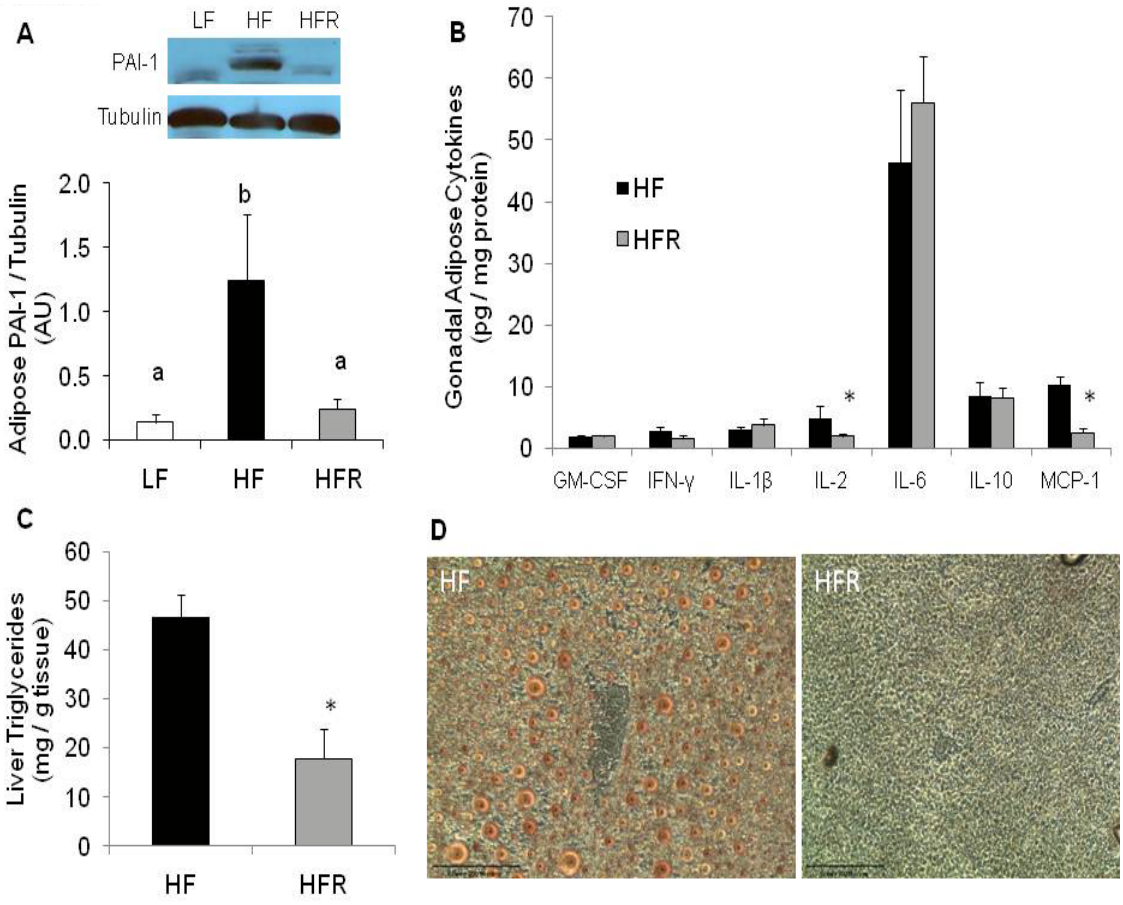

Figure 19. Gonadal adipose tissue inflammatory markers and hepatic lipid content in HF and HFR groups**.** Gonadal adipose tissue plasminogen activator inhibitor-1 (PAI-1) protein expression by western blot in C57BL/6J mice fed a high-fat (HF), low-fat (LF) or a high-fat reduced energy (HFR) diet (A). Gonadal adipose tissue cytokine levels (B), hepatic triglyceride content (C) and oil red O stained sections of liver (D) from HF and HFR groups are shown. Different letters indicate a significant difference. (n=3, 7, 4-5 and 4 from each group for A, B, C and D respectively) * $p<0.05$

Since insulin resistance and lower plasma adiponectin levels are associated with hepatic steatosis, and since the HFR group exhibited better insulin sensitivity despite lower plasma adiponectin levels when compared to the HF group, we examined whether the caloric restriction improved hepatic steatosis. Analysis of liver triglyceride content and oil red o stained sections of the liver (Figure 19C and D) showed that the HFR group had improved hepatic steatosis.

To analyze global gene expression changes in GWAT due to caloric restriction of HF diet, we performed microarray analysis. When the HF and LF groups were compared, 36 genes were differentially expressed (FDR<5%). The LF group expressed lower levels of genes involved in cytoskeleton remodeling (fibulin 5 and lysyl oxidase), Wnt signaling pathway (secreted frizzled-related sequence protein 5 - *Sfrp5*) and lipid transport (phospholipid transfer protein and lipocalin 7) and higher levels of genes involved in lipogenesis (stearoly-coenzyme a desaturase 2 - *Scd2*) when compared to the HF group (Table 8). Additionally, RT-PCR data showed that the LF group expressed lower levels of several genes involved in inflammation (toll-like receptor-1, thrombospondin-1, CD59a antigen) when compared to the HF group (Figure 20). When the HFR and HF groups were compared, 1022 genes were differentially expressed (FDR <5%). The HFR group expressed lower levels of genes involved in cell and focal adhesion and cytokine-cytokine receptor interaction. These included Cd34 antigen, thrombospondin 1, fibronectin 1 and integrin beta 1 (Table 9). The HFR group also expressed lower levels of leptin and genes involved in cytoskeleton organization and biogenesis, Wnt signaling pathway, endoplasmic reticulum (ER) associated degradation pathway and MAPK

Table 8. Genes differentially expressed between low-fat (LF) and high-fat (HF) groups

Accession ID	Gene name	Gene Symbol	Fold Change (log2)	FDR (%)
LF LOWER EXPRESSION VS HF (31 GENES)				
1416164_AT	Fibulin 5	*Fbln5*	-1.31	0.00
1423023_AT	Secreted frizzled-related sequence protein 5	*Sfrp5*	-2.54	0.00
1417963_AT	Lysyl oxidase	*Lox*	-1.19	0.00
1417963_AT	Phospholipid transfer protein	*Pltp*	-1.69	0.00
1417109_AT	Lipocalin 7	*Lcn7*	-1.21	0.00
LF HIGHER EXPRESSION VS. HF (5 GENES)				
1424474_A_AT	Calcium/calmodulin-dependent protein kinase kinase 2, beta	*Camkk2*	1.16	2.93
1426811_AT	Protein phosphatase 2, regulatory subunit b (b56), beta isoform	*Ppp2r5b*	1.20	0.00
1426258_AT	Sortilin-Related Receptor, Ldlr Class A Repeats-Containing	*Sorl1*	1.52	0.00
1415822_AT	Stearoyl-coenzyme a desaturase 2	*Scd2*	1.72	0.00
1452132_AT	Riken cdna 0610030g03 gene	*G03rik*	1.18	0.00

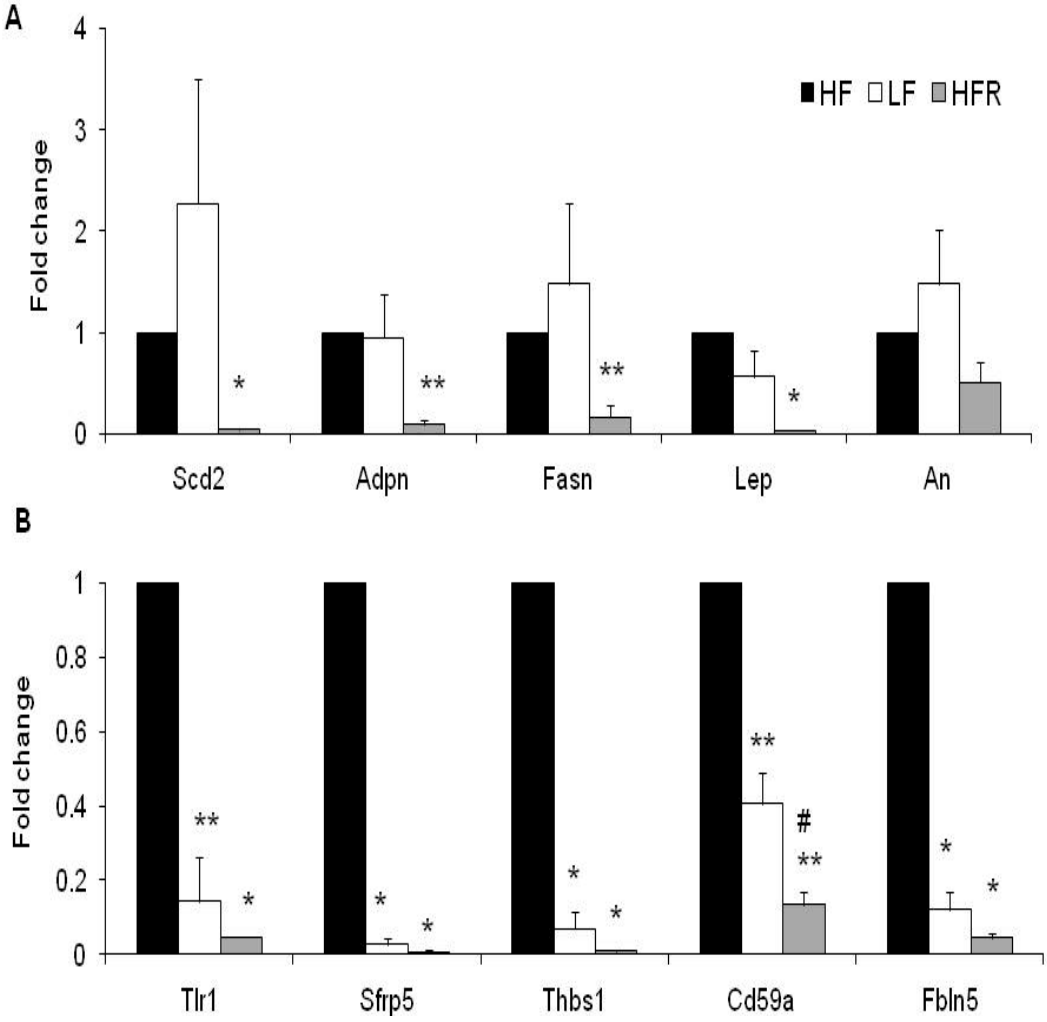

Figure 20. Gonadal adipose tissue gene expression in HF, LF and HFR groups

Gonadal adipose tissue gene expression by reverse transcriptase quantitative PCR in C57BL/6J mice fed a high-fat (HF), low-fat (LF) or a high-fat reduced energy (HFR) diet. * $p<0.001$ vs HF, ** $p<0.05$ vs HF, # $p<0.05$ vs LF (n=3 RNA samples from 3 mice in each group)

Table 9. Genes differentially expressed between high-fat reduced-energy (HFR) and high-fat (HF) groups

Accession ID	Gene name	Gene Symbol	Fold Change (log2)	FDR (%)
HFR LOWER EXPRESSION VS. HF (698 GENES)				
Cell adhesion molecules				
1416072_AT	Cd34 antigen	*Cd34*	-0.84	4.30
1422445_AT	Integrin alpha 6	*ITga6*	-0.77	4.68
1426918_AT	Integrin beta 1	*ITgb1*	-0.79	4.30
Focal adhesion				
1421811_AT	Thrombospondin 1	*Thbs1*	-3.33	1.09
1418711_AT	Platelet-derived growth factor, alpha	*Pdgfa*	-0.62	4.52
1426642_AT	Fibronectin 1	*Fn1*	-2.21	0.00
Cytokine-cytokine receptor interaction				
1451462_A_AT	Interferon (alpha and beta) receptor 2	*Ifnar2*	-0.89	4.52
1422582_AT	Leptin	*Lep*	-2.30	0.00
1417936_AT	Chemokine (c-c motif) ligand 9	*Ccl9*	-2.77	1.09
Cytoskeleton organization and biogenesis				
1456292_A_AT	Vimentin	*Vim*	-0.67	4.30
1423626_AT	Dystonin	*Dst*	-0.88	4.30
1444089_AT	Spectrin beta 2	*Spnb2*	-1.41	4.30
Wnt signaling pathway				
1448201_AT	Secreted frizzled-related sequence protein 2	*Sfrp2*	-1.51	4.30
1436075_AT	secreted frizzled-related sequence protein 5	*Sfrp5*	-2.39	0
1436791_AT	Wingless-related mmtv integration site 5a	*Wnt5a*	-1.02	4.30
ER associated degradation pathway				
1422845_AT	Calnexin	*Canx*	-0.77	4.30
1423149_AT	S-phase kinase-associated protein 1a	*Skp1a*	-0.52	4.75
1451218_AT	ER degradation enhancer, mannosidase alpha-like 1	*Edem1*	-0.73	4.30
MAPK signaling pathway				
1426648_AT	Map kinase-activated protein kinase 2	*Mapkapk2*	-0.39	4.52
1460420_A_AT	Epidermal growth factor receptor	*Edfr*	-0.44	4.75
1435646_AT	inhibitor of kappab kinase gamma	*Ikbkg*	-0.51	4.75

Table 9. Contd.

Accession ID	Gene name	Gene Symbol	Fold Change (log2)	FDR (%)
HFR HIGHER EXPRESSION VS. HF (324 GENES)				
mTOR signaling pathway				
1455981_AT	Ribosomal protein s6	*Rps6*	1.01	4.11
1426380_AT	Eukaryotic translation initiation factor 4b	*Eif4b*	0.92	4.11
Protein synthesis				
1424736_AT	Eukaryotic translation elongation factor 2	*Eef2*	0.73	3.52
1439439_X_AT	Eukaryotic translation elongation factor 1 delta	*Eef1d*	0.63	4.75
1450934_AT	Eukaryotic translation initiation factor 4a2	*Eif4a2*	0.32	3.52

Table 10. Genes differentially expressed between low-fat (LF) and high-fat reduced energy (HFR) diets

Accesion ID	Gene name	Gene Symbol	Fold Change (log2)	FDR (%)
LF HIGHER EXPRESSION VS. HFR (249 GENES)				
Lipid biosynthesis				
1415822_AT	Stearoyl-coenzyme a desaturase 2	*Scd2*	2.97	0.00
1422678_AT	Diacylglycerol o-acyltransferase 2	*Dgat2*	1.08	2.16
1451457_AT	Sterol-c5-desaturase (fungal erg3, delta-5-desaturase) homolog (s. Cerevisae)	*Sc5d*	1.19	2.16
1450646_AT	Cytochrome p450, 51	*Cyp51*	2.58	1.17
1423418_AT	Farnesyl diphosphate synthetase	*Fdps*	1.91	1.99
1420655_AT	Adiponutrin	*Adpn*	1.92	0.00
1433443_A_AT	3-hydroxy-3-methylglutaryl-coenzyme a synthase 1	*Hmgcs1*	1.28	2.16
Glucose metabolism				
1437672_AT	Insulin receptor substrate 3	*Irs3*	2.03	3.38
1416069_AT	Phosphofructokinase, platelet	*Pfkp*	1.03	4.21
1452915_AT	Protein kinase, camp dependent regulatory, type ii alpha	*Prkar2a*	1.57	0.65
LF LOWER EXPRESSION VS. HFR (563 GENES)				
Protein synthesis				
1416141_A_AT	Ribosomal protein s6	*Rps6*	-0.86	4.49
1426378_AT	Eukaryotic translation initiation factor 4b	*Eif4b*	-0.97	4.49
1416546_A_AT	Ribosomal protein l6	*Rpl6*	-0.59	4.96
Immune system process				
1460218_AT	Cd52 antigen	*Cd52*	-2.09	4.49
1456174_X_AT	N-myc downstream regulated gene 1	*Ndrg1*	-1.45	0.00
1427511_AT	Beta-2 microglobulin	*B2m*	-2.72	1.31
1422962_A_AT	Proteosome (prosome, macropain) subunit, beta type 8 (large multifunctional protease 7)	*Psmb8*	-1.42	0.00
1423135_AT	Thymus cell antigen 1, theta	*Thy1*	-1.53	2.04
1449049_AT	Toll-like receptor 1	*Tlr1*	-2.97	2.52
1421358_AT	Histocompatibility 2, m region locus 3	*H2-m3*	-1.26	3.70
1419212_AT	Icos ligand	*Cosl*	-1.77	2.04
1450696_AT	Proteosome (prosome, macropain) subunit, beta type 9 (large multifunctional protease 2)	*Psmb9*	-1.54	2.52

pathway. Conversely, The HFR group expressed higher levels of genes involved in the mTOR pathway as well as overall protein synthesis, including ribosomal protein S6. In the final comparison between LF and HFR groups, 812 genes were differentially expressed (FDR < 5%). The LF group expressed higher levels of genes involved in lipid biosynthesis when compared to the HFR group, including *Scd2*, HMG-CoA synthase 1 and adiponutrin (Table 11). The LF group also expressed higher levels of insulin receptor substrate-3 (*Irs-3*) and phosphofructokinase. In contrast, the LF group expressed lower levels of genes mediating immune response process and protein synthesis when compared to the HFR group. These included toll like receptor-1 and thymus cell antigen 1 theta. Both the LF and HFR groups expressed lower levels of genes involved in cell adhesion when compared to the HF group. Selected microarray results were validated by reverse transcriptase quantitative PCR (Figure 20). These data demonstrate an excellent correlation between changes in gene expression assessed by microarray and the real time PCR validation.

Discussion

In this study we tested the hypothesis that weight loss due to an energy restricted high-fat diet (analogous to a reduction in portion sizes) is accompanied by improvements in adipose inflammatory profiles and markers of the metabolic syndrome. While several previous studies have reported metabolic and adipose tissue gene expression changes due to energy restriction [271, 273, 411], to our knowledge this is the first study to

comprehensively investigate the long-term effects of a *reduced energy high-fat diet* on metabolic markers, adipokine secretion and adipose tissue gene expression in mice.

Although the HF diet increased adiposity compared to the LF diet in our study, there were no significant differences in fasting plasma glucose and insulin, measured after 8h of food deprivation. However, data from our lab (unpublished) and others [412] indicate that this 45% fat diet, as compared to the same LF diet causes insulin insensitivity as evidenced by higher AUC during a GTT test, and higher gonadal adipose tissue inflammation. The impaired glucose homeostasis is milder than that caused by commonly used 60% HF diets, which causes extreme changes in adipose inflammation and remodeling [381]. Thus, we believe that 45% HF diets are more appropriate when modeling human nutrition and obesity. Second, a prolonged starvation period of 8 hours prior to the measurements likely contributed to the comparable levels of insulinemia and glycemia in the HF and LF groups. Finally, it is also possible that the higher sucrose content of the LF diet (35% of energy as sucrose in the LF diet vs. 17% energy from sucrose in the HF diet) led to smaller differences in glycemia and insulinemia between groups [413]. Most importantly, our study clearly demonstrated that the HFR group exhibited lower fasting plasma glucose, insulin and HOMA-IR indicating better insulin sensitivity than the other two groups.

The HFR group also exhibited improvements in hepatic steatosis. These changes are more likely related to weight loss or the direct effect of energy restriction rather than due the fat content of the diet, consistent with findings from a recent large clinical trial involving post-menopausal women, which showed that weight loss, rather than LF intake

was associated with a reduced incidence of type 2 diabetes [312]. Thus, these findings further support that weight loss, rather than macronutrient composition of the diet is important in improving insulin sensitivity and hepatic steatosis.

It is worth noting that the HFR group exhibited evidence of better insulin sensitivity than HF despite lower plasma adiponectin levels. Previous studies on humans and rodents have yielded inconsistent results regarding the impact of weight loss on plasma adiponectin [253, 414-417]. Although our RT-PCR data shows a trend for lower adiponectin mRNA expression in the adipose tissue of the HFR group, neither these PCR data nor the microarray analyses reached statistical significance. Thus it is likely that the decrease in plasma adiponectin is due to reduced fat mass rather than due to its reduced production in the adipose tissue, as adiponectin is also expressed in fat cell differentiation-dependent manner [418]. It is also possible that the lowering of plasma adiponectin level is due to the high fat content in the diet; this is a significant finding since plasma adiponectin levels are negatively correlated with cardiovascular risk.

When other adipokines are considered, consistent with previous studies, plasma leptin level in the HFR mice reduced to about 20% to that of the HF mice [378]. A surprising finding was that the plasma PAI-1 concentration was similar between the HFR and HF groups, which were two-folds higher than the levels measured in the LF mice. Since previous studies showing improvements in plasma PAI-1 levels following energy restriction have used low-fat diets [262], this could indicate a role of fat content of the diet in modulating plasma PAI-1 levels. Despite high circulating PAI-1 levels in HFR mice, PAI-1 protein content in GWAT was reduced (Figure 19A). Thus the finding that

plasma PAI-1 levels remained elevated in the HFR mice suggests that other sources of PAI-1 such as vascular endothelium and platelets may be important in determining the final plasma levels [419]. However, no studies have investigated the secretory patterns of PAI-1 from these tissues in response to dietary fat content. Our data suggests that fat content of the diet could be a factor which influences PAI-1 secretion from non-adipose sources in normal weight individuals. .

Similar to improvements in GWAT PAI-1 levels, the HFR group also exhibited lower levels of MCP-1 and IL-2 when compared to the HF group. However, levels of several other pro-inflammatory (GM-CSF, IFN-γ, IL-1β and IL-6) and anti-inflammatory (IL-10) cytokines were similar between the HF and HFR groups. Further, GWAT gene expression by microarray analysis showed that the HFR group expressed lower levels of genes involved in cell and focal adhesion, cytokine-cytokine receptor interaction, ER associated degradation pathway, cytoskeleton organization and biogenesis, Wnt signaling pathway and MAPK pathway, when compared to the HF group. It is now established that the insulin resistance occurring in high-fat diet- induced obesity is accompanied with a chronic low-grade inflammation of the adipose tissue. This inflammatory process is characterized by CD11c+ macrophage infiltration and phenotypic switch, while recent evidence shows that adaptive immune responses of the T helper 1 (Th1) type could also be involved [93]. Lower levels of MCP-1, PAI-1, IL-2 and lower expression of genes linked to inflammation in the HFR group could indicate a partial improvement in adipose tissue inflammation by energy restriction.

The exact trigger of the adipose tissue inflammatory process in diet-induced obesity is hitherto unknown. Some have suggested that adipose tissue ER stress is a triggering event for subsequent inflammation and development of insulin resistance in obesity [64], which is reversed by weight loss [420]. The onset of ER stress is indicated by an increase in chaperon proteins followed by the unfolded protein response. In our study, the expression of calnexin, a gene encoding a chaperone protein, as well as *Edem1*, a mediator of ER stress were lower in the HFR group when compared to the HF group. This could indicate a possible reversal of ER stress in the adipose tissue following energy restriction.

Rodent studies have indicated that increasing adipose tissue mass without a similar magnitude increase in supporting vasculature could lead to tissue hypoxia, triggering the expression of inflammatory genes [62]. Similar findings have been recently reported in humans where it was shown that subcutaneous adipose tissue oxygen partial pressure negatively correlated with adiposity [421]. Thrombospondin-1 (*Tsp1*), a known inhibitor of angiogenesis, was expressed 10 times higher in HF compared to the HFR group. *Tsp1* has been previously shown to be expressed in the WAT of both humans and rodents [50, 422], correlating with both BMI and insulin resistance in humans [50]. Since thrombospondin-1 protein (TSP1) was shown to be secreted by adipocytes *in vitro*, it has been categorized as an adipokine. It is possible that TSP1 could be implicated in the suppression of angiogenesis leading to tissue hypoxia as described above. Recently, it was shown that TSP1 impairs nitric oxide signaling, possibly implicating it in

vasoconstriction [423]. Thus, TSP1 could be an important mediator of cardiovascular complications in obesity and merits further investigation.

Another interesting finding in this study was that expression of several genes involved in the Wnt signaling pathway were reduced in the HFR group when compared to the HF group. Inhibition of the Wnt signaling pathway is associated with increased adipogenesis resulting in an adipocyte phenotype [424]. In this study, the HFR group had lower expression of *Sfrp2* and *Sfrp5*, two inhibitors of canonical Wnt signaling, possibly indicating an adipogenic scenario in the HF group. Recently it was reported that secreted frizzled-related protein 5 (Sfrp5) deficient mice on a high-fat diet develop severe glucose intolerance [425]. It is possible that Sfrp5 is a factor necessary for normal adipose tissue expansion, and lower expression of this gene in the HFR group could be a response to the rapid reduction in adiposity due to caloric restriction. Along with the finding that the HFR group had lower expression of genes involved in cytoskeletal organization and biogenesis could indicate a remodeling process of the adipose tissue in the HF diet fed mice. This is in agreement with Strissel et al., who showed that in C57BL/6J mice fed a high fat diet, there is adipocyte death and remodeling occurring after 12 weeks [381].

It is interesting that despite the large difference in fat pad weights, there were relatively few genes differentially expressed between the HF and LF groups. These included a lower expression of inflammatory genes and a higher expression of lipogenic genes in the LF group when compared to the HF group. The high sucrose content in the LF diet likely contributed to the latter. In contrast, although there was no difference in the fat pad weight between the HFR and LF groups, a large number of genes were

differentially expressed between these two groups. Interestingly, several genes involved in immune function had higher expression in the HFR group when compared to the LF group. This shows that energy restriction only partially reverses the adipose inflammation induced by high-fat feeding.

We identified a large number of genes expressed at higher levels in the HFR group when compared to both HF and LF groups. A majority of these genes were involved in protein synthesis and the mTOR pathway. Several previous researchers have reported changes in adipose tissue gene expression in response to energy restriction. Higami et al. reported that there is decreased expression of genes linked to inflammation, cytoskeleton, extracellular matrix or cytoskeleton [273] and increased expression of genes associated with mitochondrial energy metabolism and macronutrient metabolism in the adipose tissue of mice subjected to caloric restriction [271]. Similar findings have been reported for humans recently [277]. Linford et al. reported that energy restriction prevented the age related downregulation of genes involved in mTOR pathway in the adipose tissue of rats [411]. While data from gene expression of other tissues including liver suggests that there is a downregulation of genes involved in synthetic activity with energy restriction [426], our data and the above suggest that the response for energy restriction could be tissue-specific.

In summary the current study provides important information regarding metabolic and genomic differences in LF/high sucrose vs. HF/Low sucrose fed ad libitum or with energy restriction; there are however a few limitations to our study. Since the weights of the groups were not exactly matched, the changes in gene expression could be related in

part to weight, and in part to the macronutrient composition of the diet. Further, it is well established now that a remodeling process occurs in the adipose tissue of DIO mice after 12 weeks of 65% high fat feeding [381], and our studies lasted 24 weeks of 45% HF feeding. Other limitations include limited cytokine and other analyses in adipose tissue from LF-fed mice due to limited tissue. Finally, we used 3 samples from each group for the microarray studies and although our stringent statistical analysis provides confidence in our differential gene expression pattern, a higher number of replicates would have been more desirable.

This study is consistent with previous findings that intake of a high-fat diet results in obesity and is associated with upregulation of genes involved in inflammation and thrombosis in the white adipose tissue. Our studies further demonstrate that energy restriction: 1. despite the high fat content of the diet, reverses diet-induced obesity with concomitant improvements in insulin sensitivity and hepatic steatosis; and 2. only partially reverses the inflammation of the WAT, without improvement in some metabolic markers involved in increasing cardiovascular risk. Our study supports the recommended low fat intake for cardiovascular health and further emphasizes the importance of considering sucrose content in the diet in the management of diabetes.

CHAPTER V
MECHANISMS OF (n-3) POLYUNSATURATED FATTY ACID-MEDIATED PREVENTION AND REVERSAL OF INSULIN RESISTANCE IN HIGH-FAT DIET-INDUCED OBESITY

Disclosure: The work described in this chapter in its entirety has been published in the following reference with minor modifications in numbering of tables and figures: "Kalupahana NS, Claycombe K, Newman SJ, Stewart T, Siriwardhana N, Matthan N, Lichtenstein A and Moustaid-Moussa N. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *Journal of Nutrition*, 2010 Nov; 140(11):1915-22"

Introduction

Obesity is a major health problem in the United States with two-thirds of the adult population being either overweight or obese [427]. It is causally linked to the metabolic syndrome, which is characterized by insulin resistance, atherogenic dyslipidemia and hypertension and also associated with a pro-inflammatory and a pro-thrombotic state. Individuals with the metabolic syndrome have a higher risk of developing type 2 diabetes and cardiovascular diseases [397]. In recent years, evidence has linked these metabolic disorders, in part, to adipose tissue endocrine function. Indeed, adipose tissue secretes numerous bioactive peptides collectively known as adipokines, which play important endocrine roles in the regulation of glucose homeostasis, blood pressure and appetite [23]. Increased adiposity leads to a chronic low-grade inflammation in the adipose tissue

resulting in increased production of pro-inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), tumor necrosis factor-α, plasminogen activator inhibitor-1 (PAI-1) [428] and angiotensin II [32] and decreased production of anti-inflammatory adipokines such as adiponectin [428]. These dysregulations of adipokine secretory patterns are identified as a link between obesity and the metabolic syndrome.

While caloric restriction leading to weight loss is the main dietary intervention to treat metabolic syndrome, other dietary interventions such as ones targeted at reducing adipose tissue inflammation independent of weight-loss are yet to be explored in detail. Long-chain (n-3) polyunsaturated fatty acids of marine origin, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to have anti-inflammatory properties [317]. *In vitro,* these (n-3) fatty acids prevent the arachidonic acid (AA)-induced increase in pro-inflammatory eicosanoids in fat cells [382] as well as inhibit the nuclear factor-κB (NF-κB) pathway activation in immune and cancer cells [429]. Since dietary intake of these fatty acids results in their enrichment in adipose tissue [430], we hypothesized that these fatty acids would prevent (P) and reverse (R) the metabolic derangements occurring in diet-induced obesity (DIO) via modulation of adipose tissue inflammation. Several previous researchers have shown that EPA and DHA prevent the development of insulin resistance in high-fat diet fed mice in an adiponectin dependent manner [323, 324, 347, 357, 431-434]. The aim of this study was to determine whether EPA can prevent and reverse the metabolic complications resulting from high saturated-fat (HF) feeding and to determine underlying mechanisms. We

hypothesized that EPA protects against HF diet-induced adiposity, insulin resistance and hepatic steatosis and that these effects are mediated in part by their anti-inflammatory effects on adipose tissue, as indicated by decreased secretion of pro-inflammatory adipokines and increased secretion of adiponectin.

Materials and Methods

Mice and diets: Male C57BL/6J mice aged 5-6 wk were purchased from the Jackson Laboratory (Bar Harbor, ME). Following a 1 wk period of acclimation, they were fed a low fat (LF) diet (10, 20 and 70% of energy from fat, proteins and carbohydrates respectively), HF diet (45, 20 and 35% of energy from fat, proteins and carbohydrates respectively) or a high saturated-fat EPA (HF-EPA-P) diet (36 g/kg EPA ethyl ester) for 11 wk. A fourth group was initially fed the HF diet for 6 wk followed by the high saturated-fat EPA diet (HF-EPA-R) for 5 wk. All four groups had 9-10 mice each. Diets were custom made by Research Diets (New Brunswick, NJ) and the detailed diet compositions are given in Table 11. Mice were housed in individual cages with free access to food and water, and subjected to 12 h light/ dark cycles. Their food intake was measured daily as the difference between the amount of food provided and the amount of food removed after accounting for any spillage. Food was stored at -20 ^{0}C and the mice were given fresh food daily. Mice were maintained on diet groups for 11 wk and weighed weekly.

Table 11. Composition of the diets for the EPA study

	LF	HF- EPA	HF
Fat , *% energy*	10	45	45
Carbohydrate, *% energy*	70	35	35
Protein, *% energy*	20	20	20
Digestible Energy, *kJ/g*	16.1	19.8	19.8
		g/kg	
Lard	19	171	207
Soybean Oil	24	29	29
EPA, Ethyl Ester	0	36	0
Mineral Mix, S10026[1]	9	12	12
DiCalcium Phosphate	12	15	15
Calcium Carbonate	5	6	6
Potassium Citrate, 1 H2O	16	19	19
Vitamin Mix, V10001[1]	9	12	12
Choline Bitartrate	2	2	2
dl-α-toco□heryl acetate	0.12	0.15	0.15
		% total fatty acids	
Saturated fats	25.1	30.0	36.3
14:0	0.5	0.7	0.8
16:0	16.5	18.7	22.6
□ 18:0	8.2	10.6	12.9
Monounsaturated□□ats	34.7	37.6	45.3
14:1	0.2	0.4	0.5
1□:1(n-9)	1.8	2.9	3.5
18:1(n-9)	32.7	34.3	41.3
Polyunsaturated fats	40.2	32.4	18.5
18:2(n-6)	34.6	13.5	14.9
18:3(n-3)	5.0	1.8	1.9
20:4(n-6)	0.7	1.3	1.6
20:5(n-3)	0.0	16.1	0.0

[1]Research Diets, Inc. (New Brunswick, NJ, USA); composition of mineral and vitamin mix as described previously by DeFuria et al. [47]

At the end of 11 wk, mice were feed-deprived for 4 h and killed using the CO_2 inhalation method. Blood was collected into tubes with EDTA, kept on ice for 10 min, centrifuged at 3,000 Xg for 20 min, and plasma samples were collected and stored at -80 ^{o}C for subsequent analyses. Epididymal (gonadal), inguinal, retroperitoneal and subscapular fat pads were dissected out, snap frozen in liquid N_2 and stored at -80^{0}C for subsequent analyses. These protocols were all approved by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville.

Glucose tolerance test: This was performed at two time points (6 wk and 10 wk into the dietary intervention) in the same set of mice. Following a 12 h period of feed-deprivation, 5 μL of tail blood was used to measure the blood glucose levels using the one touch ultra® glucometer. Next, 2 g/kg body weight of 20% D-glucose was injected intraperitoneally. Serial blood glucose measures were taken at 15, 30, 60 and 120 min after the injection.

Plasma insulin and adipokine measurements: Total protein was isolated from gonadal adipose tissue by homogenizing with modified radio-immunoprecipitation assay (RIPA) buffer containing a cocktail of protease inhibitors. Protein concentration was determined by the Bradford assay [404]. Commercially available ELISA kits were used to measure plasma insulin (Crystal Chem Inc., Downers Grove, IL) and total adiponectin (Linco Research, Billerica, MA) concentrations. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as glucose concentration (mmol/L) × insulin

concentration (mU/L) / 22.5 [19]. PAI-1, MCP-1 and IL-6 levels were measured using a commercially available microsphere based multiplexing system (Luminex xMAP). Analyte values in the adipose protein extracts were normalized to total protein concentration. All assays were performed according to the manufacturers' protocols.

RBC fatty acid profile: RBC samples from 2-3 mice per group were pooled prior to analysis. Lipids were extracted from RBC membranes [435] followed by saponification and methylation [436]. The resultant fatty acid methyl esters were quantified using an established gas chromatography method as previously described [437]. Peaks of interest were identified by comparison with authentic fatty acid standards (Nu-Chek Prep, Inc. Elysian, MN) and expressed as molar percentage (mol %) proportions of total fatty acids.

Liver triglycerides (TG): Liver TG was measured as described previously [23]. Briefly, the livers were homogenized in saline and solubilized in 1% deoxycholate. TG concentration was assayed using a colorimetric method (L-Type TG M kit, Wako chemicals USA Inc.) and normalized to liver weight.

Cell culture experiments: Adipocytes were cultured as described previously [382]. Briefly, 3T3-L1 preadipocytes were cultured in 100 mm dishes in regular growth media consisting of DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (PS). At confluence, the cells were differentiated by the addition of growth media supplemented with 250 nmol/L dexamethasone, 0.5 mmol/L Methyl

Isobutyl Xanthine and 10 nmol/L insulin for 48 h after which regular media was added. After 6-7 d, the media was replaced with serum-free media (DMEM, PS, and 1% fatty acid-free bovine serum albumin - BSA). Treatment media (above plus 10 nmol/L insulin, dimethyl sulfoxide and AA or EPA) was added and kept for 48 h. Final fatty acid concentration in the incubation media was 150 μmol/L. Both fatty acids were conjugated to BSA prior to treatment (fatty acids to BSA molar ratio: 3:1). All fatty acids were purchased from Nu-Check Prep Inc. (Elysian, MN).

Proteomics: Protein extracts from AA or EPA treated cells were labeled with cyanine (Cy) 3 or Cy 5 (green and red), respectively. Both samples were run on a 2 dimensional difference gel electrophoresis (2D-DIGE; Applied Biomics, Hayward, CA). A complete analysis of all differentially expressed proteins was obtained using Decyder software from which quantitative data was derived. Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified, obtained using the spot picker robot, and proteins within each spot were enzymatically digested and analyzed by mass spectrometry. Proteins identified from this analysis were then uploaded into DAVID Bioinformatics Resources [24], where the functional annotation chart was used to search for significantly enriched gene ontology (GO) categories.

Liver Histology: Sections from the liver were fixed in 10% buffered formalin, routinely processed, sectioned at 5 μm and stained with hematoxylin and eosin. Liver sections were examined by a pathologist who was blinded to the various treatment groups. The pattern

and degree of macrovesicular steatosis was noted and the latter was graded as none, mild, moderate or severe (0-3) [438]. The presence of microvesicular hepatosis was also noted.

Statistical Analysis: Body weight and other variables were compared for between group differences using one-way ANOVA. If the F test was significant, group means were compared using post-hoc tests. If the Levene statistic was not significant, the Tukey's post-hoc test for multiple comparisons was employed, otherwise the Games-Howell post-hoc test was used. For the glucose tolerance tests, area under the glucose curves was calculated and compared between groups. Inter-group differences for the presence of heapatic steatosis were compared using Pearson's chi-square test. The fold difference of protein expression in replicate gels in proteomic studies was compared using the one-sample t test. All analyses were performed using SPSS (ver16.0 SPSS Inc., Chicago, IL) software. The level of significance for all tests was $p<0.05$, while for the GO enrichment analysis a cut-off of false discovery rate of less than 5% was used. All data are expressed as mean ± SEM.

Results

Mouse characteristics and metabolic markers

Fatty acid analysis of red blood cells showed enrichment with dietary EPA (Table 12). As expected, the HF group gained significantly more weight ($p<0.001$) and had a significantly higher mean body weight ($p<0.001$) at the end of the 11 week study period when compared to the LF group (Figure 21, Table 13). While the HF-EPA-P group had a higher mean body weight at the end of the study compared to the LF group, this was

Table 12. Fatty acid composition of RBC in C57BL/6J mice in the LF, HF, HF-EPA-P, or HF-EPA-R groups [1]

	LF	HF-EPA-P	HF	HF-EPA-R	p value
Saturated fatty acids	46.5 ± 0.2^{b}	49.4 ± 0.1^{a}	47.1 ± 0.2^{b}	49.2 ± 0.2^{a}	<0.001
10:0	0.04 ± 0.00	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	NS
12:0	0.45 ± 0.02	0.42 ± 0.01	0.47 ± 0.04	0.39 ± 0.03	NS
14:0	0.46 ± 0.00	0.44 ± 0.01	0.41 ± 0.03	0.42 ± 0.00	NS
16:0	31.7 ± 0.14^{a}	31.3 ± 0.12^{a}	29.5 ± 0.10^{b}	31.5 ± 0.15^{a}	<0.001
18:0	12.6 ± 0.21^{b}	16.0 ± 0.13^{a}	15.5 ± 0.06^{a}	15.5 ± 0.21^{a}	<0.001
20:0	0.44 ± 0.01	0.49 ± 0.04	0.38 ± 0.02	0.43 ± 0.07	NS
22:0	0.31 ± 0.01	0.30 ± 0.02	0.32 ± 0.02	0.33 ± 0.04	NS
24:0	0.44 ± 0.02	0.47 ± 0.02	0.40 ± 0.04	0.50 ± 0.04	NS
Monounsaturated fatty acids	18.8 ± 0.1^{a}	15.0 ± 0.1^{b}	15.2 ± 0.5^{b}	15.0 ± 0.1^{b}	<0.001
14:1	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	NS
16:1(n-9)	0.40 ± 0.02^{a}	0.29 ± 0.00^{b}	0.32 ± 0.03^{ab}	0.27 ± 0.02^{b}	0.008
16:1(n-7)	1.3 ± 0.01^{a}	0.5 ± 0.02^{b}	0.6 ± 0.05^{b}	0.6 ± 0.02^{b}	<0.001
18:1(n-9)	11.8 ± 0.08	11.1 ± 0.05	11.1 ± 0.36	11.5 ± 0.11	NS
18:1(n-7)	3.3 ± 0.08^{a}	1.7 ± 0.01^{c}	2.0 ± 0.01^{b}	1.7 ± 0.06^{c}	<0.001
20:1(n-9)	1.2 ± 0.11^{a}	0.83 ± 0.08^{ab}	0.68 ± 0.08^{bc}	0.44 ± 0.07^{c}	0.002
22:1(n-9)	0.13 ± 0.01^{a}	0.09 ± 0.02^{ab}	0.08 ± 0.01^{ab}	0.06 ± 0.01^{b}	0.020
24:1(n-9)	0.53 ± 0.03	0.40 ± 0.00	0.39 ± 0.06	0.43 ± 0.03	NS
PUFA	34.7 ± 0.1^{c}	35.6 ± 0.2^{bc}	37.7 ± 0.3^{a}	35.8 ± 0.1^{ab}	<0.001
(n-3) fatty acids	6.8 ± 0.1^{b}	17.7 ± 0.2^{a}	6.8 ± 0.1^{b}	17.3 ± 0.3^{a}	<0.001
18:3(n-3)	0.13 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.12 ± 0.01	NS
20:5(n-3)	0.27 ± 0.01^{b}	8.2 ± 0.06^{a}	0.26 ± 0.05^{b}	7.9 ± 0.22^{a}	<0.001
22:5(n-3)	0.79 ± 0.01^{b}	5.2 ± 0.04^{a}	0.90 ± 0.05^{b}	4.9 ± 0.12^{a}	<0.001
22:6(n-3)	5.6 ± 0.04^{a}	4.1 ± 0.17^{b}	5.5 ± 0.09^{a}	4.3 ± 0.18^{b}	<0.001
(n-6) fatty acids	27.7 ± 0.1^{b}	17.8 ± 0.1^{c}	30.8 ± 0.4^{a}	18.3 ± 0.2^{c}	<0.001
18:2(n-6)	6.3 ± 0.18^{b}	8.6 ± 0.11^{a}	9.1 ± 0.42^{a}	8.3 ± 0.07^{a}	<0.001
18:3(n-6)	0.17 ± 0.03	0.14 ± 0.02	0.17 ± 0.01	0.14 ± 0.01	NS
20:2(n-6)	0.41 ± 0.02^{b}	0.36 ± 0.00^{b}	0.65 ± 0.02^{a}	0.36 ± 0.01^{b}	<0.001
20:3(n-6)	1.5 ± 0.02^{a}	0.60 ± 0.01^{d}	1.36 ± 0.02^{b}	0.71 ± 0.03^{c}	<0.001
20:4(n-6)	16.8 ± 0.11^{a}	7.8 ± 0.01^{b}	17.0 ± 0.65^{a}	8.4 ± 0.13^{b}	<0.001
22:2(n-6)	0.19 ± 0.05	0.06 ± 0.01	0.11 ± 0.04	0.08 ± 0.03	NS
22:4(n-6)	1.7 ± 0.02^{b}	0.34 ± 0.01^{c}	1.9 ± 0.08^{a}	0.42 ± 0.01^{c}	<0.001
22:5(n-6)	0.89 ± 0.01^{a}	0.05 ± 0.00^{b}	0.71 ± 0.06^{a}	0.11 ± 0.01^{b}	<0.001

[1]Results are mean ± SEM, n=3. Means in a row with superscripts without a common letter differ, $P < 0.05$. NS- $p>0.10$

Table 13. Mouse characteristics and metabolic markers in C57BL/6J mice in the LF, HF, HF-EPA-P, or HF-EPA-R groups [1]

Variable	LF	HF-EPA-P	HF	HF-EPA-R	p value
Dead weight, *g*	31.7 ± 1.0^{c}	35.9 ± 0.9^{b}	40.4 ± 1.2^{a}	40.6 ± 1.1^{a}	< 0.001
Total fat pad weight, *g*	3.3 ± 0.2^{c}	4.6 ± 0.3^{b}	6.0 ± 0.3^{a}	6.4 ± 0.3^{a}	<0.001
Gonadal fat pad weight, *g*	1.3 ± 0.1^{c}	1.9 ± 0.1^{b}	2.2 ± 0.1^{ab}	2.4 ± 0.1^{a}	<0.001
Inguinal fat pad weight, *g*	0.8 ± 0.1^{b}	1.1 ± 0.1^{b}	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	<0.001
Adiposity index, *%*	10.3 ± 0.5^{c}	12.8 ± 0.5^{b}	14.8 ± 0.4^{a}	15.6 ± 0.5^{a}	<0.001
Cumulative energy intake, *kJ/ 11 wk*	3784 ± 57^{b}	4765 ± 68^{a}	4807 ± 89^{a}	4834 ± 120^{a}	< 0.001
Glucose AUC at 6 wk, *min.mmol/L*	1772 ± 118^{b}	1947 ± 142^{b}	2376 ± 86^{a}	-	0.001
Glucose AUC at 11 wk, *min.mmol/L*	2099 ± 120^{b}	2253 ± 83^{b}	3289 ± 186^{a}	2943 ± 132^{a}	< 0.001
Blood glucose, *mmol/L*	10.2 ± 0.5^{b}	9.7 ± 0.4^{b}	12.2 ± 0.5^{a}	10.1 ± 0.4^{b}	0.002
Plasma insulin, *pmol/L*	69 ± 15^{b}	39 ± 13^{b}	330 ± 51^{a}	149 ± 27^{b}	< 0.001
HOMA-IR	4.5 ± 1.0^{b}	2.4 ± 0.8^{b}	25 ± 5.2^{a}	9.8 ± 2.0^{b}	< 0.001
Plasma total adiponectin, *mg/L*	27 ± 1.0^{b}	30 ± 0.8^{ab}	21 ± 0.7^{c}	31 ± 0.8^{a}	< 0.001
Plasma PAI-1, *ng/L*	382 ± 53^{b}	551 ± 52^{ab}	632 ± 69^{a}	550 ± 76^{ab}	0.027
Plasma TG, *mmol/L*	0.43 ± 0.03^{ab}	0.37 ± 0.02^{b}	0.48 ± 0.03^{a}	0.39 ± 0.03^{ab}	0.04
Gonadal adipose cytokines					
PAI-1, ng/g protein	222 ± 29^{b}	215 ± 32^{b}	400 ± 52^{a}	260 ± 35^{b}	0.005
MCP-1, ng/g protein	18 ± 2.4^{b}	22 ± 3.9^{b}	130 ± 45^{a}	72 ± 12^{ab}	0.002
Liver TG,[2] µmol/g	40 ± 4.4^{bc}	33 ± 2.1^{c}	82 ± 2.4^{a}	54 ± 6.2^{b}	<0.001

[1]Results are mean ± SEM, n = 9-10. Means in a row with superscripts without a common letter differ, P < 0.05

[2]n=4

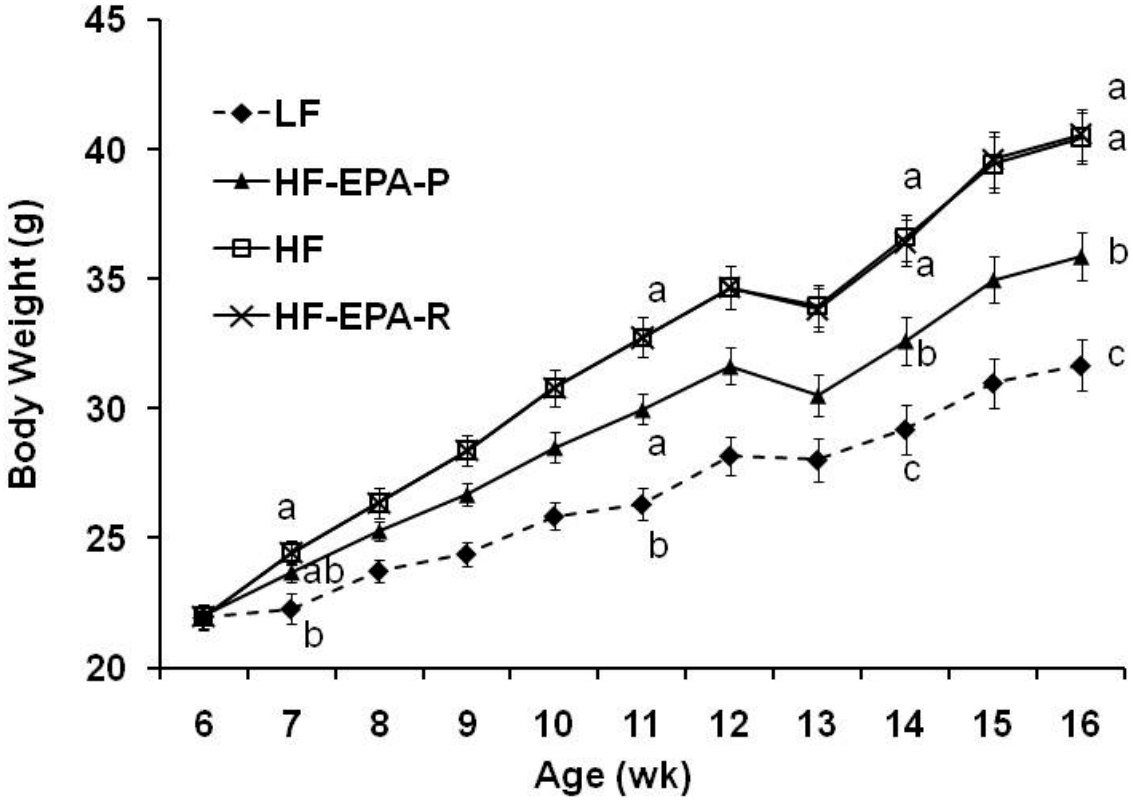

Figure 21. Effects of dietary fat content and EPA on weight gain

Change in body weight over time in C57BL/6J mice in the LF, HF, HF-EPA-P, or HF-EPA-R groups is shown. Data are presented as the mean ± SEM, n= 9-10. Labeled means at a time without a common letter differ, $P < 0.05$ (Representative differences in weight gain are shown)

significantly lower compared to the HF group ($p<0.05$). The HF-EPA-R group had a mean body weight comparable to the HF group. The total energy intake during the study period was comparable among the HF, HF-EPA-P and HF-EPA-R groups, while it was significantly lower in the LF group (Table 13). The fat pad weights and adiposity index (fat pad weight / body weight) showed a pattern similar to that of body weight, with the LF group having significantly lower values compared to the other 3 groups, and the HF-EPA-P group having significantly lower values than the HF and HF-EPA-R groups (Table 13).

The HF group developed glucose intolerance as indicated by a higher area under the glucose curve (AUC) when compared to the LF group both at 6 and 10 weeks of the dietary intervention (Figure 22, Table 13). The HF-EPA-P group maintained glucose tolerance at both time points as indicated by a similar AUC to that of the LF group despite higher body and fat pad weights in HF-EPA-P compared to the LF group. However, there was no significant difference between the AUC of the HF-EPA-R and HF groups (Figure 22C and D), indicating that EPA did not reverse the glucose intolerance caused by HF feeding. Further, the HF group also exhibited significantly higher glycemia and insulinemia following feed-deprivation, compared to the LF group (Table 13). In contrast, glycemia, insulinemia and HOMA-IR score in the HF-EPA-P and HF-EPA-R groups were similar to that of the LF group (Table 13). These data suggest that EPA prevented and reversed the insulin resistance caused by HF feeding.

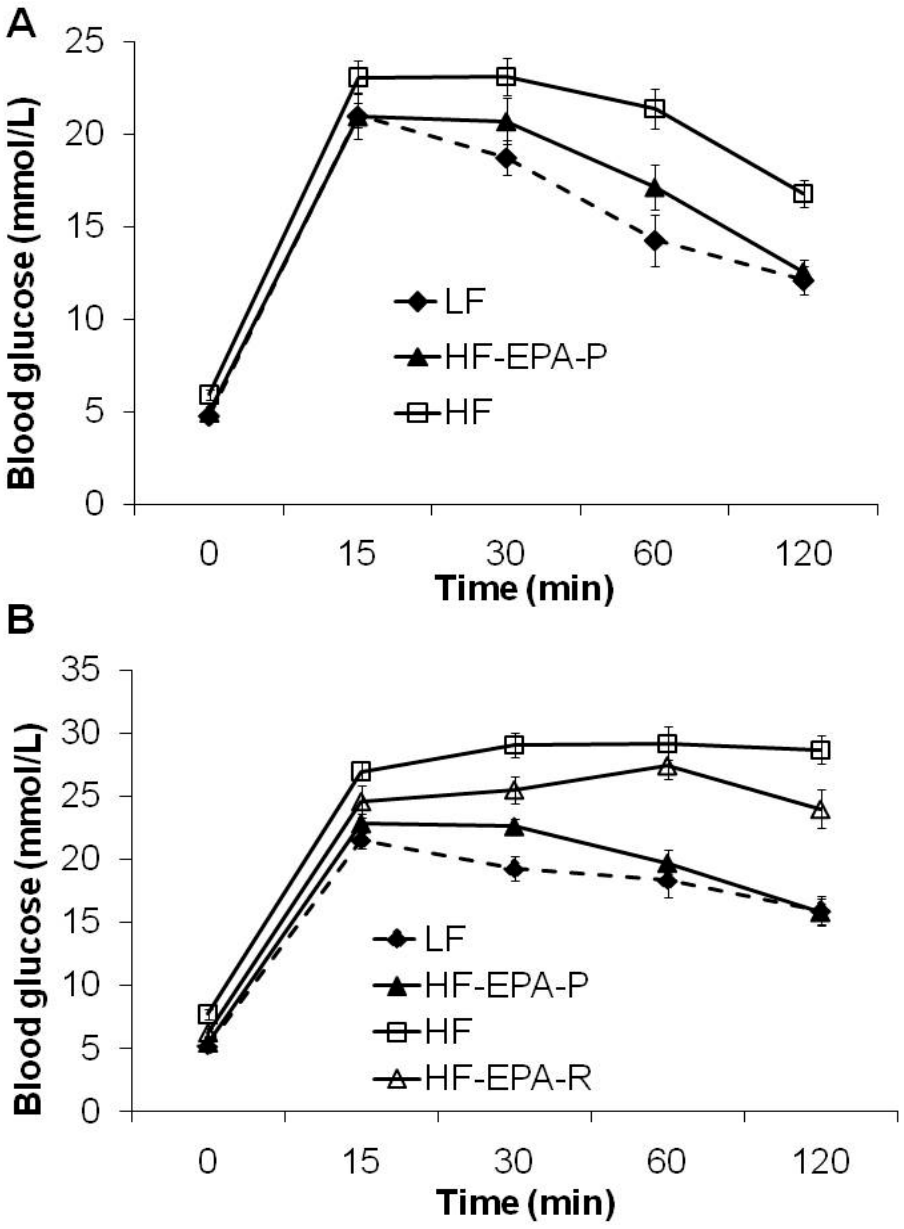

Figure 22. Effects of dietary fat content and EPA feeding on glucose tolerance

Change in blood glucose over time during the glucose tolerance test in C57BL/6J mice in the LF, HF, HF-EPA-P, or HF-EPA-R groups after 6 wk (panel A) and 10 wk (panel B) is shown. Data are presented as the mean ± SEM, n= 9-10 (20 in the HF group for A).

Plasma adiponectin concentration showed a similar pattern, with lower levels in the HF group compared to all the other three groups. Plasma PAI-1 concentration however, was higher in the HF group when compared to the LF group, while there was no significant difference in PAI-1 concentration between the HF-EPA-P and HF-EPA-R groups. Plasma triglyceride concentration was significantly lower ($p<0.05$) in the HF-EPA-P group when compared to the HF group, while the concentration in the LF and HF-EPA-R groups were not significantly different from the other two groups (Table 13). Plasma non-esterified fatty acid concentration was similar among all four groups (data not shown).

Since the development of insulin resistance in HF feeding is known to be at least in part due to adipose tissue inflammation, next we assessed some markers of adipose tissue inflammation. This confirmed that gonadal adipose tissue PAI-1 level was higher in the HF group when compared to the other three groups (Table 13). Gonadal adipose tissue MCP-1 level was highest in the HF group compared to the LF and HF-EPA-P groups, and intermediate in the HF-EPA-R group (Table 13).

Since plasma adiponectin concentration and markers of adipose tissue inflammation were comparable between the HF-fed EPA groups and the LF group, we next examined this issue using adipocytes, 3T3-L1 cells, treated with AA and EPA. Analysis of culture media from 3T3- L1 adipocytes indicated that AA-treated cells secreted less adiponectin than either control or EPA-treated cells (Figure 23A). This AA induced reduction in adiponectin secretion was prevented by co-treating with EPA. In contrast, EPA-treated cells secreted lower IL-6 levels when compared to both control, AA or AA+EPA treated adipocytes (Figure 23B).

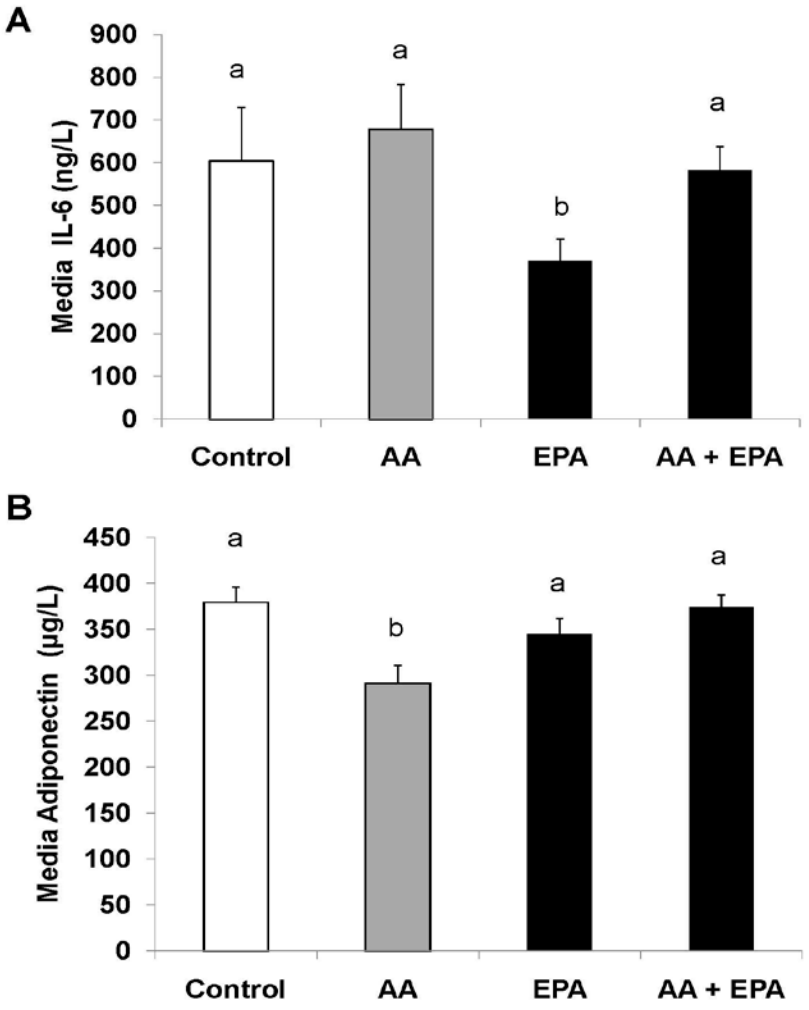

Figure 23. Effects of EPA or AA treatment on adipokine secretion

3T3-L1 adipocytes were treated with either EPA or AA. Culture media IL-6 (panel A) and adiponectin (panel B) levels are shown. Data are presented as the mean ± SEM, n= 5. Means without a common letter differ, $p<0.05$.

Given that systemic insulin resistance is also associated with hepatic steatosis, hematoxylin and eosin staining of liver was used to assess macrovesicular and microvesicular steatosis (Figure 24 for representative stained sections). This showed a significant difference among groups (44%, 11%, 70% and 44% of mice in the LF, HF-EPA-P, HF and HF-EPA-R groups, respectively, exhibited moderate to severe macrovesicular or microvesicular steatosis; $p<0.001$). Similarly, liver TG levels of the HF-EPA groups (P and R) were comparable to that of the LF group, while it was significantly lower than that of the HF group (Table 13). Both these findings suggest that EPA prevented and reversed hepatic lipid accumulation.

Protein expression in EPA and AA-treated adipocytes

To gain further understanding of global metabolic changes elicited by EPA compared to AA, we conducted proteomic studies on 3T3-L1 adipocytes treated with either EPA or AA. This showed that several proteins were differentially expressed between the two groups (Figure 25). Identification of these proteins revealed that EPA treated cells expressed higher levels of enzymes involved in carbohydrate metabolism including citrate synthase, malate deydrogenase 2 and aconitase 2 when compared to the cells treated with AA (Table 14). The EPA treated cells also expressed higher levels of proteins involved in fatty acid metabolism (fatty acid binding protein 5, pyruvate carboxylase, acyl CoA dehydrogenase, propionyl CoA carboxylase and acyl CoA thiesterase). Several other proteins involved in cellular metabolism including heat shock protein 1 expression were higher in the EPA-treated cells.

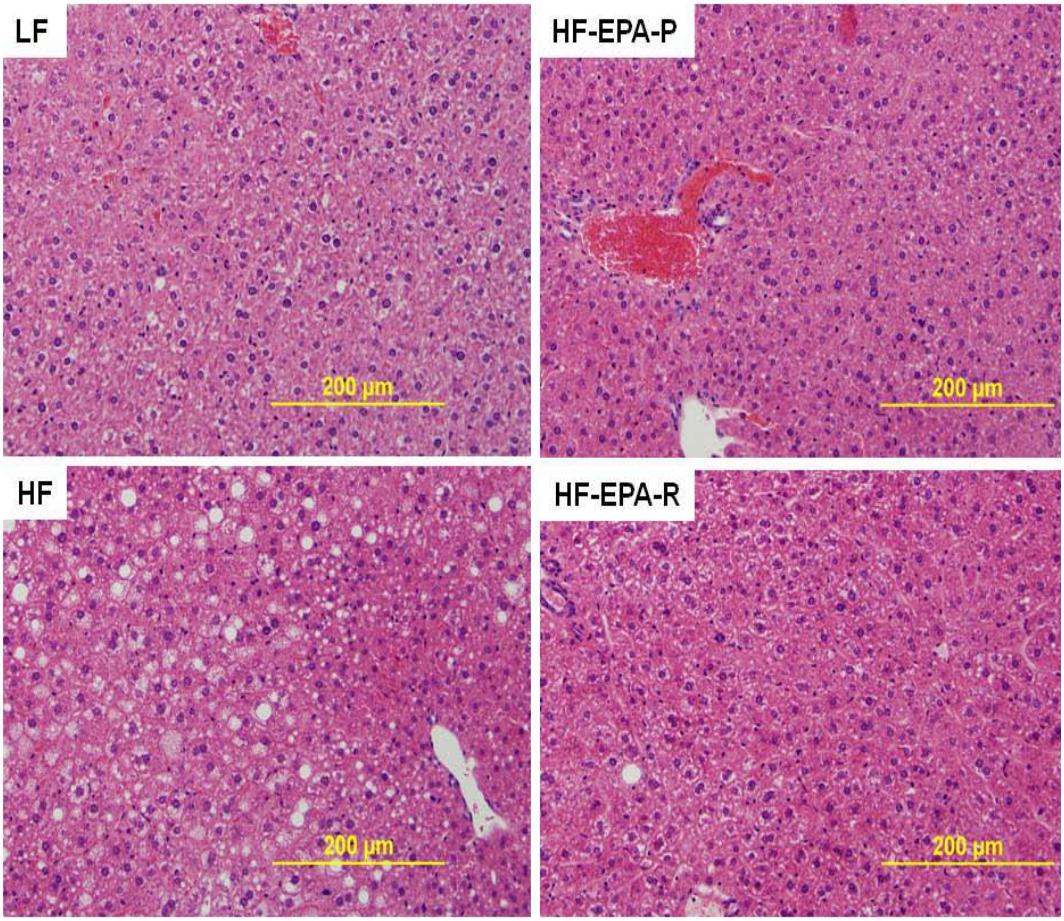

Figure 24. Effects of dietary fat content and EPA feeding on hepatic steatosis

Liver histology in C57BL/6J mice in the LF, HF, HF-EPA-P, or HF-EPA-R groups is shown. Representative hematoxylin and eosin stained sections of the liver from the four groups are shown.

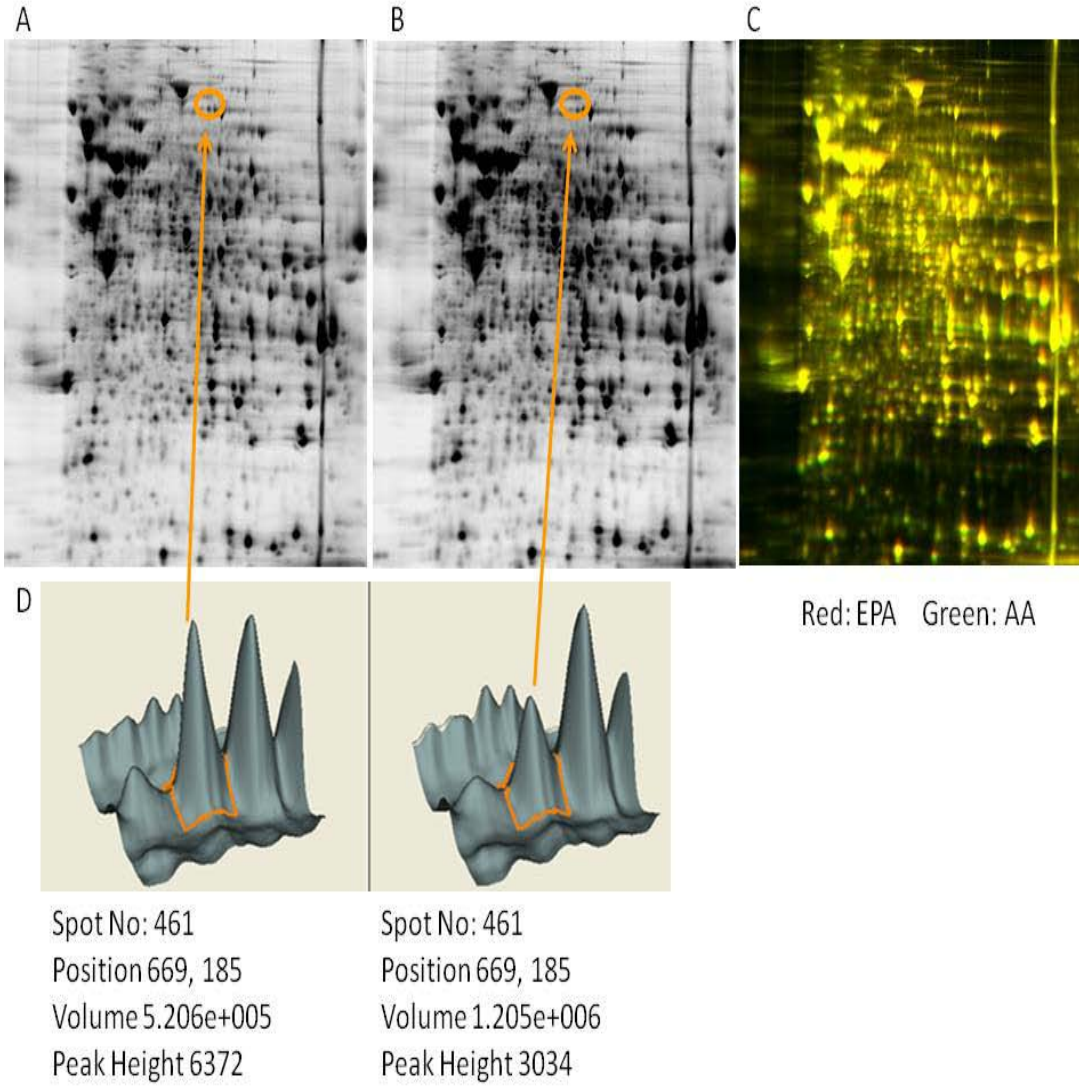

Figure 25. Proteomic studies in EPA or AA-treated adipocytes

Differential expression of proteins in EPA (panel A) or AA (panel B) treated 3T3-L1 adipocytes (panel C - overlay of 2D-DIGE) is shown. Spots were identified using Decyder software (panel D).

Table 14. Proteins expressed higher in EPA-treated compared to AA-treated adipocytes.

Protein Name	GI Accession Number	EPA/AA Spot volume ratio[1]
TCA Cycle		
Citrate synthase	13385942	2.0
Succinate-Coenzyme A ligase, ADP-forming, beta subunit	46849708	1.6
Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	21313536	1.7
Malate dehydrogenase 2, NAD (mitochondrial)	31982186	1.7
Aconitase 2, mitochondrial	18079339	1.8
Lipid metabolic process		
Pyruvate carboxylase	32822907	2.0
Fatty acid binding protein 5, epidermal	6754450	1.7
Acetyl-Coenzyme A dehydrogenase, medium chain	6680618	1.4
Propionyl Coenzyme A carboxylase, beta polypeptide	33585846	1.5
Mitochondrial acyl-coa thioesterase 1	40538846	1.5
Response to heat		
Stress-70 protein (PBP74/CSA)	903309	1.8
Heat shock protein 1 (chaperonin)	31981679	1.9
Cellular metabolic process		
Eukaryotic translation initiation factor 4E	31982407	1.4
Acetyl-Coenzyme A dehydrogenase, medium chain	6680618	1.4
Propionyl Coenzyme A carboxylase, beta polypeptide	33585846	1.5
Mitochondrial acyl-coa thioesterase 1	40538846	1.5
Isovaleryl coenzyme A dehydrogenase	9789985	1.5
Aldehyde dehydrogenase family 6, subfamily A1	23271115	1.5
Williams-Beuren syndrome chromosome region 1Homolog	15808988	1.5
Succinate-Coenzyme A ligase, ADP-forming, beta subunit	46849708	1.6
Fatty acid binding protein 5, epidermal	6754450	1.7
Malate dehydrogenase 2, NAD (mitochondrial)	31982186	1.7
Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	21313536	1.7
Aconitase 2, mitochondrial	8079339	1.8
Stress-70 protein (PBP74/CSA)	903309	1.8
Eukaryotic translation elongation factor 1 gamma	3237111	1.9
Heat shock protein 1 (chaperonin)	1981679	1.9
Pyruvate carboxylase	2822907	2.0
Citrate synthase	3385942	2.0
Glutamate dehydrogenase 1	6680027	2.2
Peptidylprolyl isomerase A	6679439	2.2

[1]Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and analyzed by mass spectrometry

Table 15. Proteins expressed lower in EPA-treated compared to AA-treated adipocytes.

Protein Name	GI Accession Number	EPA/ AA Spot Volume Ratio[1]
Cellular catabolic process		
Ubiquitin-conjugating enzyme E2L 3	6678481	0.48
Glycerol-3-phosphate dehydrogenase 1 (soluble)	6753966	0.48
Aldehyde dehydrogenase 1 family, member L2	21961590	0.53
Carboxylic acid metabolic process		
Asparaginyl-trna synthetase	29789191	0.67
Glycerol-3-phosphate dehydrogenase 1 (soluble)	6753966	0.48
Aldehyde dehydrogenase 1 family, member L2	21961590	0.53
Protein metabolic process		
Tubulin, beta, 2	22165384	0.53
Ubiquitin-conjugating enzyme E2L 3	6678481	0.48
Aldehyde dehydrogenase 1 family, member L2	21961590	0.53
Asparaginyl-trna synthetase	29789191	0.67
Clathrin, heavy polypeptide (hc)	33438248	0.59
Cofilin 1, non-muscle	55777182	0.56
Cytoskeleton organization and biogenesis		
Tubulin, beta, 2	22165384	0.53
Gamma-actin	809561	0.62
Cofilin 1, non-muscle	55777182	0.56

[1]Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and analyzed by mass spectrometry

In contrast, the EPA-treated cells expressed lower levels glycerol-3-phosphate dehydrogenase, a key enzyme in lipogenesis, when compared to the ones treated with AA (Table 15).

Discussion

It has been previously demonstrated [439, 440] that HF diet-fed mice develop higher adiposity, glucose intolerance and insulin resistance when compared to the LF diet-fed mice and EPA or DHA supplementation prevents the high-fat diet-induced increase in adiposity [321, 357, 441]. Our results now demonstrate that EPA not only *prevented,* but also *reversed,* the development of insulin resistance in response to HF feeding.

Effects of EPA on insulin resistance and adipose inflammation

The effects of EPA and DHA on preventing excess weight-gain and development of insulin resistance associated with high-fat feeding are thought to be mediated by adiponectin [347, 357]. It is not clear from data from previous studies whether the maintenance of plasma adiponectin levels was secondary to reduced adiposity. Our study showed that the HF-EPA-R group maintained normal levels of plasma adiponectin - despite similar adiposity compared to the HF group, suggesting adiposity-independent effects of EPA on adiponectin secretion. Since plasma glucose and insulin levels were comparable between the LF and both HF-EPA groups, as were plasma adiponectin

concentrations, we conclude that adiponectin is a major factor in the EPA-mediated prevention and reversal of insulin resistance.

Obesity is characterized by a chronic lowgrade inflammation in the adipose tissue [381]. Since adiponectin is exclusively secreted by adipose tissue and since the presence of pro-inflammatory cytokines are known to inhibit its synthesis and release [442], next we investigated whether EPA modulated adipose tissue inflammation induced by HF feeding. We found that gonadal adipose tissue levels of MCP-1 and PAI-1, two markers of adipose tissue inflammation, were 85% and 50% lower, respectively, in LF and EPA-P groups, compared to the HF group. Although the HF-EPA-R group exhibited adiposity similar to that of HF group, EPA prevented increased secretion of these cytokines. Consistent with these findings, *in vivo,* EPA prevented the AA induced suppression of adiponectin secretion from cultured 3T3-L1 adipocytes. EPA also reduced IL-6 secretion and other pro-inflammatory cytokines from these cells (data not shown). This evidence suggests that EPA prevented and reversed the high-fat diet induced adipose tissue inflammation.

Systemic insulin resistance and adipose tissue inflammation is associated with hepatic steatosis [443]. Similarly, we found that a higher proportion of the HF group had microscopic evidence of hepatic steatosis compared to the LF group. In contrast, the HF-EPA-P group had the lowest incidence of hepatic steatosis while the HF-EPA-R group had a value similar to the LF group. Liver TG levels also were significantly lower in the LF, HF-EPA-P and HF-EPA-R groups compared to the HF group. This suggests a protective effect of EPA on the development of HF diet-induced hepatic steatosis. These

data are consistent with previously reported hypolipidemic effects of EPA and DHA [434, 444] and may be secondary to EPA-induced hepatic AMPK activation [434] and/or higher systemic insulin sensitivity and reduced adipose tissue inflammation.

Obesity is characterized by a pro-thrombotic state associated with elevated PAI-1 concentrations. Plasma PAI-1 concentration was higher in the HF group when compared to the LF group, and intermediate between the two in the HF-EPA P and R groups. Given that gonadal adipose tissue PAI-1 levels were similar between the LF and the HF-EPA groups, it is possible that the higher plasma PAI-1 concentration of the two HF-EPA groups could be due to their higher adiposity. It is also possible that the duration of the study was too short to affect plasma PAI-1 concentration.

Effect of EPA on weight-gain and adiposity

The HF-EPA-P had lower adiposity when compared to the HF group, despite a similar energy intake. Evidence from previous studies in mouse models suggests that EPA and DHA increase lipid oxidation in the white adipose tissue [348] and small intestine [349], *in vivo,* in adipocytes [350] and myotubules [351], *in vitro,* and suppresses hepatic lipogenesis [445]. Consistent with these observations, our proteomic studies in 3T3-L1 adipocytes showed that several proteins involved in tricarboxylic acid (TCA) cycle and fatty acid β-oxidation were expressed at a higher level in the EPA-treated cells when compared to AA-treated ones. The EPA-treated cells also expressed lower levels of glycerol-3-phosphate dehydrogenase, a key lipogenic enzyme. Since EPA is known to stimulate an activator of fatty acid oxidation, AMP-activated protein kinase

(AMPK) in adipocytes *in vitro* [353], it is plausible that AMPK mediates EPA effects. Further, peroxisome proliferator-activated receptor gamma coactivator 1α, a transcription co-factor involved in mitochondrial biogenesis, was shown to be induced in the white adipose tissue of EPA/DHA supplemented mice [348]. Expression of citrate synthase, a marker of mitochondrial content, was higher in our EPA-treated adipocytes. While evidence from our study and others point towards EPA's lipid oxidizing effects, it remains to be seen whether this translates into increased energy expenditure, leading to reduced weight-gain. This is especially important to elucidate, since a recent study has shown that increased mitochondrial fatty acid oxidation does not necessarily lead to increased energy expenditure [40]. It is interesting that the HF-EPA-R and HF groups had comparable body weights. This could potentially indicate limited EPA effect on energy metabolism in already overweight (HF fed) mice. A longer intervention period would be necessary to distinguish between the effect of EPA on prevention and reversal of HF diet induced excess weight gain.

In addition to the once mentioned above, several novel proteins were identified by proteomic analysis on 3T3-L1 adipocytes treated with EPA or AA. Fatty acid binding protein 5 (mal1), is a mediator of retinoic acid-induced peroxisome proliferation-activated receptor-δ dependant fatty acid oxidation in adipocytes [41]. However, overexpression of this chaperone protein has been previously shown to be associated with insulin resistance in rodents [42]. Pyruvate carboxylase, another protein expressed higher in EPA-treated adipocytes, has been shown to be highly expressed in some rodent models of obesity [43]. Thus, it is not clear whether these *in vitro* changes of EPA-induced

protein expression will be replicated under *in vivo* conditions, and merits further investigation.

In obese rodent animal models the data are relatively consistent regarding a positive effect of EPA or DHA on insulin resistance [323, 324, 347, 431, 433]. This is not the case in humans. Most clinical studies on the effects of these fatty acids on insulin resistance have been carried out in Type-2 diabetic patients. In a recent meta-analysis of 23 randomized controlled trials, it was concluded that while EPA or DHA significantly lowered plasma TG, there was no significant effect on glycemic control [446]. Differences between the findings in obese rodents and humans may be attributable to the stage of the disorder, associations in humans have only been explored after, rather than prior, to disease onset or the absolute dose used.

In the present study we used a diet containing 36 g/kg EPA, which is equivalent to 6.75% of energy intake in those mice, fed a 45% fat diet. In comparison, the intake of EPA/DHA in the United States is about 0.1 to 0.2 g/day [319] and the current recommendations vary from 1g/day [319] to 3.5g/day [447]. There are few additional limitations in this study. First, we did not directly measure the energy expenditure of the mice, hence, are unable to attribute the lower adiposity of the HF-EPA-P group to higher energy expenditure with confidence. However, results from our proteomic and metabolic studies are consistent with increased energy expenditure. Another limitation of the study was that we did not measure high-molecular weight adiponectin, which is known to be a better associated with insulin sensitivity. Also, the prolonged period of food deprivation prior to the GTT could have adversely impacted the results. Finally, we did not use

stringent statistical criteria for our proteomic studies and the proteins that were differentially expressed need to be validated by other methods prior to confirming these changes.

In conclusion, the findings of our current study indicate that EPA both prevented and reversed the HF diet induced insulin resistance in mice. The mechanism appears to be at least in part via modulation of the adipokine secretory pattern. The relationship of EPA and insulin resistance in humans remains to be determined.

CHAPTER VI
DISCUSSION

The goal of this dissertation research was to elucidate adipose tissue function in metabolic syndrome using genetic and nutritional manipulations/approaches, with specific emphasis on the roles of inflammation and Angiotensinogen. As a genetic manipulation, we used mice overexpressing Agt in the adipose tissue to study the role of adipose RAS overexpression in the pathogenesis of insulin resistance. This was further dissected mechanistically using cultured adipocytes. Next we used energy-restricted high-fat diets and high-fat diets supplemented with EPA as dietary manipulations to prevent and reverse insulin resistance and metabolic derangements induced by high-fat feeding. Each of these studies are discussed separately in the previous chapters.

Our studies demonstrate that primary changes occurring in adipose tissue, such as adipose specific overexpression of Agt, can lead to excessive adiposity, adipose tissue inflammation and insulin resistance. This supports the current view that adipose tissue dysfunction in obesity is causally linked to the pathogenesis of insulin resistance and metabolic syndrome [84]. Moreover, we showed that dietary manipulations can improve adipose tissue inflammation and systemic insulin resistance in an adiposity-dependent (caloric restriction) as well as independent (EPA) manner. We further characterized mechanisms involved in these instances, to enhance our understanding of adipose tissue dysfunction in obesity.

Adipose tissue inflammation in obesity

A chronic low-grade inflammation occurring in adipose tissue is causally linked to the pathogenesis of insulin resistance in obesity [84]. In agreement with previous studies, we found that both mouse models of obesity we employed, i.e., aP2-Agt mice and HF diet-induced obese mice, exhibited adipose tissue inflammation and insulin resistance. Further, the improvements in insulin resistance following the dietary interventions were also accompanied by improvements in markers of adipose tissue inflammation. This highlights the importance of adipose tissue inflammation in the development of systemic insulin resistance.

The pro-inflammatory cytokine MCP-1 was consistently expressed at higher levels in adipose tissue of both mouse models of obesity. Moreover, both caloric restriction and EPA suppressed MCP-1 expression. These animal studies are further supported by *in vitro* studies showing that Ang II treatment increases MCP-1 secretion by adipocytes. Therefore, MCP-1 appears to be a key mediator of adipose tissue inflammation in obesity. Indeed, previous studies have shown that MCP-1 knockout mice are protected from HF diet-induced insulin resistance, with mice having adipose specific MCP-1 overexpression developing insulin resistance [87]. Taken together, this shows that MCP-1 is required for obesity-induced insulin resistance.

Additionally, our *in vitro* data demonstrated that Ang II induces pro-inflammatory cytokine secretion form adipocytes in an NF-kB-dependent manner. Unpublished data from our lab also shows that EPA improves adipose tissue inflammation via inhibition of the NF-kB pathway [448]. The importance of this pathway in production of

proinflammatory cytokines from immune cells is well established [449]. Here we show that this pathway is also important as a key mediator of adipocyte function. We also showed that the Ang II induced pro-inflammatory cytokine production by adipocytes was dependent upon NADPH oxidase. Activation of NADPH oxidase results in increased production of reactive oxygen species, which likely activates the NF-kB pathway [450]. Therefore, increased oxidative stress also appears to be a trigger for the activation of NF-kB pathway [451]. Taken together, this highlights NF-kB pathway as a potential target for alleviation of adipose tissue inflammation and systemic insulin resistance in obesity.

Role of hepatic steatosis in obesity-related insulin resistance

Both dietary manipulations in our studies induced improvements in hepatic steatosis. Some researchers suggest that intrahepatic fat content, rather than visceral fat content, is causally linked to the development of insulin resistance in obesity [452]. However, in both our dietary manipulations, improvement in hepatic steatosis was accompanied by improvements in adipose tissue inflammation. Further, in the EPA study, in already obese mice, EPA improved insulin sensitivity and hepatic steatosis without inducing changes in adiposity. Despite the lack of change in adiposity, these mice exhibited marked improvements in adipose tissue inflammation. Taken together, this suggests that improvements in adipose tissue function, rather than adiposity itself is related to improved insulin sensitivity and hepatic steatosis. Moreover, our unpublished studies also show that EPA-fed mice have smaller adipocytes compared to HF-fed ones,

further suggesting that improved adipose tissue function is associated with adipose tissue remodeling.

EPA supplementation also protected against HF diet-induced hypoadiponectinemia. Given that adiponectin protects against hepatic steatosis, it could be a common modulator of both hepatic steatosis and insulin sensitivity in these mice. In a previous human study which showed that intrahepatic fat, rather than visceral fat content, is important in insulin resistance, individuals with higher intrahepatic fat also had lower plasma adiponectin levels [452]. Taken together, this highlights the important role of plasma adiponectin as a key mediator of metabolic derangements in obesity.

Role of adipose RAS in insulin resistance

Individuals treated with RAS blockers are protected from the development of type-2 diabetes [119]. Moreover, systemic and adipose RAS are overactivated in several models of obesity (Table 2). However, the exact role of adipose RAS overactivation in the development of insulin resistance was hitherto unknown. We showed that primary overactivation of adipose RAS via overexpression of Agt leads to increased adiposity, adipose tissue inflammation and systemic insulin resistance. Moreover, the glucose intolerance in both wild-type and aP2-Agt mice improved following treatment with ACE inhibitor captopril. This highlights the importance of adipose tissue RAS in the pathogenesis of insulin resistance.

To further confirm whether adipose RAS is directly responsible for the pathogenesis of insulin resistance, it would be important to generate and study mice with

adipose-specific knockdown of Agt. Work in this direction is currently under way in our research group. Along these lines, preliminary unpublished data from *in vitro* studies from our lab shows that Agt silencing in cultured adipocytes significantly suppressed secretion of proinflammatory cytokines by adipocytes.

A point of contention has been whether Agt is consistently overexpressed in adipose tissue in obesity. While some human studies show a positive correlation between BMI and adipose Agt expression, some show a negative or no correlation (Table 2). Animal studies also show that adipose Agt expression is strain-dependent (Table 2). Aside from acute hormonal and nutritional control, this points toward a genetic variability in regulation of adipose Agt expression. Indeed, plasma Agt levels show a high degree of heritability in some populations [453]. Polymorphisms of Agt gene are also associated with plasma Agt levels [454]. Similarly Agt promoter variants are associated with adipose Agt expression [455]. Further characterization of RAS polymorphisms associated with adipose Agt expression would facilitate individualized genotype-based treatments in the future.

One limitation of the aP2-Agt study was that we used the adipocyte protein 2 (aP2) promoter for the overexpression of Agt in adipose tissue. While aP2 is primarily expressed in adipose tissue, evidence suggests that it is expressed in immune cells as well [456]. This could potentially lead to overexpression of Agt by these cells, and given that immune cells play a major role in adipose tissue inflammation and insulin resistance, this could act as a potential confounder in our study. Adiponectin-driven expression of

angiotensinogen or other proteins in adipose tissue would provide a better alternative to dissect the role of specific adipose genes in metabolic disorders.

Role of energy-restricted high-fat diets in improving insulin resistance

Caloric restriction leading to weight-loss is the most common form of weight loss interventions to prevent and treat obesity-associated metabolic derangements. While most health agencies recommend a low-fat diet, reduced-energy high-fat diets are also claimed to be effective in this regard. We found that weight loss induced by an energy-restricted high-fat diet was accompanied by parallel improvements in insulin resistance in mice. However this energy-restricted high-fat diet was only able to partially reverse adipose tissue inflammation. Moreover, plasma non-esterified fatty acids, adiponectin and PAI-1 levels showed no improvement compared to low-fat diets. Thus, these results also question the long term safety of these high-fat energy restricted diets, especially regarding cardiovascular risk, and support the current recommendation of low-fat diets for improvement of the metabolic profile.

Another finding of this study was that the insulin resistance, as measured by the HOMA score was comparable between the high-fat and low-fat diet-fed groups. While insulin resistance is a common feature of high-fat feeding, the development of insulin resistance in the low-fat group was likely to be due to the high sugar content of that diet. In subsequent studies, we used a low-fat diet with low sugar content, which maintained the insulin sensitivity of mice on that diet. This highlights the importance of sugar content in low-fat diets used in animal studies.

Role of EPA in improving HF diet-induced insulin resistance

We showed that EPA is able to both prevent and reverse the HF diet-induced insulin resistance in mice. Importantly, we found that EPA improved these parameters independent of loss of adiposity in already obese mice. Hence, EPA was able to dissociate increased adiposity from adipose tissue inflammation. This is an important finding, because it shows potential for development of therapeutic strategies to alleviate adipose tissue inflammation and insulin resistance without inducing weight loss.

We found that adiponectin as a key mediator of EPA-induced improvements in insulin resistance. Adiponectin is an insulin sensitizer, which improves insulin signaling in skeletal muscle via preventing serine phosphorylation of IRS-1 [457]. It also activates AMPK and induces fatty acid oxidation in skeletal muscle [458]. In our study, high-fat feeding reduced plasma adiponectin levels which was both prevented and reversed by EPA *in vivo*. Mechanistically, using cultured adipocytes, we demonstrated that AA reduced adiponectin secretion, while EPA restored it. These findings are consistent with human studies showing that EPA increases plasma adiponectin levels. In addition to its insulin-sensitizing effects, adiponectin exerts both anti-inflammatory and anti-atherogenic actions [106]. Thus, increasing plasma adiponectin levels via EPA supplementation is a promising approach to reduce the cardiovascular risk in obese individuals, especially considering other concomitant anti-inflammatory effects of this nutrient.

One limitation of this study was that the dose of EPA used was relatively higher, preventing direct extrapolation of the results to humans (equivalent dose for a human

consuming 2000 kcal/d on a 30% fat diet is 10g of EPA/day). Although the triglyceride-lowering actions of EPA are seen at around 2-3g of EPA /day, it is likely that higher doses of EPA are needed to improve insulin resistance in humans. The effect of EPA in preventing the progression of insulin resistance and pre-diabetes to type-2 diabetes in humans needs to be further examined.

CHAPTER VII
CONCLUSIONS AND RECOMMENDATIONS

General conclusions

We successfully used genetic and nutritional manipulations to dissect the role of adipose tissue dysfunction in the pathogenesis of metabolic syndrome. We showed that primary changes occurring in adipose tissue, such as overexpression of pro-inflammatory proteins, such as Agt, can lead to adipose tissue inflammation and systemic insulin resistance. We also showed that nutritional interventions targeted at reducing adipose tissue mass (caloric restriction) and adipose tissue inflammation (EPA) can both lead to improvements in systemic insulin sensitivity. These findings are summarized below:

1. Adipose specific overexpression of Agt leads to increased adiposity, glucose intolerance and systemic insulin resistance. This is at least in part due to Ang II mediated NADPH oxidase and NF-kB-dependent increases in adipose tissue inflammation.
2. Weight loss induced by an energy restricted high-fat diet is accompanied by parallel improvements in insulin resistance and hepatic steatosis. However, this diet only partially improves markers of adipose tissue inflammation and negatively impacts plasma adiponectin levels and does not reverse HF diet-induced increases in plasma non-esterified fatty acids and PAI-1.

3. EPA can both prevent and reverse high-fat diet-induced insulin resistance and hepatic steatosis in mice. Mechanistically, this is at least in part via EPA-mediated improvements in adipose tissue inflammation.

Implications and Recommendations

Based on the findings of our studies we would like to make the following recommendations. First, our studies support the current recommendation of low-fat diets for improvement of metabolic derangements associated with obesity. Our findings also support the American Heart Association's current recommendation for an increase in dietary intake of omega-3 fatty acids of marine origin.

We discovered that adipose specific RAS overactivation can lead to systemic insulin resistance. To confirm the role of adipose RAS in the pathogenesis of insulin resistance in high-fat feeding, studies using adipose-specific Agt knockout are warranted.

Our studies showed that EPA can both prevent and reverse the insulin resistance induced by high-fat diets. It is likely that EPA prevents the progression of metabolic syndrome and prediabetes to type-2 diabetes in obese humans. This hypothesis remains to be tested in humans using both tested doses as well as lower doses of EPA.

We uncovered mechanisms by which Ang II modulates adipocyte function. Further, we showed mechanisms by which EPA modulates insulin resistance. Identification of agents that modify these molecular targets could lead to development of novel therapeutic strategies to prevent and treat metabolic derangements in obesity.

LIST OF REFERENCES

1. Flegal KM, Carroll MD, Ogden CL, Curtin LR: **Prevalence and trends in obesity among US adults, 1999-2008**. *JAMA* 2010, **303**(3):235-241.
2. **Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults**. *Am J Clin Nutr* 1998, **68**(4):899-917.
3. **Obesity and overweight fact sheet** [http://www.who.int/mediacentre/factsheets/fs311/en/index.html]
4. **Prevalence of Obesity Among Children and Adolescents: United States, Trends 1963-1965 Through 2007-2008** [http://www.cdc.gov/nchs/data/hestat/obesity_child_07_08/obesity_child_07_08.htm]
5. Jacobs EJ, Newton CC, Wang Y, Patel AV, McCullough ML, Campbell PT, Thun MJ, Gapstur SM: **Waist circumference and all-cause mortality in a large US cohort**. *Arch Intern Med* 2010, **170**(15):1293-1301.
6. Van Gaal LF, Mertens IL, De Block CE: **Mechanisms linking obesity with cardiovascular disease**. *Nature* 2006, **444**(7121):875-880.
7. Kahn SE, Hull RL, Utzschneider KM: **Mechanisms linking obesity to insulin resistance and type 2 diabetes**. *Nature* 2006, **444**(7121):840-846.
8. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M: **Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies**. *Lancet* 2008, **371**(9612):569-578.
9. de Sousa AG, Cercato C, Mancini MC, Halpern A: **Obesity and obstructive sleep apnea-hypopnea syndrome**. *Obes Rev* 2008, **9**(4):340-354.
10. Lementowski PW, Zelicof SB: **Obesity and osteoarthritis**. *Am J Orthop (Belle Mead NJ)* 2008, **37**(3):148-151.
11. Deschildre A, Pin I, Gueorguieva I, de Blic J: **[Asthma and obesity in childhood: what is the link?]**. *Arch Pediatr* 2009, **16**(8):1166-1174.
12. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, Zitman FG: **Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies**. *Arch Gen Psychiatry* 2010, **67**(3):220-229.
13. Everhart JE: **Contributions of obesity and weight loss to gallstone disease**. *Ann Intern Med* 1993, **119**(10):1029-1035.
14. **Diabetes data and trends** [http://www.cdc.gov/diabetes/statistics/prev/national/figadults.htm]
15. King H, Aubert RE, Herman WH: **Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections**. *Diabetes Care* 1998, **21**(9):1414-1431.
16. Farooqi IS, O'Rahilly S: **Genetic factors in human obesity**. *Obes Rev* 2007, **8 Suppl 1**:37-40.

17. Kalupahana NS, Moustaid-Moussa N, Kim JH, Voy BH, Bassett D, Bray MS, Lightfoot TJ: **The Regulation of Physical Activity by Genetic Mechanisms: Is There a Drive to Be Active?** In: *Genetic and Molecular Aspects of Sports Performance*. Edited by Bouchard C, Hoffman E, vol. 1: Blackwell Publishing Ltd.; 2011: 283-293.
18. O'Rahilly S: **Human genetics illuminates the paths to metabolic disease**. *Nature* 2009, **462**(7271):307-314.
19. Bouchard C: **Defining the genetic architecture of the predisposition to obesity: a challenging but not insurmountable task**. *Am J Clin Nutr* 2010, **91**(1):5-6.
20. Nader PR, Bradley RH, Houts RM, McRitchie SL, O'Brien M: **Moderate-to-vigorous physical activity from ages 9 to 15 years**. *JAMA* 2008, **300**(3):295-305.
21. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr.: **Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity**. *Circulation* 2009, **120**(16):1640-1645.
22. Ibrahim MM: **Subcutaneous and visceral adipose tissue: structural and functional differences**. *Obes Rev* 2010, **11**(1):11-18.
23. Kim S, Moustaid-Moussa N: **Secretory, endocrine and autocrine/paracrine function of the adipocyte**. *The Journal of nutrition* 2000, **130**(12):3110S-3115S.
24. Alexaki VI, Notas G, Pelekanou V, Kampa M, Valkanou M, Theodoropoulos P, Stathopoulos EN, Tsapis A, Castanas E: **Adipocytes as immune cells: differential expression of TWEAK, BAFF, and APRIL and their receptors (Fn14, BAFF-R, TACI, and BCMA) at different stages of normal and pathological adipose tissue development**. *J Immunol* 2009, **183**(9):5948-5956.
25. Tran TT, Kahn CR: **Transplantation of adipose tissue and stem cells: role in metabolism and disease**. *Nat Rev Endocrinol* 2010, **6**(4):195-213.
26. Fried SK, Bunkin DA, Greenberg AS: **Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid**. *J Clin Endocrinol Metab* 1998, **83**(3):847-850.
27. Despres JP, Lemieux I: **Abdominal obesity and metabolic syndrome**. *Nature* 2006, **444**(7121):881-887.
28. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB: **The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase**. *J Clin Invest* 1995, **95**(5):2111-2119.
29. Friedman JM, Halaas JL: **Leptin and the regulation of body weight in mammals**. *Nature* 1998, **395**(6704):763-770.
30. Hu E, Liang P, Spiegelman BM: **AdipoQ is a novel adipose-specific gene dysregulated in obesity**. *J Biol Chem* 1996, **271**(18):10697-10703.

31. McTernan CL, McTernan PG, Harte AL, Levick PL, Barnett AH, Kumar S: **Resistin, central obesity, and type 2 diabetes**. *Lancet* 2002, **359**(9300):46-47.
32. Jones BH, Standridge MK, Taylor JW, Moustaid N: **Angiotensinogen gene expression in adipose tissue: analysis of obese models and hormonal and nutritional control**. *Am J Physiol* 1997, **273**(1 Pt 2):R236-242.
33. Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, Kotani H: **Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice**. *J Biol Chem* 2003, **278**(47):46654-46660.
34. Hotamisligil GS, Shargill NS, Spiegelman BM: **Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance**. *Science* 1993, **259**(5091):87-91.
35. Morange PE, Alessi MC, Verdier M, Casanova D, Magalon G, Juhan-Vague I: **PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level**. *Arterioscler Thromb Vasc Biol* 1999, **19**(5):1361-1365.
36. Mohamed-Ali V, Flower L, Sethi J, Hotamisligil G, Gray R, Humphries SE, York DA, Pinkney J: **beta-Adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies**. *J Clin Endocrinol Metab* 2001, **86**(12):5864-5869.
37. Lira FS, Rosa JC, Yamashita AS, Koyama CH, Batista ML, Jr., Seelaender M: **Endurance training induces depot-specific changes in IL-10/TNF-alpha ratio in rat adipose tissue**. *Cytokine* 2009, **45**(2):80-85.
38. Frydelund-Larsen L, Akerstrom T, Nielsen S, Keller P, Keller C, Pedersen BK: **Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise**. *Am J Physiol Endocrinol Metab* 2007, **292**(1):E24-31.
39. Castan-Laurell I, Vitkova M, Daviaud D, Dray C, Kovacikova M, Kovacova Z, Hejnova J, Stich V, Valet P: **Effect of hypocaloric diet-induced weight loss in obese women on plasma apelin and adipose tissue expression of apelin and APJ**. *Eur J Endocrinol* 2008, **158**(6):905-910.
40. Kloting N, Graham TE, Berndt J, Kralisch S, Kovacs P, Wason CJ, Fasshauer M, Schon MR, Stumvoll M, Bluher M *et al*: **Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass**. *Cell Metab* 2007, **6**(1):79-87.
41. Gealekman O, Burkart A, Chouinard M, Nicoloro SM, Straubhaar J, Corvera S: **Enhanced angiogenesis in obesity and in response to PPARgamma activators through adipocyte VEGF and ANGPTL4 production**. *Am J Physiol Endocrinol Metab* 2008, **295**(5):E1056-1064.
42. Peeraully MR, Jenkins JR, Trayhurn P: **NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines**. *Am J Physiol Endocrinol Metab* 2004, **287**(2):E331-339.
43. Juge-Aubry CE, Somm E, Chicheportiche R, Burger D, Pernin A, Cuenod-Pittet B, Quinodoz P, Giusti V, Dayer JM, Meier CA: **Regulatory effects of interleukin (IL)-1, interferon-beta, and IL-4 on the production of IL-1**

receptor antagonist by human adipose tissue. *J Clin Endocrinol Metab* 2004, **89**(6):2652-2658.

44. Kloting N, Berndt J, Kralisch S, Kovacs P, Fasshauer M, Schon MR, Stumvoll M, Bluher M: **Vaspin gene expression in human adipose tissue: association with obesity and type 2 diabetes**. *Biochem Biophys Res Commun* 2006, **339**(1):430-436.
45. Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC, Shuldiner AR, Fried SK, McLenithan JC, Gong DW: **Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action**. *Am J Physiol Endocrinol Metab* 2006, **290**(6):E1253-1261.
46. Kos K, Harte AL, James S, Snead DR, O'Hare JP, McTernan PG, Kumar S: **Secretion of neuropeptide Y in human adipose tissue and its role in maintenance of adipose tissue mass**. *Am J Physiol Endocrinol Metab* 2007, **293**(5):E1335-1340.
47. Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, Iannelli A, Staccini-Myx A, Casanova D, Ben Amor I *et al*: **Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH**. *Gastroenterology* 2006, **131**(3):788-796.
48. Bruun JM, Pedersen SB, Richelsen B: **Regulation of interleukin 8 production and gene expression in human adipose tissue in vitro**. *J Clin Endocrinol Metab* 2001, **86**(3):1267-1273.
49. Wood IS, Wang B, Jenkins JR, Trayhurn P: **The pro-inflammatory cytokine IL-18 is expressed in human adipose tissue and strongly upregulated by TNFalpha in human adipocytes**. *Biochem Biophys Res Commun* 2005, **337**(2):422-429.
50. Varma V, Yao-Borengasser A, Bodles AM, Rasouli N, Phanavanh B, Nolen GT, Kern EM, Nagarajan R, Spencer HJ, 3rd, Lee MJ *et al*: **Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance**. *Diabetes* 2008, **57**(2):432-439.
51. Becker M, Rabe K, Lebherz C, Zugwurst J, Goke B, Parhofer KG, Lehrke M, Broedl UC: **Expression of human chemerin induces insulin resistance in the skeletal muscle but does not affect weight, lipid levels, and atherosclerosis in LDL receptor knockout mice on high-fat diet**. *Diabetes* 2010, **59**(11):2898-2903.
52. Van Harmelen V, Ariapart P, Hoffstedt J, Lundkvist I, Bringman S, Arner P: **Increased adipose angiotensinogen gene expression in human obesity**. *Obes Res* 2000, **8**(4):337-341.
53. Lonnqvist F, Arner P, Nordfors L, Schalling M: **Overexpression of the obese (ob) gene in adipose tissue of human obese subjects**. *Nat Med* 1995, **1**(9):950-953.
54. Christiansen T, Richelsen B, Bruun JM: **Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects**. *Int J Obes (Lond)* 2005, **29**(1):146-150.

55. Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE, Goldstein BJ: **Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone**. *J Clin Endocrinol Metab* 2002, **87**(12):5662-5667.
56. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA *et al*: **Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance**. *J Clin Invest* 2003, **112**(12):1821-1830.
57. Wellen KE, Hotamisligil GS: **Inflammation, stress, and diabetes**. *J Clin Invest* 2005, **115**(5):1111-1119.
58. Wang P, Mariman E, Renes J, Keijer J: **The secretory function of adipocytes in the physiology of white adipose tissue**. *J Cell Physiol* 2008, **216**(1):3-13.
59. Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E, Tartare-Deckert S: **Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation**. *J Biol Chem* 2003, **278**(14):11888-11896.
60. Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR: **Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity**. *Diabetes* 2002, **51**(4):1093-1101.
61. Kos K, Wilding JP: **SPARC: a key player in the pathologies associated with obesity and diabetes**. *Nat Rev Endocrinol* 2010, **6**(4):225-235.
62. Ye J, Gao Z, Yin J, He Q: **Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice**. *Am J Physiol Endocrinol Metab* 2007, **293**(4):E1118-1128.
63. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR: **Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response**. *Diabetes* 2009, **58**(3):718-725.
64. Boden G, Duan X, Homko C, Molina EJ, Song W, Perez O, Cheung P, Merali S: **Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals**. *Diabetes* 2008, **57**(9):2438-2444.
65. Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka TA, Ozawa K, Ogawa S, Hori M, Yamasaki Y *et al*: **Involvement of endoplasmic reticulum stress in insulin resistance and diabetes**. *J Biol Chem* 2005, **280**(1):847-851.
66. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: **Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes**. *Science* 2004, **306**(5695):457-461.
67. Shen X, Zhang K, Kaufman RJ: **The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum**. *J Chem Neuroanat* 2004, **28**(1-2):79-92.

68. Magne J, Mariotti F, Fischer R, Mathe V, Tome D, Huneau JF: **Early postprandial low-grade inflammation after high-fat meal in healthy rats: possible involvement of visceral adipose tissue**. *J Nutr Biochem* 2009.
69. Kennedy A, Martinez K, Chuang CC, LaPoint K, McIntosh M: **Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications**. *J Nutr* 2009, **139**(1):1-4.
70. LaRosa PC, Miner J, Xia Y, Zhou Y, Kachman S, Fromm ME: **Trans-10, cis-12 conjugated linoleic acid causes inflammation and delipidation of white adipose tissue in mice: a microarray and histological analysis**. *Physiol Genomics* 2006, **27**(3):282-294.
71. Poirier H, Shapiro JS, Kim RJ, Lazar MA: **Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue**. *Diabetes* 2006, **55**(6):1634-1641.
72. Bes-Houtmann S, Roche R, Hoareau L, Gonthier MP, Festy F, Caillens H, Gasque P, Lefebvre d'Hellencourt C, Cesari M: **Presence of functional TLR2 and TLR4 on human adipocytes**. *Histochem Cell Biol* 2007, **127**(2):131-137.
73. Suganami T, Mieda T, Itoh M, Shimoda Y, Kamei Y, Ogawa Y: **Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation**. *Biochem Biophys Res Commun* 2007, **354**(1):45-49.
74. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD: **The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance**. *Nat Med* 2011.
75. Abe M, Matsuda M, Kobayashi H, Miyata Y, Nakayama Y, Komuro R, Fukuhara A, Shimomura I: **Effects of statins on adipose tissue inflammation: their inhibitory effect on MyD88-independent IRF3/IFN-beta pathway in macrophages**. *Arterioscler Thromb Vasc Biol* 2008, **28**(5):871-877.
76. DeFuria J, Bennett G, Strissel KJ, Perfield JW, 2nd, Milbury PE, Greenberg AS, Obin MS: **Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae**. *The Journal of nutrition* 2009, **139**(8):1510-1516.
77. Kalupahana NS, Claycombe K, Newman SJ, Stewart T, Siriwardhana N, Matthan N, Lichtenstein AH, Moustaid-Moussa N: **Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation**. *The Journal of nutrition* 2010, **140**(11):1915-1922.
78. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.: **Obesity is associated with macrophage accumulation in adipose tissue**. *J Clin Invest* 2003, **112**(12):1796-1808.
79. Gordon S: **Alternative activation of macrophages**. *Nat Rev Immunol* 2003, **3**(1):23-35.
80. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M: **The chemokine system in diverse forms of macrophage activation and polarization**. *Trends Immunol* 2004, **25**(12):677-686.

81. Zeyda M, Stulnig TM: **Adipose tissue macrophages**. *Immunol Lett* 2007, **112**(2):61-67.
82. Lumeng CN, Bodzin JL, Saltiel AR: **Obesity induces a phenotypic switch in adipose tissue macrophage polarization**. *J Clin Invest* 2007, **117**(1):175-184.
83. Prieur X, Mok CY, Velagapudi VR, Nunez V, Fuentes L, Montaner D, Ishikawa K, Camacho A, Barbarroja N, O'Rahilly S *et al*: **Differential Lipid Partitioning Between Adipocytes and Tissue Macrophages Modulates Macrophage Lipotoxicity and M2/M1 Polarization in Obese Mice**. *Diabetes* 2011.
84. Olefsky JM, Glass CK: **Macrophages, inflammation, and insulin resistance**. *Annu Rev Physiol* 2010, **72**:219-246.
85. Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR: **Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes**. *Diabetes* 2008, **57**(12):3239-3246.
86. Sato C, Shikata K, Hirota D, Sasaki M, Nishishita S, Miyamoto S, Kodera R, Ogawa D, Tone A, Kataoka HU *et al*: **P-selectin glycoprotein ligand-1 deficiency is protective against obesity-related insulin resistance**. *Diabetes* 2011, **60**(1):189-199.
87. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K *et al*: **MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity**. *J Clin Invest* 2006, **116**(6):1494-1505.
88. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr.: **CCR2 modulates inflammatory and metabolic effects of high-fat feeding**. *J Clin Invest* 2006, **116**(1):115-124.
89. Surmi BK, Webb CD, Ristau AC, Hasty AH: **Absence of macrophage inflammatory protein-1{alpha} does not impact macrophage accumulation in adipose tissue of diet-induced obese mice**. *Am J Physiol Endocrinol Metab* 2010, **299**(3):E437-445.
90. Skurk T, Alberti-Huber C, Herder C, Hauner H: **Relationship between adipocyte size and adipokine expression and secretion**. *J Clin Endocrinol Metab* 2007, **92**(3):1023-1033.
91. Eiras S, Teijeira-Fernandez E, Salgado-Somoza A, Couso E, Garcia-Caballero T, Sierra J, Juanatey JR: **Relationship between epicardial adipose tissue adipocyte size and MCP-1 expression**. *Cytokine* 2010, **51**(2):207-212.
92. Lumeng CN, Maillard I, Saltiel AR: **T-ing up inflammation in fat**. *Nat Med* 2009, **15**(8):846-847.
93. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S *et al*: **CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity**. *Nat Med* 2009, **15**(8):914-920.
94. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S *et al*: **Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters**. *Nat Med* 2009, **15**(8):930-939.

95. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, Dorfman R, Wang Y, Zielenski J, Mastronardi F *et al*: **Normalization of obesity-associated insulin resistance through immunotherapy**. *Nat Med* 2009, **15**(8):921-929.
96. Schenk S, Saberi M, Olefsky JM: **Insulin sensitivity: modulation by nutrients and inflammation**. *J Clin Invest* 2008, **118**(9):2992-3002.
97. Stumvoll M, Goldstein BJ, van Haeften TW: **Type 2 diabetes: principles of pathogenesis and therapy**. *Lancet* 2005, **365**(9467):1333-1346.
98. Taniguchi CM, Emanuelli B, Kahn CR: **Critical nodes in signalling pathways: insights into insulin action**. *Nat Rev Mol Cell Biol* 2006, **7**(2):85-96.
99. Cheng Z, Tseng Y, White MF: **Insulin signaling meets mitochondria in metabolism**. *Trends Endocrinol Metab* 2010, **21**(10):589-598.
100. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E: **SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice**. *J Biol Chem* 2001, **276**(51):47944-47949.
101. Zick Y: **Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance**. *Sci STKE* 2005, **2005**(268):pe4.
102. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE: **Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB**. *Nat Med* 2005, **11**(2):183-190.
103. Rui L, Yuan M, Frantz D, Shoelson S, White MF: **SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2**. *J Biol Chem* 2002, **277**(44):42394-42398.
104. Ueki K, Fruman DA, Yballe CM, Fasshauer M, Klein J, Asano T, Cantley LC, Kahn CR: **Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling**. *J Biol Chem* 2003, **278**(48):48453-48466.
105. Kalupahana NS, Voy BH, Fletcher S, Stewart T, Kim JH, Quignard-Boulange A, Wasserman D, Moustaid-Moussa N: **Mechanisms linking overproduction of angiotensinogen by adipose tissue to inflammation, glucose intolerance and insulin resistance**. In: *27th annual scientific meeting of The Obesity society*. Washington, DC; 2009.
106. Luo N, Liu J, Chung BH, Yang Q, Klein RL, Garvey WT, Fu Y: **Macrophage adiponectin expression improves insulin sensitivity and protects against inflammation and atherosclerosis**. *Diabetes* 2010, **59**(4):791-799.
107. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: **A central role for JNK in obesity and insulin resistance**. *Nature* 2002, **420**(6913):333-336.
108. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M: **IKK-beta links inflammation to obesity-induced insulin resistance**. *Nat Med* 2005, **11**(2):191-198.
109. Zhang X, Xu A, Chung SK, Cresser JH, Sweeney G, Wong RL, Lin A, Lam KS: **Selective inactivation of c-Jun NH2-terminal kinase in adipose tissue protects**

against diet-induced obesity and improves insulin sensitivity in both liver and skeletal muscle in mice. *Diabetes* 2011, **60**(2):486-495.

110. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N *et al*: **The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity**. *Nat Med* 2001, **7**(8):941-946.
111. Lindsay RS, Funahashi T, Hanson RL, Matsuzawa Y, Tanaka S, Tataranni PA, Knowler WC, Krakoff J: **Adiponectin and development of type 2 diabetes in the Pima Indian population**. *Lancet* 2002, **360**(9326):57-58.
112. Brooks GC, Blaha MJ, Blumenthal RS: **Relation of C-reactive protein to abdominal adiposity**. *Am J Cardiol* 2010, **106**(1):56-61.
113. Tang T, Zhang J, Yin J, Staszkiewicz J, Gawronska-Kozak B, Jung DY, Ko HJ, Ong H, Kim JK, Mynatt R *et al*: **Uncoupling of inflammation and insulin resistance by NF-kappaB in transgenic mice through elevated energy expenditure**. *J Biol Chem* 2010, **285**(7):4637-4644.
114. Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, Balletshofer B, Machicao F, Fritsche A, Haring HU: **Identification and characterization of metabolically benign obesity in humans**. *Arch Intern Med* 2008, **168**(15):1609-1616.
115. Gustafson B, Gogg S, Hedjazifar S, Jenndahl L, Hammarstedt A, Smith U: **Inflammation and impaired adipogenesis in hypertrophic obesity in man**. *Am J Physiol Endocrinol Metab* 2009.
116. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: **An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma)**. *J Biol Chem* 1995, **270**(22):12953-12956.
117. Samuel VT, Petersen KF, Shulman GI: **Lipid-induced insulin resistance: unravelling the mechanism**. *Lancet* 2010, **375**(9733):2267-2277.
118. Stratford S, DeWald DB, Summers SA: **Ceramide dissociates 3'-phosphoinositide production from pleckstrin homology domain translocation**. *Biochem J* 2001, **354**(Pt 2):359-368.
119. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM: **Renin-angiotensin system and cardiovascular risk**. *Lancet* 2007, **369**(9568):1208-1219.
120. Vermes E, Ducharme A, Bourassa MG, Lessard M, White M, Tardif JC: **Enalapril reduces the incidence of diabetes in patients with chronic heart failure: insight from the Studies Of Left Ventricular Dysfunction (SOLVD)**. *Circulation* 2003, **107**(9):1291-1296.
121. Bosch J, Yusuf S, Gerstein HC, Pogue J, Sheridan P, Dagenais G, Diaz R, Avezum A, Lanas F, Probstfield J *et al*: **Effect of ramipril on the incidence of diabetes**. *N Engl J Med* 2006, **355**(15):1551-1562.
122. Hansson L, Lindholm LH, Niskanen L, Lanke J, Hedner T, Niklason A, Luomanmaki K, Dahlof B, de Faire U, Morlin C *et al*: **Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril**

Prevention Project (CAPPP) randomised trial. *Lancet* 1999, **353**(9153):611-616.

123. Yasue S, Masuzaki H, Okada S, Ishii T, Kozuka C, Tanaka T, Fujikura J, Ebihara K, Hosoda K, Katsurada A *et al*: **Adipose tissue-specific regulation of angiotensinogen in obese humans and mice: impact of nutritional status and adipocyte hypertrophy**. *Am J Hypertens* 2010, **23**(4):425-431.
124. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD: **Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin**. *J Clin Invest* 2002, **109**(11):1417-1427.
125. Karlsson C, Lindell K, Ottosson M, Sjostrom L, Carlsson B, Carlsson LM: **Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II**. *J Clin Endocrinol Metab* 1998, **83**(11):3925-3929.
126. Strazzullo P, Iacone R, Iacoviello L, Russo O, Barba G, Russo P, D'Orazio A, Barbato A, Cappuccio FP, Farinaro E *et al*: **Genetic variation in the renin-angiotensin system and abdominal adiposity in men: the Olivetti Prospective Heart Study**. *Ann Intern Med* 2003, **138**(1):17-23.
127. Takakura Y, Yoshida T, Yoshioka K, Umekawa T, Kogure A, Toda H, Kagawa K, Fukui S, Yoshikawa T: **Angiotensinogen gene polymorphism (Met235Thr) influences visceral obesity and insulin resistance in obese Japanese women**. *Metabolism* 2006, **55**(6):819-824.
128. Prat-Larquemin L, Oppert JM, Clement K, Hainault I, Basdevant A, Guy-Grand B, Quignard-Boulange A: **Adipose angiotensinogen secretion, blood pressure, and AGT M235T polymorphism in obese patients**. *Obes Res* 2004, **12**(3):556-561.
129. Goossens GH, Jocken JW, Blaak EE, Schiffers PM, Saris WH, van Baak MA: **Endocrine role of the renin-angiotensin system in human adipose tissue and muscle: effect of beta-adrenergic stimulation**. *Hypertension* 2007, **49**(3):542-547.
130. Engeli S, Bohnke J, Gorzelniak K, Janke J, Schling P, Bader M, Luft FC, Sharma AM: **Weight loss and the renin-angiotensin-aldosterone system**. *Hypertension* 2005, **45**(3):356-362.
131. Cooper R, Forrester T, Ogunbiyi O, Muffinda J: **Angiotensinogen levels and obesity in four black populations. ICSHIB Investigators**. *J Hypertens* 1998, **16**(5):571-575.
132. Schorr U, Blaschke K, Turan S, Distler A, Sharma AM: **Relationship between angiotensinogen, leptin and blood pressure levels in young normotensive men**. *J Hypertens* 1998, **16**(10):1475-1480.
133. Bloem LJ, Manatunga AK, Tewksbury DA, Pratt JH: **The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children**. *J Clin Invest* 1995, **95**(3):948-953.
134. Umemura S, Nyui N, Tamura K, Hibi K, Yamaguchi S, Nakamaru M, Ishigami T, Yabana M, Kihara M, Inoue S *et al*: **Plasma angiotensinogen concentrations in obese patients**. *Am J Hypertens* 1997, **10**(6):629-633.

135. Cooper R, McFarlane-Anderson N, Bennett FI, Wilks R, Puras A, Tewksbury D, Ward R, Forrester T: **ACE, angiotensinogen and obesity: a potential pathway leading to hypertension**. *J Hum Hypertens* 1997, **11**(2):107-111.
136. Faloia E, Gatti C, Camilloni MA, Mariniello B, Sardu C, Garrapa GG, Mantero F, Giacchetti G: **Comparison of circulating and local adipose tissue renin-angiotensin system in normotensive and hypertensive obese subjects**. *J Endocrinol Invest* 2002, **25**(4):309-314.
137. Licata G, Scaglione R, Ganguzza A, Corrao S, Donatelli M, Parrinello G, Dichiara MA, Merlino G, Cecala MG: **Central obesity and hypertension. Relationship between fasting serum insulin, plasma renin activity, and diastolic blood pressure in young obese subjects**. *Am J Hypertens* 1994, **7**(4 Pt 1):314-320.
138. Egan BM, Stepniakowski K, Goodfriend TL: **Renin and aldosterone are higher and the hyperinsulinemic effect of salt restriction greater in subjects with risk factors clustering**. *Am J Hypertens* 1994, **7**(10 Pt 1):886-893.
139. Uckaya G, Ozata M, Sonmez A, Kinalp C, Eyileten T, Bingol N, Koc B, Kocabalkan F, Ozdemir IC: **Plasma leptin levels strongly correlate with plasma renin activity in patients with essential hypertension**. *Horm Metab Res* 1999, **31**(7):435-438.
140. Giacchetti G, Faloia E, Sardu C, Camilloni MA, Mariniello B, Gatti C, Garrapa GG, Guerrieri M, Mantero F: **Gene expression of angiotensinogen in adipose tissue of obese patients**. *Int J Obes Relat Metab Disord* 2000, **24 Suppl 2**:S142-143.
141. van Harmelen V, Elizalde M, Ariapart P, Bergstedt-Lindqvist S, Reynisdottir S, Hoffstedt J, Lundkvist I, Bringman S, Arner P: **The association of human adipose angiotensinogen gene expression with abdominal fat distribution in obesity**. *Int J Obes Relat Metab Disord* 2000, **24**(6):673-678.
142. Gorzelniak K, Engeli S, Janke J, Luft FC, Sharma AM: **Hormonal regulation of the human adipose-tissue renin-angiotensin system: relationship to obesity and hypertension**. *J Hypertens* 2002, **20**(5):965-973.
143. Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA: **Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension**. *Am J Physiol Regul Integr Comp Physiol* 2004, **287**(4):R943-949.
144. Kobori H, Katsurada A, Miyata K, Ohashi N, Satou R, Saito T, Hagiwara Y, Miyashita K, Navar LG: **Determination of plasma and urinary angiotensinogen levels in rodents by newly developed ELISA**. *Am J Physiol Renal Physiol* 2008, **294**(5):F1257-1263.
145. Coelho MS, Lopes KL, Freitas Rde A, de Oliveira-Sales EB, Bergasmaschi CT, Campos RR, Casarini DE, Carmona AK, Araujo Mda S, Heimann JC *et al*: **High sucrose intake in rats is associated with increased ACE2 and angiotensin-(1-7) levels in the adipose tissue**. *Regul Pept* 2010, **162**(1-3):61-67.
146. Dobrian AD, Davies MJ, Prewitt RL, Lauterio TJ: **Development of hypertension in a rat model of diet-induced obesity**. *Hypertension* 2000, **35**(4):1009-1015.

147. Granger JP, West D, Scott J: **Abnormal pressure natriuresis in the dog model of obesity-induced hypertension**. *Hypertension* 1994, **23**(1 Suppl):I8-11.
148. Harker CT, O'Donnell MP, Kasiske BL, Keane WF, Katz SA: **The renin-angiotensin system in the type II diabetic obese Zucker rat**. *J Am Soc Nephrol* 1993, **4**(6):1354-1361.
149. Alonso-Galicia M, Brands MW, Zappe DH, Hall JE: **Hypertension in obese Zucker rats. Role of angiotensin II and adrenergic activity**. *Hypertension* 1996, **28**(6):1047-1054.
150. Frederich RC, Jr., Kahn BB, Peach MJ, Flier JS: **Tissue-specific nutritional regulation of angiotensinogen in adipose tissue**. *Hypertension* 1992, **19**(4):339-344.
151. Hainault I, Nebout G, Turban S, Ardouin B, Ferre P, Quignard-Boulange A: **Adipose tissue-specific increase in angiotensinogen expression and secretion in the obese (fa/fa) Zucker rat**. *Am J Physiol Endocrinol Metab* 2002, **282**(1):E59-66.
152. Rahmouni K, Mark AL, Haynes WG, Sigmund CD: **Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity**. *Am J Physiol Endocrinol Metab* 2004, **286**(6):E891-895.
153. Juan CC, Au LC, Fang VS, Kang SF, Ko YH, Kuo SF, Hsu YP, Kwok CF, Ho LT: **Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids**. *Biochem Biophys Res Commun* 2001, **289**(5):1328-1333.
154. Giacchetti G, Sechi LA, Griffin CA, Don BR, Mantero F, Schambelan M: **The tissue renin-angiotensin system in rats with fructose-induced hypertension: overexpression of type 1 angiotensin II receptor in adipose tissue**. *J Hypertens* 2000, **18**(6):695-702.
155. Tamura K, Umemura S, Yamakawa T, Nyui N, Hibi K, Watanabe Y, Ishigami T, Yabana M, Tanaka S, Sekihara H *et al*: **Modulation of tissue angiotensinogen gene expression in genetically obese hypertensive rats**. *Am J Physiol* 1997, **272**(6 Pt 2):R1704-1711.
156. Cassis LA, Fettinger MJ, Roe AL, Shenoy UR, Howard G: **Characterization and regulation of angiotensin II receptors in rat adipose tissue. Angiotensin receptors in adipose tissue**. *Adv Exp Med Biol* 1996, **396**:39-47.
157. Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, Quignard-Boulange A, Negrel R, Ailhaud G, Seydoux J *et al*: **Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation**. *Faseb J* 2001, **15**(14):2727-2729.
158. Cassis L, Helton M, English V, Burke G: **Angiotensin II regulates oxygen consumption**. *Am J Physiol Regul Integr Comp Physiol* 2002, **282**(2):R445-453.
159. Brink M, Wellen J, Delafontaine P: **Angiotensin II causes weight loss and decreases circulating insulin-like growth factor I in rats through a pressor-independent mechanism**. *J Clin Invest* 1996, **97**(11):2509-2516.
160. Mathai ML, Naik S, Sinclair AJ, Weisinger HS, Weisinger RS: **Selective reduction in body fat mass and plasma leptin induced by angiotensin-**

converting enzyme inhibition in rats. *Int J Obes (Lond)* 2008, **32**(10):1576-1584.

161. Lee MH, Song HK, Ko GJ, Kang YS, Han SY, Han KH, Kim HK, Han JY, Cha DR: **Angiotensin receptor blockers improve insulin resistance in type 2 diabetic rats by modulating adipose tissue**. *Kidney Int* 2008, **74**(7):890-900.
162. Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, Botion LM, Bader M, Alenina N, Santos RA: **Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism**. *Diabetes* 2008, **57**(2):340-347.
163. Buchanan TA, Thawani H, Kades W, Modrall JG, Weaver FA, Laurel C, Poppiti R, Xiang A, Hsueh W: **Angiotensin II increases glucose utilization during acute hyperinsulinemia via a hemodynamic mechanism**. *J Clin Invest* 1993, **92**(2):720-726.
164. Fliser D, Arnold U, Kohl B, Hartung R, Ritz E: **Angiotensin II enhances insulin sensitivity in healthy volunteers under euglycemic conditions**. *J Hypertens* 1993, **11**(9):983-988.
165. Morris AD, Petrie JR, Ueda S, Connell JM, Elliott HL, Small M, Donnelly R: **Pressor and subpressor doses of angiotensin II increase insulin sensitivity in NIDDM. Dissociation of metabolic and blood pressure effects**. *Diabetes* 1994, **43**(12):1445-1449.
166. Jonk AM, Houben AJ, Schaper NC, de Leeuw PW, Serne EH, Smulders YM, Stehouwer CD: **Angiotensin II enhances insulin-stimulated whole-body glucose disposal but impairs insulin-induced capillary recruitment in healthy volunteers**. *J Clin Endocrinol Metab* 2010, **95**(8):3901-3908.
167. Ogihara T, Asano T, Ando K, Chiba Y, Sakoda H, Anai M, Shojima N, Ono H, Onishi Y, Fujishiro M *et al*: **Angiotensin II-induced insulin resistance is associated with enhanced insulin signaling**. *Hypertension* 2002, **40**(6):872-879.
168. Ran J, Hirano T, Adachi M: **Chronic ANG II infusion increases plasma triglyceride level by stimulating hepatic triglyceride production in rats**. *Am J Physiol Endocrinol Metab* 2004, **287**(5):E955-961.
169. Cassis LA, Marshall DE, Fettinger MJ, Rosenbluth B, Lodder RA: **Mechanisms contributing to angiotensin II regulation of body weight**. *Am J Physiol* 1998, **274**(5 Pt 1):E867-876.
170. Sloniger JA, Saengsirisuwan V, Diehl CJ, Dokken BB, Lailerd N, Lemieux AM, Kim JS, Henriksen EJ: **Defective insulin signaling in skeletal muscle of the hypertensive TG(mREN2)27 rat**. *Am J Physiol Endocrinol Metab* 2005, **288**(6):E1074-1081.
171. Soltani-Bejnood M, Fletcher S, Morris J, Das S, Voy BH, Moustaid-Moussa N: **Overexpression of renin in the liver impairs glucose tolerance**. *FASEB J* 2007, **21**(6):A831-.
172. Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA, Gironacci MM, Turyn D, Dominici FP: **Chronic infusion of angiotensin-(1-7) improves insulin resistance and hypertension induced by a high-fructose diet in rats**. *Am J Physiol Endocrinol Metab* 2009, **296**(2):E262-271.

173. Takahashi N, Li F, Hua K, Deng J, Wang CH, Bowers RR, Bartness TJ, Kim HS, Harp JB: **Increased energy expenditure, dietary fat wasting, and resistance to diet-induced obesity in mice lacking renin**. *Cell Metab* 2007, **6**(6):506-512.
174. Massiera F, Seydoux J, Geloen A, Quignard-Boulange A, Turban S, Saint-Marc P, Fukamizu A, Negrel R, Ailhaud G, Teboul M: **Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity**. *Endocrinology* 2001, **142**(12):5220-5225.
175. Jayasooriya AP, Mathai ML, Walker LL, Begg DP, Denton DA, Cameron-Smith D, Egan GF, McKinley MJ, Rodger PD, Sinclair AJ *et al*: **Mice lacking angiotensin-converting enzyme have increased energy expenditure, with reduced fat mass and improved glucose clearance**. *Proc Natl Acad Sci U S A* 2008, **105**(18):6531-6536.
176. Kouyama R, Suganami T, Nishida J, Tanaka M, Toyoda T, Kiso M, Chiwata T, Miyamoto Y, Yoshimasa Y, Fukamizu A *et al*: **Attenuation of diet-induced weight gain and adiposity through increased energy expenditure in mice lacking angiotensin II type 1a receptor**. *Endocrinology* 2005, **146**(8):3481-3489.
177. Tomono Y, Iwai M, Inaba S, Mogi M, Horiuchi M: **Blockade of AT1 receptor improves adipocyte differentiation in atherosclerotic and diabetic models**. *Am J Hypertens* 2008, **21**(2):206-212.
178. Yvan-Charvet L, Even P, Bloch-Faure M, Guerre-Millo M, Moustaid-Moussa N, Ferre P, Quignard-Boulange A: **Deletion of the angiotensin type 2 receptor (AT2R) reduces adipose cell size and protects from diet-induced obesity and insulin resistance**. *Diabetes* 2005, **54**(4):991-999.
179. Yvan-Charvet L, Massiera F, Lamande N, Ailhaud G, Teboul M, Moustaid-Moussa N, Gasc JM, Quignard-Boulange A: **Deficiency of angiotensin type 2 receptor rescues obesity but not hypertension induced by overexpression of angiotensinogen in adipose tissue**. *Endocrinology* 2009, **150**(3):1421-1428.
180. Bonnet F, Patel S, Laville M, Balkau B, Favuzzi A, Monti LD, Lalic N, Walker M: **Influence of the ACE gene insertion/deletion polymorphism on insulin sensitivity and impaired glucose tolerance in healthy subjects**. *Diabetes Care* 2008, **31**(4):789-794.
181. Cong ND, Hamaguchi K, Saikawa T, Hara M, Sakata T: **The I/D polymorphism of angiotensin-converting enzyme gene but not the angiotensinogen gene is associated with insulin response to oral glucose in Japanese**. *Proc Soc Exp Biol Med* 1999, **220**(1):46-51.
182. Han T, Wang X, Cui Y, Ye H, Tong X, Piao M: **Relationship between angiotensin-converting enzyme gene insertion or deletion polymorphism and insulin sensitivity in healthy newborns**. *Pediatrics* 2007, **119**(6):1089-1094.
183. Pollex RL, Hanley AJ, Zinman B, Harris SB, Khan HM, Hegele RA: **Metabolic syndrome in aboriginal Canadians: prevalence and genetic associations**. *Atherosclerosis* 2006, **184**(1):121-129.

184. Schlemm L, Haumann HM, Ziegner M, Stirnberg B, Sohn A, Alter M, Pfab T, Kalache KD, Guthmann F, Hocher B: **New evidence for the fetal insulin hypothesis: fetal angiotensinogen M235T polymorphism is associated with birth weight and elevated fetal total glycated hemoglobin at birth**. *J Hypertens* 2010, **28**(4):732-739.
185. Miyanaga K, Fukuo K, Akasaka H, Katsuya T, Fukada R, Rakugi H, Kazumi T: **C allele of angiotensin II type 1 receptor gene A1166C polymorphism affects plasma adiponectin concentrations in healthy young Japanese women**. *Hypertens Res* 2009, **32**(10):901-905.
186. Mehri S, Koubaa N, Hammami S, Mahjoub S, Chaaba R, Nakbi A, Zouari B, Abid M, Ben Arab S, Baudin B *et al*: **Genotypic interactions of renin-angiotensin system genes with diabetes type 2 in a Tunisian population**. *Life Sci* 2010, **87**(1-2):49-54.
187. Yusuf S, Gerstein H, Hoogwerf B, Pogue J, Bosch J, Wolffenbuttel BH, Zinman B: **Ramipril and the development of diabetes**. *JAMA* 2001, **286**(15):1882-1885.
188. Henriksen EJ, Jacob S, Kinnick TR, Teachey MK, Krekler M: **Selective angiotensin II receptor receptor antagonism reduces insulin resistance in obese Zucker rats**. *Hypertension* 2001, **38**(4):884-890.
189. Iwai M, Kanno H, Tomono Y, Inaba S, Senba I, Furuno M, Mogi M, Horiuchi M: **Direct renin inhibition improved insulin resistance and adipose tissue dysfunction in type 2 diabetic KK-A(y) mice**. *J Hypertens* 2010, **28**(7):1471-1481.
190. Juan CC, Chien Y, Wu LY, Yang WM, Chang CL, Lai YH, Ho PH, Kwok CF, Ho LT: **Angiotensin II enhances insulin sensitivity in vitro and in vivo**. *Endocrinology* 2005, **146**(5):2246-2254.
191. Lastra G, Habibi J, Whaley-Connell AT, Manrique C, Hayden MR, Rehmer J, Patel K, Ferrario C, Sowers JR: **Direct renin inhibition improves systemic insulin resistance and skeletal muscle glucose transport in a transgenic rodent model of tissue renin overexpression**. *Endocrinology* 2009, **150**(6):2561-2568.
192. Sloniger JA, Saengsirisuwan V, Diehl CJ, Kim JS, Henriksen EJ: **Selective angiotensin II receptor antagonism enhances whole-body insulin sensitivity and muscle glucose transport in hypertensive TG(mREN2)27 rats**. *Metabolism* 2005, **54**(12):1659-1668.
193. Wasserman DH: **Four grams of glucose**. *Am J Physiol Endocrinol Metab* 2009, **296**(1):E11-21.
194. Richey JM, Ader M, Moore D, Bergman RN: **Angiotensin II induces insulin resistance independent of changes in interstitial insulin**. *Am J Physiol* 1999, **277**(5 Pt 1):E920-926.
195. Wei Y, Sowers JR, Nistala R, Gong H, Uptergrove GM, Clark SE, Morris EM, Szary N, Manrique C, Stump CS: **Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells**. *J Biol Chem* 2006, **281**(46):35137-35146.

196. Wei Y, Sowers JR, Clark SE, Li W, Ferrario CM, Stump CS: **Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase**. *Am J Physiol Endocrinol Metab* 2008, **294**(2):E345-351.
197. Henriksen EJ: **Improvement of insulin sensitivity by antagonism of the renin-angiotensin system**. *Am J Physiol Regul Integr Comp Physiol* 2007, **293**(3):R974-980.
198. Marrero MB, Fulton D, Stepp D, Stern DM: **Angiotensin II-induced insulin resistance and protein tyrosine phosphatases**. *Arterioscler Thromb Vasc Biol* 2004, **24**(11):2009-2013.
199. Calegari VC, Alves M, Picardi PK, Inoue RY, Franchini KG, Saad MJ, Velloso LA: **Suppressor of cytokine signaling-3 Provides a novel interface in the cross-talk between angiotensin II and insulin signaling systems**. *Endocrinology* 2005, **146**(2):579-588.
200. Mitsuishi M, Miyashita K, Muraki A, Itoh H: **Angiotensin II reduces mitochondrial content in skeletal muscle and affects glycemic control**. *Diabetes* 2009, **58**(3):710-717.
201. Henriksen EJ, Jacob S: **Angiotensin converting enzyme inhibitors and modulation of skeletal muscle insulin resistance**. *Diabetes Obes Metab* 2003, **5**(4):214-222.
202. Rao RH: **Pressor doses of angiotensin II increase hepatic glucose output and decrease insulin sensitivity in rats**. *J Endocrinol* 1996, **148**(2):311-318.
203. Toblli JE, Munoz MC, Cao G, Mella J, Pereyra L, Mastai R: **ACE inhibition and AT1 receptor blockade prevent fatty liver and fibrosis in obese Zucker rats**. *Obesity (Silver Spring)* 2008, **16**(4):770-776.
204. Lau T, Carlsson PO, Leung PS: **Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets**. *Diabetologia* 2004, **47**(2):240-248.
205. Bindom SM, Lazartigues E: **The sweeter side of ACE2: physiological evidence for a role in diabetes**. *Mol Cell Endocrinol* 2009, **302**(2):193-202.
206. Bindom SM, Hans CP, Xia H, Boulares AH, Lazartigues E: **Angiotensin I-converting enzyme type 2 (ACE2) gene therapy improves glycemic control in diabetic mice**. *Diabetes* 2010, **59**(10):2540-2548.
207. Kim S, Voy BH, Huang T, Koontz J, Quignard-Boulange A, Hayser P, Harp J, Moustaid-Moussa N: **Angiotensin II regulates adipocyte metabolism via insulin signaling molecules**. *Adipocytes* 2005, **1**(4):239-248.
208. Harte A, McTernan P, Chetty R, Coppack S, Katz J, Smith S, Kumar S: **Insulin-mediated upregulation of the renin angiotensin system in human subcutaneous adipocytes is reduced by rosiglitazone**. *Circulation* 2005, **111**(15):1954-1961.
209. Ran J, Hirano T, Fukui T, Saito K, Kageyama H, Okada K, Adachi M: **Angiotensin II infusion decreases plasma adiponectin level via its type 1 receptor in rats: an implication for hypertension-related insulin resistance**. *Metabolism* 2006, **55**(4):478-488.

210. Serazin-Leroy V, Morot M, de Mazancourt P, Giudicelli Y: **Androgen regulation and site specificity of angiotensinogen gene expression and secretion in rat adipocytes**. *Am J Physiol Endocrinol Metab* 2000, **279**(6):E1398-1405.
211. Chen X, Zhang SL, Pang L, Filep JG, Tang SS, Ingelfinger JR, Chan JS: **Characterization of a putative insulin-responsive element and its binding protein(s) in rat angiotensinogen gene promoter: regulation by glucose and insulin**. *Endocrinology* 2001, **142**(6):2577-2585.
212. Brasier AR, Li J: **Mechanisms for inducible control of angiotensinogen gene transcription**. *Hypertension* 1996, **27**(3 Pt 2):465-475.
213. Gabriely I, Yang XM, Cases JA, Ma XH, Rossetti L, Barzilai N: **Hyperglycemia modulates angiotensinogen gene expression**. *Am J Physiol Regul Integr Comp Physiol* 2001, **281**(3):R795-802.
214. Einstein FH, Fishman S, Bauman J, Thompson RF, Huffman DM, Atzmon G, Barzilai N, Muzumdar RH: **Enhanced activation of a "nutrient-sensing" pathway with age contributes to insulin resistance**. *Faseb J* 2008, **22**(10):3450-3457.
215. Goossens GH, Blaak EE, Saris WH, van Baak MA: **Angiotensin II-induced effects on adipose and skeletal muscle tissue blood flow and lipolysis in normal-weight and obese subjects**. *J Clin Endocrinol Metab* 2004, **89**(6):2690-2696.
216. Jones BH, Standridge MK, Moustaid N: **Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells**. *Endocrinology* 1997, **138**(4):1512-1519.
217. Danforth E, Jr.: **Failure of adipocyte differentiation causes type II diabetes mellitus?** *Nat Genet* 2000, **26**(1):13.
218. Furuhashi M, Ura N, Takizawa H, Yoshida D, Moniwa N, Murakami H, Higashiura K, Shimamoto K: **Blockade of the renin-angiotensin system decreases adipocyte size with improvement in insulin sensitivity**. *J Hypertens* 2004, **22**(10):1977-1982.
219. Munoz MC, Giani JF, Dominici FP, Turyn D, Toblli JE: **Long-term treatment with an angiotensin II receptor blocker decreases adipocyte size and improves insulin signaling in obese Zucker rats**. *J Hypertens* 2009, **27**(12):2409-2420.
220. Zorad S, Dou JT, Benicky J, Hutanu D, Tybitanclova K, Zhou J, Saavedra JM: **Long-term angiotensin II AT1 receptor inhibition produces adipose tissue hypotrophy accompanied by increased expression of adiponectin and PPARgamma**. *Eur J Pharmacol* 2006, **552**(1-3):112-122.
221. Schupp M, Janke J, Clasen R, Unger T, Kintscher U: **Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity**. *Circulation* 2004, **109**(17):2054-2057.
222. Schupp M, Lee LD, Frost N, Umbreen S, Schmidt B, Unger T, Kintscher U: **Regulation of peroxisome proliferator-activated receptor gamma activity by losartan metabolites**. *Hypertension* 2006, **47**(3):586-589.

223. Darimont C, Vassaux G, Ailhaud G, Negrel R: **Differentiation of preadipose cells: paracrine role of prostacyclin upon stimulation of adipose cells by angiotensin-II**. *Endocrinology* 1994, **135**(5):2030-2036.
224. Matsushita K, Wu Y, Okamoto Y, Pratt RE, Dzau VJ: **Local renin angiotensin expression regulates human mesenchymal stem cell differentiation to adipocytes**. *Hypertension* 2006, **48**(6):1095-1102.
225. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM: **Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors**. *Diabetes* 2002, **51**(6):1699-1707.
226. Brucher R, Cifuentes M, Acuna MJ, Albala C, Rojas CV: **Larger anti-adipogenic effect of angiotensin II on omental preadipose cells of obese humans**. *Obesity (Silver Spring)* 2007, **15**(7):1643-1646.
227. Fuentes P, Acuna MJ, Cifuentes M, Rojas CV: **The anti-adipogenic effect of angiotensin II on human preadipose cells involves ERK1,2 activation and PPARG phosphorylation**. *J Endocrinol* 2010, **206**(1):75-83.
228. Saiki A, Koide N, Watanabe F, Murano T, Miyashita Y, Shirai K: **Suppression of lipoprotein lipase expression in 3T3-L1 cells by inhibition of adipogenic differentiation through activation of the renin-angiotensin system**. *Metabolism* 2008, **57**(8):1093-1100.
229. Mehta PK, Griendling KK: **Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system**. *Am J Physiol Cell Physiol* 2007, **292**(1):C82-97.
230. Skurk T, van Harmelen V, Hauner H: **Angiotensin II stimulates the release of interleukin-6 and interleukin-8 from cultured human adipocytes by activation of NF-kappaB**. *Arterioscler Thromb Vasc Biol* 2004, **24**(7):1199-1203.
231. Asamizu S, Urakaze M, Kobashi C, Ishiki M, Norel Din AK, Fujisaka S, Kanatani Y, Bukahari A, Senda S, Suzuki H *et al*: **Angiotensin II enhances the increase in monocyte chemoattractant protein-1 production induced by tumor necrosis factor-{alpha} from 3T3-L1 preadipocytes**. *J Endocrinol* 2009, **202**(2):199-205.
232. Wolf AM, Woodworth KA: **Obesity prevention: recommended strategies and challenges**. *Am J Med* 2009, **122**(4 Suppl 1):S19-23.
233. Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD: **Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer**. *Am J Med* 2002, **113 Suppl 9B**:71S-88S.
234. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM: **Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin**. *N Engl J Med* 2002, **346**(6):393-403.
235. Shai I, Schwarzfuchs D, Henkin Y, Shahar DR, Witkow S, Greenberg I, Golan R, Fraser D, Bolotin A, Vardi H *et al*: **Weight loss with a low-carbohydrate, Mediterranean, or low-fat diet**. *N Engl J Med* 2008, **359**(3):229-241.

236. Jazet IM, Schaart G, Gastaldelli A, Ferrannini E, Hesselink MK, Schrauwen P, Romijn JA, Maassen JA, Pijl H, Ouwens DM *et al*: **Loss of 50% of excess weight using a very low energy diet improves insulin-stimulated glucose disposal and skeletal muscle insulin signalling in obese insulin-treated type 2 diabetic patients**. *Diabetologia* 2008, **51**(2):309-319.
237. Weiss EP, Holloszy JO: **Improvements in body composition, glucose tolerance, and insulin action induced by increasing energy expenditure or decreasing energy intake**. *The Journal of nutrition* 2007, **137**(4):1087-1090.
238. Rector RS, Warner SO, Liu Y, Hinton PS, Sun GY, Cox RH, Stump CS, Laughlin MH, Dellsperger KC, Thomas TR: **Exercise and diet induced weight loss improves measures of oxidative stress and insulin sensitivity in adults with characteristics of the metabolic syndrome**. *Am J Physiol Endocrinol Metab* 2007, **293**(2):E500-506.
239. Isbell JM, Tamboli RA, Hansen EN, Saliba J, Dunn JP, Phillips SE, Marks-Shulman PA, Abumrad NN: **The importance of caloric restriction in the early improvements in insulin sensitivity after Roux-en-Y gastric bypass surgery**. *Diabetes Care* 2010, **33**(7):1438-1442.
240. Rezzi S, Martin FP, Shanmuganayagam D, Colman RJ, Nicholson JK, Weindruch R: **Metabolic shifts due to long-term caloric restriction revealed in nonhuman primates**. *Exp Gerontol* 2009, **44**(5):356-362.
241. Viljanen AP, Lautamaki R, Jarvisalo M, Parkkola R, Huupponen R, Lehtimaki T, Ronnemaa T, Raitakari OT, Iozzo P, Nuutila P: **Effects of weight loss on visceral and abdominal subcutaneous adipose tissue blood-flow and insulin-mediated glucose uptake in healthy obese subjects**. *Ann Med* 2009, **41**(2):152-160.
242. Mitterberger MC, Mattesich M, Klaver E, Lechner S, Engelhardt T, Larcher L, Pierer G, Piza-Katzer H, Zwerschke W: **Adipokine profile and insulin sensitivity in formerly obese women subjected to bariatric surgery or diet-induced long-term caloric restriction**. *J Gerontol A Biol Sci Med Sci* 2010, **65**(9):915-923.
243. Harvie MN, Pegington M, Mattson MP, Frystyk J, Dillon B, Evans G, Cuzick J, Jebb SA, Martin B, Cutler RG *et al*: **The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women**. *Int J Obes (Lond)* 2010.
244. Sheu WH, Chang TM, Lee WJ, Ou HC, Wu CM, Tseng LN, Lang HF, Wu CS, Wan CJ, Lee IT: **Effect of weight loss on proinflammatory state of mononuclear cells in obese women**. *Obesity (Silver Spring)* 2008, **16**(5):1033-1038.
245. Anderlova K, Kremen J, Dolezalova R, Housova J, Haluzikova D, Kunesova M, Haluzik M: **The influence of very-low-calorie-diet on serum leptin, soluble leptin receptor, adiponectin and resistin levels in obese women**. *Physiol Res* 2006, **55**(3):277-283.
246. Arvidsson E, Viguerie N, Andersson I, Verdich C, Langin D, Arner P: **Effects of different hypocaloric diets on protein secretion from adipose tissue of obese women**. *Diabetes* 2004, **53**(8):1966-1971.

247. Polak J, Kovacova Z, Jacek M, Klimcakova E, Kovacikova M, Vitkova M, Kuda O, Sebela M, Samcova E, Stich V: **An increase in plasma adiponectin multimeric complexes follows hypocaloric diet-induced weight loss in obese and overweight pre-menopausal women**. *Clin Sci (Lond)* 2007, **112**(11):557-565.
248. Christiansen T, Paulsen SK, Bruun JM, Pedersen SB, Richelsen B: **Exercise training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects: a 12-week randomized intervention study**. *Am J Physiol Endocrinol Metab* 2010, **298**(4):E824-831.
249. Esposito K, Pontillo A, Di Palo C, Giugliano G, Masella M, Marfella R, Giugliano D: **Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial**. *JAMA* 2003, **289**(14):1799-1804.
250. Madsen EL, Rissanen A, Bruun JM, Skogstrand K, Tonstad S, Hougaard DM, Richelsen B: **Weight loss larger than 10% is needed for general improvement of levels of circulating adiponectin and markers of inflammation in obese subjects: a 3-year weight loss study**. *Eur J Endocrinol* 2008, **158**(2):179-187.
251. Kovacova Z, Vitkova M, Kovacikova M, Klimcakova E, Bajzova M, Hnevkovska Z, Rossmeislova L, Stich V, Langin D, Polak J: **Secretion of adiponectin multimeric complexes from adipose tissue explants is not modified by very low calorie diet**. *Eur J Endocrinol* 2009, **160**(4):585-592.
252. Polak J, Kovacova Z, Holst C, Verdich C, Astrup A, Blaak E, Patel K, Oppert JM, Langin D, Martinez JA *et al*: **Total adiponectin and adiponectin multimeric complexes in relation to weight loss-induced improvements in insulin sensitivity in obese women: the NUGENOB study**. *Eur J Endocrinol* 2008, **158**(4):533-541.
253. Abbasi F, Chang SA, Chu JW, Ciaraldi TP, Lamendola C, McLaughlin T, Reaven GM, Reaven PD: **Improvements in insulin resistance with weight loss, in contrast to rosiglitazone, are not associated with changes in plasma adiponectin or adiponectin multimeric complexes**. *Am J Physiol Regul Integr Comp Physiol* 2006, **290**(1):R139-144.
254. Garaulet M, Viguerie N, Porubsky S, Klimcakova E, Clement K, Langin D, Stich V: **Adiponectin gene expression and plasma values in obese women during very-low-calorie diet. Relationship with cardiovascular risk factors and insulin resistance**. *J Clin Endocrinol Metab* 2004, **89**(2):756-760.
255. Bruun JM, Verdich C, Toubro S, Astrup A, Richelsen B: **Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men**. *Eur J Endocrinol* 2003, **148**(5):535-542.
256. Salas-Salvado J, Bullo M, Garcia-Lorda P, Figueredo R, Del Castillo D, Bonada A, Balanza R: **Subcutaneous adipose tissue cytokine production is not responsible for the restoration of systemic inflammation markers during weight loss**. *Int J Obes (Lond)* 2006, **30**(12):1714-1720.

257. Ryan AS, Nicklas BJ: **Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women**. *Diabetes Care* 2004, **27**(7):1699-1705.
258. Vitkova M, Klimcakova E, Kovacikova M, Valle C, Moro C, Polak J, Hanacek J, Capel F, Viguerie N, Richterova B *et al*: **Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity**. *J Clin Endocrinol Metab* 2007, **92**(6):2330-2335.
259. Agueda M, Lasa A, Simon E, Ares R, Larrarte E, Labayen I: **Association of circulating visfatin concentrations with insulin resistance and low-grade inflammation after dietary energy restriction in Spanish obese non-diabetic women: Role of body composition changes**. *Nutr Metab Cardiovasc Dis* 2010.
260. Lasa A, Churruca I, Simon E, Macarulla MT, Fernandez-Quintela A, Rodriguez VM, Portillo MP: **Effects of high-fat high-sucrose feeding, energy restriction, and trans-10,cis-12 conjugated linoleic acid on visfatin and apelin in hamsters**. *J Am Coll Nutr* 2009, **28**(6):627-635.
261. Chiba T, Ezaki O: **Dietary restriction suppresses inflammation and delays the onset of stroke in stroke-prone spontaneously hypertensive rats**. *Biochem Biophys Res Commun* 2010, **399**(1):98-103.
262. Clifton PM, Keogh JB, Foster PR, Noakes M: **Effect of weight loss on inflammatory and endothelial markers and FMD using two low-fat diets**. *Int J Obes (Lond)* 2005, **29**(12):1445-1451.
263. Solomon TP, Sistrun SN, Krishnan RK, Del Aguila LF, Marchetti CM, O'Carroll SM, O'Leary VB, Kirwan JP: **Exercise and diet enhance fat oxidation and reduce insulin resistance in older obese adults**. *J Appl Physiol* 2008, **104**(5):1313-1319.
264. Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S, Smith SR, Alfonso A, Ravussin E: **Effect of calorie restriction with or without exercise on insulin sensitivity, beta-cell function, fat cell size, and ectopic lipid in overweight subjects**. *Diabetes Care* 2006, **29**(6):1337-1344.
265. Varady KA, Tussing L, Bhutani S, Braunschweig CL: **Degree of weight loss required to improve adipokine concentrations and decrease fat cell size in severely obese women**. *Metabolism* 2009, **58**(8):1096-1101.
266. Hellstrom L, Reynisdottir S, Langin D, Rossner S, Arner P: **Regulation of lipolysis in fat cells of obese women during long-term hypocaloric diet**. *Int J Obes Relat Metab Disord* 1996, **20**(8):745-752.
267. Stich V, Marion-Latard F, Hejnova J, Viguerie N, Lefort C, Suljkovicova H, Langin D, Lafontan M, Berlan M: **Hypocaloric diet reduces exercise-induced alpha 2-adrenergic antilipolytic effect and alpha 2-adrenergic receptor mRNA levels in adipose tissue of obese women**. *J Clin Endocrinol Metab* 2002, **87**(3):1274-1281.

268. Franck N, Gummesson A, Jernas M, Glad C, Svensson PA, Guillot G, Rudemo M, Nystrom FH, Carlsson LM, Olsson B: **Identification of adipocyte genes regulated by caloric intake**. *J Clin Endocrinol Metab* 2011, **96**(2):E413-418.
269. Valle A, Sastre-Serra J, Roca P, Oliver J: **Modulation of white adipose tissue proteome by aging and calorie restriction**. *Aging Cell* 2010, **9**(5):882-894.
270. Mangravite LM, Dawson K, Davis RR, Gregg JP, Krauss RM: **Fatty acid desaturase regulation in adipose tissue by dietary composition is independent of weight loss and is correlated with the plasma triacylglycerol response**. *Am J Clin Nutr* 2007, **86**(3):759-767.
271. Higami Y, Pugh TD, Page GP, Allison DB, Prolla TA, Weindruch R: **Adipose tissue energy metabolism: altered gene expression profile of mice subjected to long-term caloric restriction**. *Faseb J* 2004, **18**(2):415-417.
272. Zhu M, Lee GD, Ding L, Hu J, Qiu G, de Cabo R, Bernier M, Ingram DK, Zou S: **Adipogenic signaling in rat white adipose tissue: modulation by aging and calorie restriction**. *Exp Gerontol* 2007, **42**(8):733-744.
273. Higami Y, Barger JL, Page GP, Allison DB, Smith SR, Prolla TA, Weindruch R: **Energy restriction lowers the expression of genes linked to inflammation, the cytoskeleton, the extracellular matrix, and angiogenesis in mouse adipose tissue**. *The Journal of nutrition* 2006, **136**(2):343-352.
274. Clement K, Viguerie N, Poitou C, Carette C, Pelloux V, Curat CA, Sicard A, Rome S, Benis A, Zucker JD *et al*: **Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects**. *Faseb J* 2004, **18**(14):1657-1669.
275. Vieira VJ, Valentine RJ, Wilund KR, Antao N, Baynard T, Woods JA: **Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice**. *Am J Physiol Endocrinol Metab* 2009, **296**(5):E1164-1171.
276. Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, Ferrante AW, Jr.: **Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue**. *J Clin Invest* 2010, **120**(10):3466-3479.
277. Capel F, Klimcakova E, Viguerie N, Roussel B, Vitkova M, Kovacikova M, Polak J, Kovacova Z, Galitzky J, Maoret JJ *et al*: **Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization**. *Diabetes* 2009, **58**(7):1558-1567.
278. Tsutsumi A, Motoshima H, Kondo T, Kawasaki S, Matsumura T, Hanatani S, Igata M, Ishii N, Kinoshita H, Kawashima J *et al*: **Caloric restriction decreases ER stress in liver and adipose tissue in ob/ob mice**. *Biochem Biophys Res Commun* 2011, **404**(1):339-344.
279. Davidson RT, Arias EB, Cartee GD: **Calorie restriction increases muscle insulin action but not IRS-1-, IRS-2-, or phosphotyrosine-PI 3-kinase**. *Am J Physiol Endocrinol Metab* 2002, **282**(2):E270-276.

280. Wang ZQ, Floyd ZE, Qin J, Liu X, Yu Y, Zhang XH, Wagner JD, Cefalu WT: **Modulation of skeletal muscle insulin signaling with chronic caloric restriction in cynomolgus monkeys**. *Diabetes* 2009, **58**(7):1488-1498.
281. McCurdy CE, Cartee GD: **Akt2 is essential for the full effect of calorie restriction on insulin-stimulated glucose uptake in skeletal muscle**. *Diabetes* 2005, **54**(5):1349-1356.
282. McCurdy CE, Davidson RT, Cartee GD: **Brief calorie restriction increases Akt2 phosphorylation in insulin-stimulated rat skeletal muscle**. *Am J Physiol Endocrinol Metab* 2003, **285**(4):E693-700.
283. Tucker MZ, Turcotte LP: **Brief food restriction increases FA oxidation and glycogen synthesis under insulin-stimulated conditions**. *Am J Physiol Regul Integr Comp Physiol* 2002, **282**(4):R1210-1218.
284. Toledo FG, Watkins S, Kelley DE: **Changes induced by physical activity and weight loss in the morphology of intermyofibrillar mitochondria in obese men and women**. *J Clin Endocrinol Metab* 2006, **91**(8):3224-3227.
285. Kelley DE, Goodpaster BH: **Skeletal muscle triglyceride. An aspect of regional adiposity and insulin resistance**. *Diabetes Care* 2001, **24**(5):933-941.
286. Haugaard SB, Vaag A, Hoy CE, Madsbad S: **Desaturation of skeletal muscle structural and depot lipids in obese individuals during a very-low-calorie diet intervention**. *Obesity (Silver Spring)* 2007, **15**(1):117-125.
287. Haugaard SB, Madsbad S, Hoy CE, Vaag A: **Dietary intervention increases n-3 long-chain polyunsaturated fatty acids in skeletal muscle membrane phospholipids of obese subjects. Implications for insulin sensitivity**. *Clin Endocrinol (Oxf)* 2006, **64**(2):169-178.
288. Liu X, Liu M, Zhang J, Bai X, Ramos F, Van Remmen H, Richardson A, Liu FY, Dong LQ, Liu F: **Downregulation of Grb2 contributes to the insulin-sensitizing effect of calorie restriction**. *Am J Physiol Endocrinol Metab* 2009, **296**(5):E1067-1075.
289. Viljanen AP, Iozzo P, Borra R, Kankaanpaa M, Karmi A, Lautamaki R, Jarvisalo M, Parkkola R, Ronnemaa T, Guiducci L *et al*: **Effect of weight loss on liver free fatty acid uptake and hepatic insulin resistance**. *J Clin Endocrinol Metab* 2009, **94**(1):50-55.
290. Catalano KJ, Stefanovski D, Bergman RN: **Critical role of the mesenteric depot versus other intra-abdominal adipose depots in the development of insulin resistance in young rats**. *Diabetes* 2010, **59**(6):1416-1423.
291. Larson-Meyer DE, Newcomer BR, Heilbronn LK, Volaufova J, Smith SR, Alfonso AJ, Lefevre M, Rood JC, Williamson DA, Ravussin E: **Effect of 6-month calorie restriction and exercise on serum and liver lipids and markers of liver function**. *Obesity (Silver Spring)* 2008, **16**(6):1355-1362.
292. Barzilai N, Banerjee S, Hawkins M, Chen W, Rossetti L: **Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat**. *J Clin Invest* 1998, **101**(7):1353-1361.
293. Zheng Y, Zhang W, Pendleton E, Leng S, Wu J, Chen R, Sun XJ: **Improved insulin sensitivity by calorie restriction is associated with reduction of ERK**

and p70S6K activities in the liver of obese Zucker rats. *J Endocrinol* 2009, **203**(3):337-347.

294. Kelly KR, Brooks LM, Solomon TP, Kashyap SR, O'Leary VB, Kirwan JP: **The glucose-dependent insulinotropic polypeptide and glucose-stimulated insulin response to exercise training and diet in obesity**. *Am J Physiol Endocrinol Metab* 2009, **296**(6):E1269-1274.
295. Escalante-Pulido M, Escalante-Herrera A, Milke-Najar ME, Alpizar-Salazar M: **Effects of weight loss on insulin secretion and in vivo insulin sensitivity in obese diabetic and non-diabetic subjects**. *Diabetes Nutr Metab* 2003, **16**(5-6):277-283.
296. Fontana L, Partridge L, Longo VD: **Extending healthy life span--from yeast to humans**. *Science* 2010, **328**(5976):321-326.
297. Fontana L, Klein S: **Aging, adiposity, and calorie restriction**. *JAMA* 2007, **297**(9):986-994.
298. Rochon J, Bales CW, Ravussin E, Redman LM, Holloszy JO, Racette SB, Roberts SB, Das SK, Romashkan S, Galan KM *et al*: **Design and conduct of the CALERIE study: comprehensive assessment of the long-term effects of reducing intake of energy**. *J Gerontol A Biol Sci Med Sci* 2011, **66**(1):97-108.
299. Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F *et al*: **Ribosomal protein S6 kinase 1 signaling regulates mammalian life span**. *Science* 2009, **326**(5949):140-144.
300. Bluher M, Kahn BB, Kahn CR: **Extended longevity in mice lacking the insulin receptor in adipose tissue**. *Science* 2003, **299**(5606):572-574.
301. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA: **Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase**. *Science* 2004, **305**(5682):390-392.
302. Lomb DJ, Laurent G, Haigis MC: **Sirtuins regulate key aspects of lipid metabolism**. *Biochim Biophys Acta* 2010, **1804**(8):1652-1657.
303. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL *et al*: **Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan**. *Nature* 2003, **425**(6954):191-196.
304. Wang F, Nguyen M, Qin FX, Tong Q: **SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction**. *Aging Cell* 2007, **6**(4):505-514.
305. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR *et al*: **SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation**. *Nature* 2010, **464**(7285):121-125.
306. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K *et al*: **Resveratrol improves health and survival of mice on a high-calorie diet**. *Nature* 2006, **444**(7117):337-342.

307. Gardner CD, Kiazand A, Alhassan S, Kim S, Stafford RS, Balise RR, Kraemer HC, King AC: **Comparison of the Atkins, Zone, Ornish, and LEARN diets for change in weight and related risk factors among overweight premenopausal women: the A TO Z Weight Loss Study: a randomized trial**. *JAMA* 2007, **297**(9):969-977.
308. Raatz SK, Torkelson CJ, Redmon JB, Reck KP, Kwong CA, Swanson JE, Liu C, Thomas W, Bantle JP: **Reduced glycemic index and glycemic load diets do not increase the effects of energy restriction on weight loss and insulin sensitivity in obese men and women**. *The Journal of nutrition* 2005, **135**(10):2387-2391.
309. Ornish D, Scherwitz LW, Billings JH, Brown SE, Gould KL, Merritt TA, Sparler S, Armstrong WT, Ports TA, Kirkeeide RL *et al*: **Intensive lifestyle changes for reversal of coronary heart disease**. *JAMA* 1998, **280**(23):2001-2007.
310. Nordmann AJ, Nordmann A, Briel M, Keller U, Yancy WS, Jr., Brehm BJ, Bucher HC: **Effects of low-carbohydrate vs low-fat diets on weight loss and cardiovascular risk factors: a meta-analysis of randomized controlled trials**. *Arch Intern Med* 2006, **166**(3):285-293.
311. Papakonstantinou E, Triantafillidou D, Panagiotakos DB, Koutsovasilis A, Saliaris M, Manolis A, Melidonis A, Zampelas A: **A high-protein low-fat diet is more effective in improving blood pressure and triglycerides in calorie-restricted obese individuals with newly diagnosed type 2 diabetes**. *Eur J Clin Nutr* 2010, **64**(6):595-602.
312. Tinker LF, Bonds DE, Margolis KL, Manson JE, Howard BV, Larson J, Perri MG, Beresford SA, Robinson JG, Rodriguez B *et al*: **Low-fat dietary pattern and risk of treated diabetes mellitus in postmenopausal women: the Women's Health Initiative randomized controlled dietary modification trial**. *Arch Intern Med* 2008, **168**(14):1500-1511.
313. Axen KV, Axen K: **Longitudinal adaptations to very low-carbohydrate weight-reduction diet in obese rats: body composition and glucose tolerance**. *Obesity (Silver Spring)* 2010, **18**(8):1538-1544.
314. Jornayvaz FR, Jurczak MJ, Lee HY, Birkenfeld AL, Frederick DW, Zhang D, Zhang XM, Samuel VT, Shulman GI: **A high-fat, ketogenic diet causes hepatic insulin resistance in mice, despite increasing energy expenditure and preventing weight gain**. *Am J Physiol Endocrinol Metab* 2010, **299**(5):E808-815.
315. Capel F, Viguerie N, Vega N, Dejean S, Arner P, Klimcakova E, Martinez JA, Saris WH, Holst C, Taylor M *et al*: **Contribution of energy restriction and macronutrient composition to changes in adipose tissue gene expression during dietary weight-loss programs in obese women**. *J Clin Endocrinol Metab* 2008, **93**(11):4315-4322.
316. Lofgren P, Andersson I, Wahrenberg H, Hoffstedt J: **No difference in lipolysis or glucose transport of subcutaneous fat cells between moderate-fat and low-fat hypocaloric diets in obese women**. *Horm Metab Res* 2005, **37**(12):734-740.
317. Chapkin RS, Kim W, Lupton JR, McMurray DN: **Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation**. *Prostaglandins Leukot Essent Fatty Acids* 2009, **81**(2-3):187-191.

318. Flachs P, Rossmeisl M, Bryhn M, Kopecky J: **Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism**. *Clin Sci (Lond)* 2009, **116**(1):1-16.
319. Kris-Etherton PM, Harris WS, Appel LJ: **Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease**. *Circulation* 2002, **106**(21):2747-2757.
320. Mori TA, Bao DQ, Burke V, Puddey IB, Watts GF, Beilin LJ: **Dietary fish as a major component of a weight-loss diet: effect on serum lipids, glucose, and insulin metabolism in overweight hypertensive subjects**. *Am J Clin Nutr* 1999, **70**(5):817-825.
321. Ruzickova J, Rossmeisl M, Prazak T, Flachs P, Sponarova J, Veck M, Tvrzicka E, Bryhn M, Kopecky J: **Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue**. *Lipids* 2004, **39**(12):1177-1185.
322. Gonzalez-Periz A, Horrillo R, Ferre N, Gronert K, Dong B, Moran-Salvador E, Titos E, Martinez-Clemente M, Lopez-Parra M, Arroyo V *et al*: **Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins**. *Faseb J* 2009, **23**(6):1946-1957.
323. Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG, Pascoe WS: **Fish oil prevents insulin resistance induced by high-fat feeding in rats**. *Science* 1987, **237**(4817):885-888.
324. Ghafoorunissa, Ibrahim A, Rajkumar L, Acharya V: **Dietary (n-3) long chain polyunsaturated fatty acids prevent sucrose-induced insulin resistance in rats**. *The Journal of nutrition* 2005, **135**(11):2634-2638.
325. Kabir M, Skurnik G, Naour N, Pechtner V, Meugnier E, Rome S, Quignard-Boulange A, Vidal H, Slama G, Clement K *et al*: **Treatment for 2 mo with n 3 polyunsaturated fatty acids reduces adiposity and some atherogenic factors but does not improve insulin sensitivity in women with type 2 diabetes: a randomized controlled study**. *Am J Clin Nutr* 2007, **86**(6):1670-1679.
326. Rivellese AA, Maffettone A, Iovine C, Di Marino L, Annuzzi G, Mancini M, Riccardi G: **Long-term effects of fish oil on insulin resistance and plasma lipoproteins in NIDDM patients with hypertriglyceridemia**. *Diabetes Care* 1996, **19**(11):1207-1213.
327. Nettleton JA, Katz R: **n-3 long-chain polyunsaturated fatty acids in type 2 diabetes: a review**. *J Am Diet Assoc* 2005, **105**(3):428-440.
328. Adkins Y, Kelley DS: **Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids**. *J Nutr Biochem* 2010, **21**(9):781-792.
329. Simopoulos AP: **Evolutionary Aspects of Diet: The Omega-6/Omega-3 Ratio and the Brain**. *Mol Neurobiol* 2011.
330. Simopoulos AP: **The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases**. *Exp Biol Med (Maywood)* 2008, **233**(6):674-688.
331. Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR: **Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century**. *Am J Clin Nutr* 2011.

332. Aarsetoey H, Aarsetoey R, Lindner T, Staines H, Harris WS, Nilsen DW: **Low Levels of the Omega-3 Index are Associated with Sudden Cardiac Arrest and Remain Stable in Survivors in the Subacute Phase**. *Lipids* 2011, **46**(2):151-161.
333. Rustan AC, Nossen JO, Christiansen EN, Drevon CA: **Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase**. *J Lipid Res* 1988, **29**(11):1417-1426.
334. Saraswathi V, Gao L, Morrow JD, Chait A, Niswender KD, Hasty AH: **Fish oil increases cholesterol storage in white adipose tissue with concomitant decreases in inflammation, hepatic steatosis, and atherosclerosis in mice**. *The Journal of nutrition* 2007, **137**(7):1776-1782.
335. Pettinelli P, Del Pozo T, Araya J, Rodrigo R, Araya AV, Smok G, Csendes A, Gutierrez L, Rojas J, Korn O *et al*: **Enhancement in liver SREBP-1c/PPAR-alpha ratio and steatosis in obese patients: correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion**. *Biochim Biophys Acta* 2009, **1792**(11):1080-1086.
336. Sekiya M, Yahagi N, Matsuzaka T, Najima Y, Nakakuki M, Nagai R, Ishibashi S, Osuga J, Yamada N, Shimano H: **Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression**. *Hepatology* 2003, **38**(6):1529-1539.
337. Madsen L, Rustan AC, Vaagenes H, Berge K, Dyroy E, Berge RK: **Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference**. *Lipids* 1999, **34**(9):951-963.
338. Teran-Garcia M, Adamson AW, Yu G, Rufo C, Suchankova G, Dreesen TD, Tekle M, Clarke SD, Gettys TW: **Polyunsaturated fatty acid suppression of fatty acid synthase (FASN): evidence for dietary modulation of NF-Y binding to the Fasn promoter by SREBP-1c**. *Biochem J* 2007, **402**(3):591-600.
339. Dentin R, Benhamed F, Pegorier JP, Foufelle F, Viollet B, Vaulont S, Girard J, Postic C: **Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation**. *J Clin Invest* 2005, **115**(10):2843-2854.
340. Hein GJ, Bernasconi AM, Montanaro MA, Pellon-Maison M, Finarelli G, Chicco A, Lombardo YB, Brenner RR: **Nuclear receptors and hepatic lipidogenic enzyme response to a dyslipidemic sucrose-rich diet and its reversal by fish oil n-3 polyunsaturated fatty acids**. *Am J Physiol Endocrinol Metab* 2010, **298**(3):E429-439.
341. Svegliati-Baroni G, Candelaresi C, Saccomanno S, Ferretti G, Bachetti T, Marzioni M, De Minicis S, Nobili L, Salzano R, Omenetti A *et al*: **A model of insulin resistance and nonalcoholic steatohepatitis in rats: role of peroxisome proliferator-activated receptor-alpha and n-3 polyunsaturated fatty acid treatment on liver injury**. *Am J Pathol* 2006, **169**(3):846-860.

342. Neschen S, Moore I, Regittnig W, Yu CL, Wang Y, Pypaert M, Petersen KF, Shulman GI: **Contrasting effects of fish oil and safflower oil on hepatic peroxisomal and tissue lipid content**. *Am J Physiol Endocrinol Metab* 2002, **282**(2):E395-401.
343. Suchankova G, Tekle M, Saha AK, Ruderman NB, Clarke SD, Gettys TW: **Dietary polyunsaturated fatty acids enhance hepatic AMP-activated protein kinase activity in rats**. *Biochem Biophys Res Commun* 2005, **326**(4):851-858.
344. Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, Rossbacher JC, Moore IK, Regittnig W, Munoz DS *et al*: **n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner**. *Diabetes* 2007, **56**(4):1034-1041.
345. Jelenik T, Rossmeisl M, Kuda O, Jilkova ZM, Medrikova D, Kus V, Hensler M, Janovska P, Miksik I, Baranowski M *et al*: **AMP-activated protein kinase alpha2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids**. *Diabetes* 2010, **59**(11):2737-2746.
346. Couet C, Delarue J, Ritz P, Antoine JM, Lamisse F: **Effect of dietary fish oil on body fat mass and basal fat oxidation in healthy adults**. *Int J Obes Relat Metab Disord* 1997, **21**(8):637-643.
347. Flachs P, Mohamed-Ali V, Horakova O, Rossmeisl M, Hosseinzadeh-Attar MJ, Hensler M, Ruzickova J, Kopecky J: **Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet**. *Diabetologia* 2006, **49**(2):394-397.
348. Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, Ruzickova J, Sponarova J, Drahota Z, Vlcek C *et al*: **Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat**. *Diabetologia* 2005, **48**(11):2365-2375.
349. van Schothorst EM, Flachs P, Franssen-van Hal NL, Kuda O, Bunschoten A, Molthoff J, Vink C, Hooiveld GJ, Kopecky J, Keijer J: **Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet**. *BMC Genomics* 2009, **10**:110.
350. Guo W, Xie W, Lei T, Hamilton JA: **Eicosapentaenoic acid, but not oleic acid, stimulates beta-oxidation in adipocytes**. *Lipids* 2005, **40**(8):815-821.
351. Wensaas AJ, Rustan AC, Just M, Berge RK, Drevon CA, Gaster M: **Fatty acid incubation of myotubes from humans with type 2 diabetes leads to enhanced release of beta-oxidation products because of impaired fatty acid oxidation: effects of tetradecylthioacetic acid and eicosapentaenoic acid**. *Diabetes* 2009, **58**(3):527-535.
352. Figueras M, Olivan M, Busquets S, Lopez-Soriano FJ, Argiles JM: **Effects of eicosapentaenoic Acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status**. *Obesity (Silver Spring)* 2011, **19**(2):362-369.
353. Lorente-Cebrian S, Bustos M, Marti A, Martinez JA, Moreno-Aliaga MJ: **Eicosapentaenoic acid stimulates AMP-activated protein kinase and**

increases visfatin secretion in cultured murine adipocytes. *Clin Sci (Lond)* 2009, **117**(6):243-249.

354. Ide T: **Interaction of fish oil and conjugated linoleic acid in affecting hepatic activity of lipogenic enzymes and gene expression in liver and adipose tissue**. *Diabetes* 2005, **54**(2):412-423.
355. Krebs JD, Browning LM, McLean NK, Rothwell JL, Mishra GD, Moore CS, Jebb SA: **Additive benefits of long-chain n-3 polyunsaturated fatty acids and weight-loss in the management of cardiovascular disease risk in overweight hyperinsulinaemic women**. *Int J Obes (Lond)* 2006, **30**(10):1535-1544.
356. Itoh M, Suganami T, Satoh N, Tanimoto-Koyama K, Yuan X, Tanaka M, Kawano H, Yano T, Aoe S, Takeya M *et al*: **Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects**. *Arterioscler Thromb Vasc Biol* 2007, **27**(9):1918-1925.
357. Kuda O, Jelenik T, Jilkova Z, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J *et al*: **n-3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet**. *Diabetologia* 2009, **52**(5):941-951.
358. Neschen S, Morino K, Rossbacher JC, Pongratz RL, Cline GW, Sono S, Gillum M, Shulman GI: **Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-gamma-dependent mechanism in mice**. *Diabetes* 2006, **55**(4):924-928.
359. Perez-Echarri N, Perez-Matute P, Marcos-Gomez B, Baena MJ, Marti A, Martinez JA, Moreno-Aliaga MJ: **Differential inflammatory status in rats susceptible or resistant to diet-induced obesity: effects of EPA ethyl ester treatment**. *Eur J Nutr* 2008, **47**(7):380-386.
360. Perez-Echarri N, Perez-Matute P, Marcos-Gomez B, Martinez JA, Moreno-Aliaga MJ: **Effects of eicosapentaenoic acid ethyl ester on visfatin and apelin in lean and overweight (cafeteria diet-fed) rats**. *Br J Nutr* 2009, **101**(7):1059-1067.
361. Puglisi MJ, Hasty AH, Saraswathi V: **The role of adipose tissue in mediating the beneficial effects of dietary fish oil**. *J Nutr Biochem* 2011, **22**(2):101-108.
362. Todoric J, Loffler M, Huber J, Bilban M, Reimers M, Kadl A, Zeyda M, Waldhausl W, Stulnig TM: **Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids**. *Diabetologia* 2006, **49**(9):2109-2119.
363. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM: **GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects**. *Cell* 2010, **142**(5):687-698.
364. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H *et al*: **Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40**. *Nature* 2003, **422**(6928):173-176.

365. Schwab JM, Chiang N, Arita M, Serhan CN: **Resolvin E1 and protectin D1 activate inflammation-resolution programmes**. *Nature* 2007, **447**(7146):869-874.
366. White PJ, Arita M, Taguchi R, Kang JX, Marette A: **Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice**. *Diabetes* 2010, **59**(12):3066-3073.
367. Aas V, Rokling-Andersen MH, Kase ET, Thoresen GH, Rustan AC: **Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells**. *J Lipid Res* 2006, **47**(2):366-374.
368. Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW: **Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid**. *Diabetes* 1991, **40**(2):280-289.
369. Kusunoki M, Tsutsumi K, Hara T, Ogawa H, Nakamura T, Miyata T, Sakakibara F, Fukuzawa Y, Suga T, Kato K *et al*: **Ethyl icosapentate (omega-3 fatty acid) causes accumulation of lipids in skeletal muscle but suppresses insulin resistance in OLETF rats. Otsuka Long-Evans Tokushima Fatty**. *Metabolism* 2003, **52**(1):30-34.
370. Sinha S, Perdomo G, Brown NF, O'Doherty RM: **Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B**. *J Biol Chem* 2004, **279**(40):41294-41301.
371. Kobori H, Nangaku M, Navar LG, Nishiyama A: **The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease**. *Pharmacol Rev* 2007, **59**(3):251-287.
372. Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado Mda G, Murari A, Botion LM, Alenina N, Bader M, Santos RA: **Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7)**. *Arterioscler Thromb Vasc Biol* 2010, **30**(5):953-961.
373. Braga MF, Leiter LA: **Role of renin-angiotensin system blockade in patients with diabetes mellitus**. *Am J Cardiol* 2009, **104**(6):835-839.
374. Nagel JM, Tietz AB, Goke B, Parhofer KG: **The effect of telmisartan on glucose and lipid metabolism in nondiabetic, insulin-resistant subjects**. *Metabolism* 2006, **55**(9):1149-1154.
375. Chu KY, Lau T, Carlsson PO, Leung PS: **Angiotensin II type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes**. *Diabetes* 2006, **55**(2):367-374.
376. Tataranni PA, Ortega E: **A burning question: does an adipokine-induced activation of the immune system mediate the effect of overnutrition on type 2 diabetes?** *Diabetes* 2005, **54**(4):917-927.
377. Engeli S, Negrel R, Sharma AM: **Physiology and pathophysiology of the adipose tissue renin-angiotensin system**. *Hypertension* 2000, **35**(6):1270-1277.

378. Kim S, Soltani-Bejnood M, Quignard-Boulange A, Massiera F, Teboul M, Ailhaud G, Kim JH, Moustaid-Moussa N, Voy BH: **The adipose Renin-Angiotensin system modulates systemic markers of insulin sensitivity and activates the intrarenal Renin-Angiotensin system**. *J Biomed Biotechnol* 2006, **2006**(5):27012.
379. Kalupahana NS, Voy BH, Saxton AM, Moustaid-Moussa N: **Energy-Restricted High-Fat Diets Only Partially Improve Markers of Systemic and Adipose Tissue Inflammation**. *Obesity (Silver Spring)* 2010.
380. Ayala JE, Bracy DP, McGuinness OP, Wasserman DH: **Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse**. *Diabetes* 2006, **55**(2):390-397.
381. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, Greenberg AS, Obin MS: **Adipocyte death, adipose tissue remodeling, and obesity complications**. *Diabetes* 2007, **56**(12):2910-2918.
382. Wortman P, Miyazaki Y, Kalupahana NS, Kim S, Hansen-Petrik M, Saxton AM, Claycombe KJ, Voy BH, Whelan J, Moustaid-Moussa N: **n3 and n6 polyunsaturated fatty acids differentially modulate prostaglandin E secretion but not markers of lipogenesis in adipocytes**. *Nutr Metab (Lond)* 2009, **6**:5.
383. Macotela Y, Boucher J, Tran TT, Kahn CR: **Sex and depot differences in adipocyte insulin sensitivity and glucose metabolism**. *Diabetes* 2009, **58**(4):803-812.
384. Aksnes TA, Seljeflot I, Torjesen PA, Hoieggen A, Moan A, Kjeldsen SE: **Improved insulin sensitivity by the angiotensin II-receptor blocker losartan is not explained by adipokines, inflammatory markers, or whole blood viscosity**. *Metabolism* 2007, **56**(11):1470-1477.
385. Goodpaster BH, Thaete FL, Kelley DE: **Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus**. *Am J Clin Nutr* 2000, **71**(4):885-892.
386. Kremen J, Dolinkova M, Krajickova J, Blaha J, Anderlova K, Lacinova Z, Haluzikova D, Bosanska L, Vokurka M, Svacina S *et al*: **Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance**. *J Clin Endocrinol Metab* 2006, **91**(11):4620-4627.
387. Roubicek T, Dolinkova M, Blaha J, Haluzikova D, Bosanska L, Mraz M, Kremen J, Haluzik M: **Increased angiotensinogen production in epicardial adipose tissue during cardiac surgery: possible role in a postoperative insulin resistance**. *Physiol Res* 2008, **57**(6):911-917.
388. Dietze D, Koenen M, Rohrig K, Horikoshi H, Hauner H, Eckel J: **Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes**. *Diabetes* 2002, **51**(8):2369-2376.
389. Folli F, Kahn CR, Hansen H, Bouchie JL, Feener EP: **Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk**. *J Clin Invest* 1997, **100**(9):2158-2169.

390. Tsuchiya K, Yoshimoto T, Hirono Y, Tateno T, Sugiyama T, Hirata Y: **Angiotensin II induces monocyte chemoattractant protein-1 expression via a nuclear factor-kappaB-dependent pathway in rat preadipocytes**. *Am J Physiol Endocrinol Metab* 2006, **291**(4):E771-778.
391. Chipitsyna G, Gong Q, Gray CF, Haroon Y, Kamer E, Arafat HA: **Induction of monocyte chemoattractant protein-1 expression by angiotensin II in the pancreatic islets and beta-cells**. *Endocrinology* 2007, **148**(5):2198-2208.
392. Derosa G, Maffioli P, Salvadeo SA, Ferrari I, Gravina A, Mereu R, Palumbo I, Fogari E, D'Angelo A, Cicero AF: **Differential effects of candesartan and olmesartan on adipose tissue activity biomarkers in type II diabetic hypertensive patients**. *Hypertens Res* 2010, **33**(8):790-795.
393. Soejima H, Ogawa H, Yasue H, Kaikita K, Takazoe K, Nishiyama K, Misumi K, Miyamoto S, Yoshimura M, Kugiyama K *et al*: **Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction**. *J Am Coll Cardiol* 1999, **34**(4):983-988.
394. Wei Y, Whaley-Connell AT, Chen K, Habibi J, Uptergrove GM, Clark SE, Stump CS, Ferrario CM, Sowers JR: **NADPH oxidase contributes to vascular inflammation, insulin resistance, and remodeling in the transgenic (mRen2) rat**. *Hypertension* 2007, **50**(2):384-391.
395. Tang X, Edwards EM, Holmes BB, Falck JR, Campbell WB: **Role of phospholipase C and diacylglyceride lipase pathway in arachidonic acid release and acetylcholine-induced vascular relaxation in rabbit aorta**. *Am J Physiol Heart Circ Physiol* 2006, **290**(1):H37-45.
396. Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N: **Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism**. *The Journal of nutrition* 2002, **132**(6):1135-1140.
397. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr. *et al*: **Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement**. *Circulation* 2005, **112**(17):2735-2752.
398. Weiss EP, Racette SB, Villareal DT, Fontana L, Steger-May K, Schechtman KB, Klein S, Holloszy JO: **Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial**. *Am J Clin Nutr* 2006, **84**(5):1033-1042.
399. Miller RS, Becker KG, Prabhu V, Cooke DW: **Adipocyte gene expression is altered in formerly obese mice and as a function of diet composition**. *The Journal of nutrition* 2008, **138**(6):1033-1038.
400. Kennedy AR, Pissios P, Otu H, Roberson R, Xue B, Asakura K, Furukawa N, Marino FE, Liu FF, Kahn BB *et al*: **A high-fat, ketogenic diet induces a unique metabolic state in mice**. *Am J Physiol Endocrinol Metab* 2007, **292**(6):E1724-1739.

401. Ledikwe JH, Ello-Martin JA, Rolls BJ: **Portion sizes and the obesity epidemic**. *The Journal of nutrition* 2005, **135**(4):905-909.
402. Lee S, Muniyappa R, Yan X, Chen H, Yue LQ, Hong EG, Kim JK, Quon MJ: **Comparison between surrogate indexes of insulin sensitivity and resistance and hyperinsulinemic euglycemic clamp estimates in mice**. *Am J Physiol Endocrinol Metab* 2008, **294**(2):E261-270.
403. Chan MY, Zhao Y, Heng CK: **Sequential responses to high-fat and high-calorie feeding in an obese mouse model**. *Obesity (Silver Spring)* 2008, **16**(5):972-978.
404. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Anal Biochem* 1976, **72**:248-254.
405. Miao B, Zondlo S, Gibbs S, Cromley D, Hosagrahara VP, Kirchgessner TG, Billheimer J, Mukherjee R: **Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator**. *J Lipid Res* 2004, **45**(8):1410-1417.
406. Mark M, Teletin M, Antal C, Wendling O, Auwerx J, Heikkinen S, Khetchoumian K, Argmann CA, Dgheem M: **Histopathology in mouse metabolic investigations**. *Curr Protoc Mol Biol* 2007, **Chapter 29**:Unit 29B 24.
407. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.
408. Efron B, Tibshirani R: **Empirical bayes methods and false discovery rates for microarrays**. *Genet Epidemiol* 2002, **23**(1):70-86.
409. Huang da W, Sherman BT, Lempicki RA: **Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources**. *Nat Protoc* 2009, **4**(1):44-57.
410. Hochberg Y, Benjamini Y: **More powerful procedures for multiple significance testing**. *Stat Med* 1990, **9**(7):811-818.
411. Linford NJ, Beyer RP, Gollahon K, Krajcik RA, Malloy VL, Demas V, Burmer GC, Rabinovitch PS: **Transcriptional response to aging and caloric restriction in heart and adipose tissue**. *Aging Cell* 2007, **6**(5):673-688.
412. Grove KL, Fried SK, Greenberg AS, Xiao XQ, Clegg DJ: **A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice**. *Int J Obes (Lond)* 2010, **34**(6):989-1000.
413. Chen GC, Huang CY, Chang MY, Chen CH, Chen SW, Huang CJ, Chao PM: **Two unhealthy dietary habits featuring a high fat content and a sucrose-containing beverage intake, alone or in combination, on inducing metabolic syndrome in Wistar rats and C57BL/6J mice**. *Metabolism* 2009.
414. Wang Z, Masternak MM, Al-Regaiey KA, Bartke A: **Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice**. *Endocrinology* 2007, **148**(6):2845-2853.
415. Coppola A, Marfella R, Coppola L, Tagliamonte E, Fontana D, Liguori E, Cirillo T, Cafiero M, Natale S, Astarita C: **Effect of weight loss on coronary**

circulation and adiponectin levels in obese women. *Int J Cardiol* 2009, **134**(3):414-416.

416. Maestu J, Jurimae J, Valter I, Jurimae T: **Increases in ghrelin and decreases in leptin without altering adiponectin during extreme weight loss in male competitive bodybuilders**. *Metabolism* 2008, **57**(2):221-225.
417. Silha JV, Weiler HA, Murphy LJ: **Plasma adipokines and body composition in response to modest dietary manipulations in the mouse**. *Obesity (Silver Spring)* 2006, **14**(8):1320-1329.
418. Korner A, Wabitsch M, Seidel B, Fischer-Posovszky P, Berthold A, Stumvoll M, Bluher M, Kratzsch J, Kiess W: **Adiponectin expression in humans is dependent on differentiation of adipocytes and down-regulated by humoral serum components of high molecular weight**. *Biochem Biophys Res Commun* 2005, **337**(2):540-550.
419. Brogren H, Sihlbom C, Wallmark K, Lonn M, Deinum J, Karlsson L, Jern S: **Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin**. *Thromb Res* 2008, **122**(2):271-281.
420. Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, Klein S: **Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss**. *Diabetes* 2009, **58**(3):693-700.
421. Pasarica M, Rood J, Ravussin E, Schwarz JM, Smith SR, Redman LM: **Reduced oxygenation in human obese adipose tissue is associated with impaired insulin suppression of lipolysis**. *J Clin Endocrinol Metab* 2010, **95**(8):4052-4055.
422. Urs S, Smith C, Campbell B, Saxton AM, Taylor J, Zhang B, Snoddy J, Jones Voy B, Moustaid-Moussa N: **Gene expression profiling in human preadipocytes and adipocytes by microarray analysis**. *The Journal of nutrition* 2004, **134**(4):762-770.
423. Isenberg JS, Frazier WA, Roberts DD: **Thrombospondin-1: a physiological regulator of nitric oxide signaling**. *Cell Mol Life Sci* 2008, **65**(5):728-742.
424. Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, Harrison SD, MacDougald OA: **Regulation of Wnt signaling during adipogenesis**. *J Biol Chem* 2002, **277**(34):30998-31004.
425. Ouchi N, Higuchi A, Ohashi K, Oshima Y, Gokce N, Shibata R, Akasaki Y, Shimono A, Walsh K: **Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity**. *Science* 2010, **329**(5990):454-457.
426. Selman C, Kerrison ND, Cooray A, Piper MD, Lingard SJ, Barton RH, Schuster EF, Blanc E, Gems D, Nicholson JK *et al*: **Coordinated multitissue transcriptional and plasma metabonomic profiles following acute caloric restriction in mice**. *Physiol Genomics* 2006, **27**(3):187-200.
427. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM: **Prevalence of overweight and obesity in the United States, 1999-2004**. *JAMA* 2006, **295**(13):1549-1555.
428. Greenberg AS, Obin MS: **Obesity and the role of adipose tissue in inflammation and metabolism**. *Am J Clin Nutr* 2006, **83**(2):461S-465S.

429. Zhao Y, Joshi-Barve S, Barve S, Chen LH: **Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation**. *J Am Coll Nutr* 2004, **23**(1):71-78.
430. Popp-Snijders C, Blonk MC: **Omega-3 fatty acids in adipose tissue of obese patients with non-insulin-dependent diabetes mellitus reflect long-term dietary intake of eicosapentaenoic and docosahexaenoic acid**. *Am J Clin Nutr* 1995, **61**(2):360-365.
431. Mori Y, Murakawa Y, Yokoyama J, Tajima N, Ikeda Y, Nobukata H, Ishikawa T, Shibutani Y: **Effect of highly purified eicosapentaenoic acid ethyl ester on insulin resistance and hypertension in Dahl salt-sensitive rats**. *Metabolism* 1999, **48**(9):1089-1095.
432. Rossi AS, Lombardo YB, Lacorte JM, Chicco AG, Rouault C, Slama G, Rizkalla SW: **Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats**. *Am J Physiol Regul Integr Comp Physiol* 2005, **289**(2):R486-R494.
433. Robbez Masson V, Lucas A, Gueugneau AM, Macaire JP, Paul JL, Grynberg A, Rousseau D: **Long-chain (n-3) polyunsaturated fatty acids prevent metabolic and vascular disorders in fructose-fed rats**. *The Journal of nutrition* 2008, **138**(10):1915-1922.
434. Saraswathi V, Morrow JD, Hasty AH: **Dietary fish oil exerts hypolipidemic effects in lean and insulin sensitizing effects in obese LDLR-/- mice**. *The Journal of nutrition* 2009, **139**(12):2380-2386.
435. Folch J, Lees M, Sloane Stanley GH: **A simple method for the isolation and purification of total lipides from animal tissues**. *J Biol Chem* 1957, **226**(1):497-509.
436. Morrison WR, Smith LM: **Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride--Methanol**. *J Lipid Res* 1964, **5**:600-608.
437. Matthan NR, Dillard A, Lecker JL, Ip B, Lichtenstein AH: **Effects of dietary palmitoleic acid on plasma lipoprotein profile and aortic cholesterol accumulation are similar to those of other unsaturated fatty acids in the F1B golden Syrian hamster**. *J Nutr* 2009, **139**(2):215-221.
438. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR: **Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions**. *Am J Gastroenterol* 1999, **94**(9):2467-2474.
439. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN: **Diet-induced type II diabetes in C57BL/6J mice**. *Diabetes* 1988, **37**(9):1163-1167.
440. Wueest S, Rapold RA, Schumann DM, Rytka JM, Schildknecht A, Nov O, Chervonsky AV, Rudich A, Schoenle EJ, Donath MY *et al*: **Deletion of Fas in adipocytes relieves adipose tissue inflammation and hepatic manifestations of obesity in mice**. *J Clin Invest* 2010, **120**(1):191-202.
441. Samane S, Christon R, Dombrowski L, Turcotte S, Charrouf Z, Lavigne C, Levy E, Bachelard H, Amarouch H, Marette A *et al*: **Fish oil and argan oil intake**

differently modulate insulin resistance and glucose intolerance in a rat model of dietary-induced obesity. *Metabolism* 2009, **58**(7):909-919.

442. Chazenbalk G, Trivax BS, Yildiz BO, Bertolotto C, Mathur R, Heneidi S, Azziz R: **Regulation of Adiponectin Secretion by Adipocytes in the Polycystic Ovary Syndrome: Role of Tumor Necrosis Factor-{alpha}**. *J Clin Endocrinol Metab* 2010.
443. Alkhouri N, Gornicka A, Berk MP, Thapaliya S, Dixon LJ, Kashyap S, Schauer PR, Feldstein AE: **Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis**. *J Biol Chem* 2010, **285**(5):3428-3438.
444. Rossmeisl M, Jelenik T, Jilkova Z, Slamova K, Kus V, Hensler M, Medrikova D, Povysil C, Flachs P, Mohamed-Ali V *et al*: **Prevention and reversal of obesity and glucose intolerance in mice by DHA derivatives**. *Obesity (Silver Spring)* 2009, **17**(5):1023-1031.
445. Arai T, Kim HJ, Chiba H, Matsumoto A: **Anti-obesity effect of fish oil and fish oil-fenofibrate combination in female KK mice**. *J Atheroscler Thromb* 2009, **16**(5):674-683.
446. Hartweg J, Perera R, Montori V, Dinneen S, Neil HA, Farmer A: **Omega-3 polyunsaturated fatty acids (PUFA) for type 2 diabetes mellitus**. *Cochrane Database Syst Rev* 2008(1):CD003205.
447. Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE: **Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity**. *Am J Clin Nutr* 2006, **83**(6 Suppl):1483S-1493S.
448. Siriwardhana N, Fletcher S, Xin W, Kalupahana NS, Mukherjee A, Smolensky D, Layman R, Wortman P, Zhao L, Saxton A *et al*: **Eicosapentaenoic acid (EPA) regulates angiotensinogen and other inflammatory adipokines.** . In: *Annual meeting of The Obesity Society.* San Diego, CA; 2010.
449. Wan F, Lenardo MJ: **The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives**. *Cell Res* 2010, **20**(1):24-33.
450. Iles KE, Forman HJ: **Macrophage signaling and respiratory burst**. *Immunol Res* 2002, **26**(1-3):95-105.
451. Ogihara T, Asano T, Katagiri H, Sakoda H, Anai M, Shojima N, Ono H, Fujishiro M, Kushiyama A, Fukushima Y *et al*: **Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase**. *Diabetologia* 2004, **47**(5):794-805.
452. Fabbrini E, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, Okunade A, Klein S: **Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity**. *Proc Natl Acad Sci U S A* 2009, **106**(36):15430-15435.
453. Watkins WS, Rohrwasser A, Peiffer A, Leppert MF, Lalouel JM, Jorde LB: **AGT genetic variation, plasma AGT, and blood pressure: An analysis of the Utah Genetic Reference Project pedigrees**. *Am J Hypertens* 2010, **23**(8):917-923.
454. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM *et al*: **Molecular basis of human hypertension: role of angiotensinogen**. *Cell* 1992, **71**(1):169-180.

455. Sarzani R, Bordicchia M, Marcucci P, Minardi D, Muzzonigro G, Dessi-Fulgheri P, Rappelli A: **Angiotensinogen promoter variants influence gene expression in human kidney and visceral adipose tissue**. *J Hum Hypertens* 2010, **24**(3):213-219.
456. Pelton PD, Zhou L, Demarest KT, Burris TP: **PPARgamma activation induces the expression of the adipocyte fatty acid binding protein gene in human monocytes**. *Biochem Biophys Res Commun* 1999, **261**(2):456-458.
457. Wang C, Mao X, Wang L, Liu M, Wetzel MD, Guan KL, Dong LQ, Liu F: **Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1**. *J Biol Chem* 2007, **282**(11):7991-7996.
458. Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH, Kim JB: **Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha**. *Diabetes* 2006, **55**(9):2562-2570.

VITA

Nishan Sudheera Kalupahana was born July 6, 1974 in Kurunegala, Sri Lanka. He received the Bachelor of Medicine and Surgery degree in 2001 and Master of Philosophy degree in 2006 from the University of Peradeniya, Sri Lanka. He served as a medical officer at the Department of Health, Sri Lanka, and a lecturer at Peradeniya Medical School, Sri Lanka before moving to Knoxville, TN to pursue a doctoral degree at the University of Tennessee. During this graduate work, he was awarded the American Heart Association pre-doctoral fellowship in 2009, the American Society for Nutrition graduate student research award in 2010, the University of Tennessee Chancellor's Honors in 2010 and Gamma Sigma Delta graduate student award of merit in 2010. He also served as a graduate teaching assistant (Department of Nutrition) and research assistant (Department of Animal Science); and as a graduate assistant for the University of Tennessee, Obesity Research Center, Knoxville, TN, USA.