DIETARY L-ARGININE SUPPLEMENTATION REDUCES FAT MASS IN DIET-INDUCED OBESE RATS

A Dissertation

by

WENJUAN SHI JOBGEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Nutrition

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ABSTRACT

Dietary L-arginine Supplementation Reduces Fat Mass in Diet-Induced Obese Rats. (August 2007)

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This study was conducted to test the hypothesis that dietary arginine supplementation reduces fat mass in diet-induced obese rats. Male Sprague-Dawley rats were fed either low- or high-fat diets for 15 wks (16 rats/diet). Thereafter, lean or obese rats continued to be fed their same respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) (n=8/treatment group). Twelve weeks after the initiation of the arginine treatment, rats were euthanized to obtain tissues for biochemical analyses. Results were statistically analyzed as a 2x2 factorial experimental design using ANOVA. High-fat diet increased the mass of white adipose tissues at different anatomical locations by 49-96% compared to the low-fat diet. Concentrations of serum cholesterol as well as lipids in skeletal muscle and liver were higher in obese rats than in lean rats. L-Arginine supplementation reduced white adipose tissue mass by 20-40% while increasing brown adipose tissue mass by 15-20%. In addition, arginine treatment decreased adipocyte size, serum concentrations of glucose, triglycerides and leptin, improved glucose tolerance, and enhanced glucose and oleic

acid oxidation in skeletal muscles. The mRNA levels for hepatic fatty acid synthase and stearoyl-CoA desaturase were reduced, but mRNA levels for hepatic AMP-activated protein kinase (AMPK), PPARγ coactivator-1 α and carnitine palmitoyltransferase I (CPT-I) as well as muscle CPT-I were increased in response to the arginine treatment. Subsequent experiments were conducted with cell models to define the direct effects of arginine on energy-substrate metabolism in insulin-sensitive cells. In BNL CL.2 mouse hepatocytes, C2C12 mouse myotubes and 3T3-L1 mouse adipocytes, increasing extracellular concentrations of arginine from 0 to 400 μM increased AMPK expression as well as glucose and oleic acid oxidation. Inhibition of nitric oxide synthesis moderately attenuated the arginine-stimulated increases of substrate oxidation as well as AMPK and ACC phosphorylation in BNL CL.2 cells, but had no effect in C2C12 and 3T3-L1 cells. Collectively, these results suggest that arginine increases AMPK expression and energy-substrate oxidation in a cell-specific manner, thereby reducing fat mass in diet-induced obese rats. The findings have important implications for treating obesity in humans and companion animals as well as decreasing fat deposition in livestock species.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Fat deposition in humans or animals depends on the balance between dietary caloric intake and whole-body energy expenditure. A chronic imbalance in energy metabolism (more energy input than energy output) due to complex genetic and/or environmental factors results in excess fat accretion or obesity in humans, which is currently a major public health problem worldwide (Hill *et al.* 2003; Bell *et al.* 2001). The 1999-2000 National Health and Nutrition Examination Survey found that 65% and 31% of the U.S. adult population were overweight (a body mass index > 25 kg/m^2) and obese (a body mass index > 30 kg/m^2), respectively (Hill *et al.* 2003). The prevalence of overweight and obesity increased by 16% and 35%, respectively, compared with the survey conducted between 1988 and 1994. Children and adolescents have not been immune to this epidemic, as 15% of them in the U.S. are obese, representing a 36% increase within the past decade (Hill *et al.* 2003). Most other countries are also experiencing the obesity crisis. For example, the prevalence of overweight in China doubled in women and almost tripled in men between 1989 and 1997 (Bell *et al.* 2001). Worldwide, more than 300 million adults are obese and over one billion are overweight (Hill *et al.* 2003). Obesity is a major risk factor for insulin resistance, type II diabetes, atherosclerosis,

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stroke, hypertension, impaired vascular function, sleep disorders, and some types of cancer (including colon and breast cancers) (Nisoli & Carruba, 2004). Consequently, obesity claims an increasing number of lives and contributes to tremendous costs of health care worldwide. In the U.S. alone, about 300,000 people die of obesity-related diseases every year, the incidence of type II diabetes among children has increased 10-fold over the past decade, and obesity accounts for 6-8% of all health care expenditures (Hill *et al.* 2003). Unfortunately, clinicians have few tools to fight the obesity epidemic, because current anti-obesity drugs are not highly effective and are fraught with side effects (Nisoli & Carruba, 2004). Because even a modest (5-7%) weight loss in obese subjects is associated with a significant improvement in risk factors for cardiovascular disease and diabetes (Nisoli & Carruba, 2004), identifying new means to reduce body fat will be extremely beneficial for human health.

Nitric oxide (NO), a signaling molecule that regulates nutrient metabolism, is a free radical produced from L-arginine by various isoforms of NO synthase (NOS). This synthetic reaction occurs in virtually all mammalian cells and tissues, including adipocytes, brain, endothelial cells, heart, hepatocytes, macrophages, and skeletal muscle (Lee *et al.* 2003; Wu & Morris, 1998; Wu *et al.* 2001; Wu & Meininger, 2002). As a small, hydrophilic gas molecule, NO readily diffuses into cells and acts on the heme of target molecules. Through multiple cGMP-dependent pathways, NO plays a crucial role in regulating vascular tone, neurotransmission, host immunity, and whole-body homeostasis (Alderton *et al.* 2001; Shi *et al.* 2004). We recently found that dietary supplementation with L-arginine reduced fat mass and enhanced expression of key genes

responsible for glucose and fatty acid oxidation in the Zucker diabetic fatty (ZDF) rat, an animal model of type II diabetes secondary to a defect in the leptin receptor (Fu *et al.* 2005). Physiological levels of NO stimulate glucose uptake and oxidation as well as fatty acid oxidation in insulin-sensitive tissues (muscle, heart, liver, and adipose tissue), inhibit the synthesis of glucose, glycogen and fat in target tissues (e.g., liver and adipose), and enhance lipolysis in adipocytes. The underlying mechanisms may involve multiple cGMP-dependent pathways: 1) phosphorylation of AMP-activated protein kinase (AMPK); 2) phosphorylation of hormone-sensitive lipase and perilipins; 3) increased expression of peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1α); and 4) increased blood flow to tissues. Modulation of the arginine-NO pathway may provide a potentially novel means to reduce fat mass in obese subjects.

NO synthesis in animal cells

NO synthases

The biosynthesis of NO by NOS requires L-arginine (a basic amino acid at physiological pH) and oxygen as substrates, as well as tetrahydrobiopterin (BH4), NADPH, Ca^{2+}, calmodulin, FMN, and FAD (Wu & Morris, 1998). There are three isoforms of the NOS: nNOS (type-1 NOS), which was first discovered in neuronal tissues; iNOS (type-2 NOS), which was originally found to be inducible under certain conditions in macrophages and hepatocytes; and eNOS (type-3 NOS), which was first identified in endothelial cells. These NOS isoforms are encoded by three different genes, and their nucleotide sequences are 51-57% homologous (Alderton *et al.* 2001). NOS can be

present in plasma membrane, cytoplasm, nucleus, rough endoplasmic reticulum, and mitochondria of cells. Notably, there is complex intracellular compartmentation for the NOS isoforms in animal cells and tissues (Buchwalow *et al.* 2005). In general, eNOS, nNOS, and iNOS are localized primarily in plasma membrane and cytoplasm, mitochondria and cytoplasm, and cytoplasm, respectively. The intracellular compartmentation of NOS isoforms may play a role in regulating NO synthesis in animal cells. While nNOS and eNOS are expressed constitutively at low levels in a variety of cell types and tissues, iNOS is normally not expressed at a significant level in cells or tissues (Wu & Morris, 1998). When induced by certain immunological stimuli (including inflammatory cytokines or bacterial endotoxin), iNOS is highly expressed in many cell types and produces a large amount of NO. Due to its high affinity for calcium and tight binding with calmodulin, iNOS is fully active in the absence of exogenous Ca^{2+} or calmodulin. All of the NOS isoforms can be induced under certain stimuli through transcriptional and translational mechanisms (Wu & Morris, 1998).

A small quantity of NO is produced by eNOS and nNOS, relative to the amount of NO generated by iNOS. For example, NO production by unstimulated bovine venular endothelial cells is 0.24 nmol/10^6 cells/h (Wu *et al.* 2001), which is only approximately 7% of that by LPS-activated RAW 264.7 macrophages (3.3 nmol/10^6 cells/h) (Meininger *et al.* 2000). Similarly, *in vivo* systemic production of NO is low in rats (e.g., 15 μmol/kg body wt/24 h), but increases 15-fold in response to immunological challenge (Wu *et al.* 1999a). Compelling evidence shows that NO synthesis is regulated not only by the amount and/or phosphorylation of the NOS protein but also by the availability of

cofactors (particularly NADPH, BH4, and Ca^{2+}) and arginine (Wu & Meininger, 2002). Arginine, BH4, and heme promote formation of and also stabilize the active dimeric form of all isoforms of the NOS (Alderton *et al.* 2001). Although the K_m values of NOS for arginine are 3 to 20 μM depending on the isoform, increasing extracellular arginine concentrations from 0.05 to 5 mM dose-dependently increases NO synthesis in a variety of cells, including activated macrophages and endothelial cells (Wu & Morris, 1998). It is now clear that arginine increases the transcription of iNOS in macrophages (Lee *et al.* 2003) and BH4 synthesis in endothelial cells (Shi *et al.* 2004), resulting in increased NO production in both cell types. Additionally, arginine-dependent NO synthesis is affected by various amino acids, including glutamine, lysine, glutamate, phenylalanine, and taurine (Wu & Meininger, 2002).

NOS expression in insulin-sensitive tissues

Because of an important role for skeletal muscle, adipose tissue, heart, and liver in NO synthesis (Kobzik *et al.* 1994; Nakane *et al.* 1993; Bates *et al.* 1996; Kobzik *et al.* 1995; Gath *et al.* 1996; Punkt *et al.* 2002) and the metabolism of energy substrates (Nisoli & Carruba, 2004; Digirolamo *et al.* 1993), here we review briefly the pertinent literature related to NOS expression in these insulin-sensitive tissues. In mammalian skeletal and cardiac muscle, all three isoforms of the NOS are constitutively expressed (Kobzik *et al.* 1994; Nakane *et al.* 1993; Bates *et al.* 1996; Kobzik *et al.* 1995; Gath *et al.* 1996; Punkt *et al.* 2002). The NOS isoforms are present mainly in sarcolemma (plasma membrane), sarcoplasm (cytoplasm), sarcoplasmic reticulum, and mitochondria of muscle fibers (Buchwalow *et al.* 2005; Frandsen *et al.* 1996). In addition to myocytes, all three NOS

isoforms can be detected in capillaries and arterioles of the skeletal musculature (Buchwalow *et al.* 2005). The expression and activity of the NOS in skeletal muscle are regulated by several factors, which include fiber type, contraction, developmental stage, and pathological state (Punkt *et al.* 2002; Kapur *et al.* 1997; Stamler & Meissner, 2001). Brown adipose tissue contains both eNOS and iNOS in the cytoplasm and nucleus, as well as eNOS in the plasma membrane (Giordano *et al.* 2002). In contrast, both eNOS and iNOS appear to be absent from the nucleus but are present in the cytoplasm and plasma membrane of white adipose tissue (Elizalde *et al.* 2000; Ribiere *et al.* 1996). Interestingly, nNOS is weakly expressed only in the cytoplasm of brown and white adipose tissues and is absent from mitochondria or plasma membranes (Elizalde *et al.* 2000; Ribiere *et al.* 1996; Giordano *et al.* 2002). In liver, eNOS is uniformly distributed in hepatocytes and in the endothelium of hepatic arteries, terminal hepatic venules, sinusoids, and the epithelium of biliary ducts, whereas iNOS is constitutively expressed primarily in the cytoplasm of periportal hepatocytes (McNaughton *et al.* 2002). While still under debate, most biochemical and genetic studies have shown that nNOS is the isoform found in the mitochondria of brain, heart, kidneys, liver, and skeletal muscle (Ghafourifar & Cadenas, 2005). Thus, the insulin-sensitive tissues are significant sites of NO production in the body, and the compartmentation of NO synthesis is likely related to specific cell functions.

NO targets

The target cells for NO action can be the NO-producing cell itself, neighboring cells via its direct release, or distant cells via transport by glutathione and red blood cells (Stamler

& Meissner; 2001). A soluble guanylyl cyclase is a major receptor for physiological levels of NO (Denninger & Marletta, 1999). The membrane-permeable NO binds to the prosthetic heme group of the enzyme, thereby activating the production of cyclic guanosine-3',5'-monophosphate (cGMP) from guanosine triphosphate (GTP) in target cells. cGMP is an intracellular signaling molecule, which plays an important role in regulating various cellular events through activation of cGMP-dependent protein kinase (PKG), cGMP-gated ion channels, and cGMP-dependent phosphodiesterases (Ghafourifar & Cadenas, 2005; Denninger & Marletta, 1999; Russwurm & Koesling, 2002). As a highly reactive free radical molecule, high levels of NO produced by iNOS can oxidize biomolecules (including proteins, fatty acids, and DNA), thereby damaging cell membranes, inhibiting vital biochemical reactions (e.g., the mitochondrial Krebs cycle and respiratory chain), and even causing cell death (Fang *et al.* 2002). Therefore, effects of NO on cell metabolism critically depend on its concentrations at the reaction site.

Interorgan metabolism of energy substrates

Small intestine

The metabolism of dietary energy substrates in animals and humans requires the cooperation of multiple organs and tissues, particularly the small intestine, liver, skeletal muscle, heart, kidneys, brain, and adipose tissue. The small intestine is responsible for the terminal digestion of fat, protein, and carbohydrate in monogastric animals, whereas fermentation of these substances occurs in the rumen of ruminants to produce short-chain fatty acids (acetate, propionate, and butyrate). The small intestine is also the

primary site for the absorption of fatty acids, glucose, amino acids, and other nutrients in both monogastric and ruminant animals (reviewed in Wu, 1998). In most mammals (including humans, pigs, cattle, and rats), extensive catabolism of dietary glutamine, glutamate, and aspartate takes place in enterocytes (Wu, 1998). Glutamine, glutamate, and proline are substrates for the net production of both citrulline and arginine in neonates and of citrulline in adults (Wu & Morris, 1998). The circulating citrulline is taken up by extraintestinal and extrahepatic tissues (primarily the kidneys) and by almost all cell types (except for hepatocytes) for conversion into arginine by argininosuccinate synthase and argininosuccinate lyase (Wu & Morris, 1998). The small intestine also plays an important role in the catabolism of many dietary essential and nonessential amino acids (including branched-chain amino acids, lysine, phenylalanine, methionine, glycine, and serine), although the responsible cell types have not yet been identified (Wu, 1998). Interestingly, oxidation of glucose and long-chain fatty acids is limited in the small intestine under fed conditions. After feeding, the small intestine releases amino acids, glucose, and lipoproteins (mainly as chylomicrons and very-low-density-lipoprotein, VLDL) (Jungas *et al.* 1992). Amino acids and glucose enter the portal vein through the mucosal vasculature, and then reach the liver. In mammals, triacylglycerols (TAG) packaged as soluble chylomicrons and VLDL leave the small intestine via exocytosis, are transported through lymph vessels, and enter blood circulation through the thoracic duct and cranial vena cava. In birds, the intestinal lymphatic system is poorly developed and the VLDL synthesized in the avian small intestine are absorbed directly into the portal vein. Absorption of long-chain fatty acids (LCFA) and release of

chylomicrons by the small intestine of ruminants are much lower in comparison with monogastric animals, because the lower content of dietary fat (< 5% of dry matter intake) is necessary to prevent interference with ruminal microbial metabolism (Bergen & Mersmann, 2005). Lipoprotein lipase (LPL), which is present on the wall of blood capillaries in peripheral tissues, hydrolyzes the TAG of chylomicrons and VLDL to form free fatty acids, glycerol, and low-density-lipoprotein (LDL). LDL is the major carrier of cholesterol in humans, pigs, rabbits, sheep, and guinea pigs (Bergen & Mersmann, 2005). During fasting, ketone bodies produced by the liver are the major energy substrates for the small intestine of most animals except for pigs that have limited hepatic ketogenesis and thus very low plasma levels of these fuels.

Liver

Although the liver represents only 2-3% of the total body weight, it accounts for approximately 20% of the total energy expenditure under basal conditions (Jungas *et al.* 1992). Gluconeogenesis (formation of glucose from noncarbohydrate precursors), ketogenesis (production of acetoacetate and β-hydroxybutyrate), amino acid oxidation, urea cycle, as well as syntheses of cholesterol, bile acids, and VLDL occur primarily in the liver. The liver also synthesizes and releases high-density-lipoproteins (HDL) into the circulation. Importantly, this organ is a major site for the oxidation of fatty acids in all animals studied, including humans, pigs, cattle, rats, mice, and chickens (Bergen & Mersmann, 2005). During fasting, the rates of gluconeogenesis (from amino acids, lactate and glycerol in all animals and also from propionate in ruminants), glycogenolysis, and ketogenesis (from acetyl-CoA) in liver increase markedly to supply

glucose and ketone bodies to the brain, peripheral tissues, and cells of the immune system as well as glucose for red blood cells (Jungas *et al.* 1992). Because of the blood-brain barrier, plasma long-chain fatty acids cannot be utilized for oxidation by the brain (Bergen & Mersmann, 2005), and instead ketone bodies serve as major energy substrates for this tissue when plasma glucose level is reduced (e.g., < 4.5 mM in adult humans) during fasting (Wu & Marliss, 1992). In the fed state, glucose, which enters the portal circulation from the small intestine (with exception of roughage-fed ruminants), is either stored as glycogen or metabolized via the pentose cycle and fatty acid synthesis in the liver, or it enters the systemic circulation for utilization by extrahepatic cells and tissues (Wu & Marliss, 1992). In humans, cats, dogs, rabbits, rodents, and avian species (e.g., chickens and ducks), the liver is a major site for *de novo* fatty acid synthesis from glucose and amino acids, whereas, in pigs and ruminants, this synthetic event is virtually absent from the liver.

Skeletal muscle and heart

Skeletal muscle is quantitatively the largest organ in the body, representing about 40-45% of the total body mass and accounting for 30% of the resting metabolic rate in adults (Ceddia, 2005). Skeletal and cardiac muscles are the major sites for the oxidation of fatty acids and glucose, the transamination of branched-chain amino acids (leucine, isoleucine, and valine), and the release of glutamine and alanine (major glucogenic substrates) (Wu & Marliss, 1992). Glutamine and alanine derive their α-amino groups from branched-chain amino acids, and their carbon skeletons primarily from glucose. Thus, muscle plays an important role in the regulation of plasma levels of glucose and

fatty acids. The fatty acids utilized by skeletal muscle and heart can be derived from either the lipolysis of TAG in adipose tissue or the hydrolysis of the circulating TAG-rich chylomicrons and VLDL by LPL (Rasmussen & Wolfe, 1999). Among animal tissues, LPL is expressed mainly in muscle and adipose tissue, where the enzyme binds to the capillary endothelium (Frayn *et al*. 2005). Interestingly, the muscle and adipose-tissue LPL are regulated differentially by hormones and nutritional factors. Fasting increases LPL activity in the muscle to facilitate TAG hydrolysis, but decreases LPL activity in adipose tissue to reduce its uptake of TAG and thus fat storage (Fried *et al.* 1993a). Acetyl-CoA, a common intermediate of glucose, fatty acid and amino acid oxidation, either enters the Krebs cycle for complete oxidation or is utilized for lipogenesis in skeletal and cardiac muscles, depending on the intracellular energy status and activity of acetyl-CoA carboxylase (ACC) (Jungas *et al.* 1992). In the muscles, as in the liver, malonyl-CoA, a product of the ACC reaction, is an allosteric inhibitor of carnitine palmitoyl transferase-I (CPT-I), the enzyme that transfers the long-chain fatty acyl-CoA into mitochondria for β-oxidation (Rasmussen & Wolfe, 1999). Intracellular concentrations of malonyl-CoA are regulated not only by its synthesis from acetyl-CoA but also by its decarboxylation. Therefore, conditions that result in reduced or increased intracellular levels of malonyl-CoA favor LCFA oxidation or lipogenesis, respectively (Rasmussen & Wolfe, 1999; Ruderman *et al.* 2003). Thus, the level of malonyl-CoA is decreased in skeletal and cardiac muscles during exercise or fasting, thereby promoting fatty acid oxidation in these tissues (Rasmussen & Wolfe, 1999).

Adipose tissues

There are white and brown adipose tissues in animals, the amounts varying with species, developmental stage, and environment (Rothwell & Stock, 1985). Most mammals are born with significant amounts of both white and brown adipose tissues, with an exception of pigs that possess no brown adipose tissue at birth or in adult life (Mersmann, 1974). With an increase in age, the amounts of white adipose tissue normally increase. For example, in pigs, fat content is only 1% at term birth and reaches 12% by 14 days of age (Wu *et al.* 2004a). In contrast, the amounts of brown adipose tissue normally decrease in rodents with increasing age, and virtually disappear shortly after birth in large mammals. Thus, brown adipose tissue is present at relatively low levels in adult rats and is virtually absent from adult humans. Interestingly, under certain conditions (e.g., an increase in NO production, PGC-1α expression, or eukaryotic-translation-initiation-factor-4E binding protein), white adipose tissue may be converted into brown adipose tissue that expresses high levels of uncoupling protein (UCP)-1 (Nisoli & Carruba, 2004). Because of the presence of large amounts of mitochondria and uncoupling proteins, brown adipose tissue is a major site for heat production in newborn mammals and may play an important role in the oxidation of energy substrates in adults.

White adipose tissue is the major site responsible for the substantial change in whole body fat, and plays an important role in energy balance. Adipose tissue fat accretion depends on the balance between the rates of accumulation and breakdown of TAG in adipocytes (Frayn *et al.* 2005). Significant oxidation of glucose and fatty acids occurs in

adipose tissues of animals (Digirolamo *et al.* 1993; Fu *et al.* 2005) and humans (Wu & Marliss, 1992). Whether *de novo* fatty acid synthesis occurs in adipose tissue depends on animal species and age/obesity. For example, fatty acid synthesis is nearly absent from the adipose tissue of humans, chickens or adult rats. Interestingly, white adipose tissue is the major site for *de novo* fatty acid synthesis in pigs and ruminants under normal fed conditions. White adipose tissue, like the liver, is the primary site for *de novo* lipogenesis in dogs, cats, rabbits, and young rodents (Bergen & Mersmann, 2005). In some monogastric animals (e.g., pigs and young rats) and ruminants (e.g., cattle and sheep), glucose and acetate are the major precursors for lipogenesis in adipose tissue, respectively (Bergen & Mersmann, 2005; Wu & Marliss, 1992). As noted above, in fed animals, adipose LPL hydrolyzes chylomicrons and VLDL to produce free fatty acids for TAG synthesis and storage in adipocytes. There are fat depot-specific differences in LPL expression in humans in response to hormonal and nutritional changes (Fried *et al.* 1993a). Interestingly, Kratky *et al.* (2005) recently reported that there was an alternative lipase called endothelial lipase in LPL-deficient mouse adipose tissue that could provide free fatty acids to adipocytes for re-esterification. This endothelial lipase appears to be a phospholipase that utilizes HDL as its main substrate.

During food deprivation, adipose-tissue TAG is hydrolyzed by hormone-sensitive lipase (HSL) and monoacylglycerol lipase into glycerol and free fatty acids. HSL, often considered to be a key regulatory enzyme in lipolysis, is present in the cytoplasm under basal, unstimulated conditions. Interestingly, another lipase, adipose triglyceride lipase localized in lipid droplets, was recently discovered to play a role in initiating the

hydrolysis of TAG to produce diacylglycerol, the preferred substrate for HSL (Zimmermann *et al.* 2004). The adipose triglyceride lipase is highly expressed in the white adipose tissue of humans and mice, but its expression in domestic animals remains to be established. Finally, monoglyceride lipase hydrolyzes monoacyglycerol to form glycerol and free fatty acids, thereby completing the hydrolysis of TAG (Frayn *et al.* 2005; Zechner *et al.* 2005). Owing to a lack of glycerol kinase in adipose tissue, glycerol is not utilized by this tissue but is released from adipocytes into the circulation. The TAG-derived glycerol can be used for hepatic and renal gluconeogenesis. Long-chain free fatty acids released from TAG hydrolysis can be oxidized to provide ATP in adipocytes or are released from the cells. Because of their hydrophobic properties, long-chain fatty acids are transported in blood by albumin and oxidized by most tissues to provide ATP except in the brain (Frayn, 2002). Because white adipose tissue represents a significant portion (15-40% depending on sex and obesity) of total body mass, the metabolism of adipose tissue likely contributes significantly to the whole body homeostasis of glucose and fatty acids (Ceddia, 2005; Shepherd *et al.* 1993). Thus, selective overexpression of the glucose transporter GLUT-4 in adipose tissue enhances glucose transport by adipocytes and improves whole-body insulin sensitivity in mice (Shepherd *et al.* 1993).

Regulation of lipolysis and fatty acid oxidation

Lipolysis and its regulation

Lipolysis is an early, critical event in the utilization of the stored TAG in adipose tissue and other tissues. Lipolysis is tightly regulated by hormones and perilipin. Perilipins are

proteins that coat the surface of lipid droplets in adipocytes and have a protective effect on lipolysis in their non-phosphorylated state (Holm, 2003). The perilipin is part of the PAT-family of proteins, which include perilipin, adipophilin (adipose differentiation-related protein), and TIP47 (the tail-interacting protein of 47 kDa) (Holm, 2003). These proteins are associated with the neutral lipid droplets and share similarities in their nucleotide sequences (Tansey *et al.* 2004). At present, the precise roles of adipophilin and TIP47 in fat metabolism are not known. Upon activation of the lipolysis cascade by adrenergic or other agonists, both HSL and perilipins are phosphorylated by cAMP-dependent protein kinase (PKA), and HSL is also phosphorylated by cGMP-dependent protein kinase (PKG) and AMPK. Phosphorylation activates HSL and also promotes the interaction of perilipins (particularly perilipin A) with HSL. Such an association triggers the translocation of HSL from the cytosolic compartment to the lipid droplet surface to initiate lipolysis (Tansey *et al.* 2004). Glucagon (in young rodents but not in humans) and β-adrenergic agonists (including catecholamines) increase intracellular cAMP levels by activating adenylyl cyclase, whereas insulin reduces intracellular cAMP concentrations by stimulating phosphodiesterase-3B activity (Holm, 2003). Likewise, guanylyl-cyclase activators (e.g., NO and CO) and Viagra (sildenafil citrate) increase intracellular cGMP levels in many cell types (including vascular smooth muscle cells and endothelial cells) by increasing cGMP production and inhibiting phosphodiesterase-5 activity, respectively.

Fatty acid oxidation and its regulation

The mitochondrial ß-oxidation pathway is quantitatively the major pathway for oxidation of short (<C_8), medium (C_8-C_{12}), and long (C_{14}-C_{20}) chain fatty acids in animals. Long-chain fatty acyl-CoA enters mitochondria through the carnitine palmitoyltransferase system. Mitochondrial β-oxidation of long-chain fatty acyl-CoA yields acetyl-CoA, which is oxidized via the mitochondrial Krebs cycle and electron transport system to yield CO_2, water, and ATP. ß-Oxidation of long chain (C_{14-20}) and very long-chain (> C_{20}) fatty acids, as well as dicarboxylic acids, branched-chain fatty acids, and bile acid derivatives, can also occur in peroxisomes, which is independent of the carnitine transport system. Peroxisomal systems I and II are responsible for ß-oxidation of straight, very-long-chain fatty acids and branched, very-long-chain fatty acids, respectively. The short-chain fatty acids produced via the peroxisomal systems enter mitochondria for complete oxidation. The mitochondrial ß-oxidation of fatty acids is enhanced in response to mitochondrial biogenesis induced by transcription factors, such as nuclear respiration factor (NRF)-1, NRF-2, PPARα, and mitochondrial transcription factor A (mtTFA). PGC-1α upregulates the expression of these transcription factors and uncoupling proteins in a cGMP-dependent mechanism (Nisoli *et al.* 2003). Thus, PGC-1α is a master regulator of mitochondrial biogenesis and oxidative phosphorylation. Like PPARα, PPARβ (also called PPARδ) enhances fatty acid oxidation in tissues (e.g., liver, heart, and skeletal muscle) through increases in expression of CPT-1 and enzymes of the β-oxidation pathway (Desvergne *et al.* 2004). PPARγ is expressed predominantly in adipose tissue, and plays an important role in

adipogenesis and lipid storage through increases in LPL expression and TAG synthesis. Synthetic ligands for PPARα, PPARβ, and PPARγ include hypolipidemic drugs (fibrates), HDL, and hypoglycemic drugs (thiazolidinediones, TZDs), respectively (Desvergne *et al.* 2004). Natural/biological PPAR ligands include long-chain and very long-chain fatty acids, high-fat diet, the adrenal steroid dehydroepiandrosterone, and eicosanoids derived from arachidonic acid via the lipoxygenase and cyclooxygenase pathways (Kota *et al.* 2005). The activated PPAR interacts with retinoid X receptor (RXR) to form PPAR/RXR heterodimers, which bind to the peroxisome proliferator response elements localized in numerous gene promotors to increase expression of specific genes. Like any metabolic pathway, although protein phosphorylation and allosteric regulation of key enzymes provide a rapid mechanism for regulation of lipolysis and fatty acid oxidation via changes in the specific activity of an enzyme, gene expression is the most effective mechanism for long-term regulation of these events through alterations in the amount of enzyme protein.

NO and AMPK

AMPK and substrate oxidation

AMP-activated protein kinase (AMPK) plays a crucial role in regulating glucose and fatty acid metabolism in animals. It is a heterotrimeric enzyme consisting of 3 subunits: a catalytic α subunit as well as regulatory β and γ units (Kahn *et al.* 2005a). AMPK acts as a sensor for cellular energy and is activated by an increased [AMP]/[ATP] ratio. Activation of AMPK occurs via phosphorylation by an established upstream AMPK kinase, LKB1. Adiponectin and leptin exert their physiological effects on cells (e.g.,

adipocytes) through activation of AMPK (Kahn *et al.* 2005a). The overall effect of AMPK activation is to switch off the ATP-consuming pathways such as lipogenesis or gluconeogenesis while switching on the ATP-producing pathways such as fatty acid and glucose oxidation. Available evidence suggests that AMPK activity is rapidly altered through protein phosphorylation and dephosphorylation, but a change in its protein level may be regulated at the gene transcriptional level and/or via protein degradation.

Multiple mechanisms are responsible for the action of AMPK on the metabolism of energy substrates. The activation of AMPK stimulates GLUT-4 translocation and glucose transport in both skeletal muscle and cardiac muscle (Hayashi *et al.* 1998; Russell *et al.* 1999; Li *et al.* 2004). This results in an increase in whole-body glucose utilization. In addition, activated AMPK phosphorylates and, thus, inactivates ACC. As a result, the intracellular malonyl-CoA level is reduced, which allows for an increase in fatty acid oxidation (Winder & Hardie, 1996). AMPK also activates malonyl-CoA decarboxylase, the enzyme responsible for the degradation of malonyl-CoA (Park *et al.* 2002a). Glycerol-3-phosphate acyltransferase, the enzyme that catalyzes the first reaction of TAG synthesis, is another target regulated by AMPK. In liver, adipose tissue and muscle, AMPK inhibits glycerol-3-phosphate acyltransferase activity (Park *et al.* 2002a; Muoio *et al.* 1999). AMPK may also enhance expression of PGC-1α in cells (Nisoli *et al.* 2003), thereby promoting mitochondrial biogenesis and oxidative phosphorylation. Finally, AMPK may increase mTOR activity and expression, thereby increasing the synthesis of adipophilin in adipocytes (Chung *et al.* 2005). This may result in reduced expression of perilipin, and, therefore, adipophilin can bind to the

neutral lipid droplets to facilitate TAG hydrolysis. Thus, through multiple mechanisms, AMPK stimulates the catabolism and oxidation of energy substrates.

NO and AMPK

There is an emerging body of evidence indicating that there is a relationship between NO and AMPK expression/activity in cells (Chen *et al.* 1999; Chen *et al.* 2000; Fryer *et al.* 2000; Shearer *et al.* 2004; Zou *et al.* 2003; Pilon *et al.* 2004; Zou *et al.* 2004). Such a relationship cooperatively promotes glucose and fatty acid oxidation. There is ample evidence showing that AMPK regulates NO production in cells. For example, eNOS is phosphorylated by AMPK at position Ser^{1177} (Chen *et al.* 1999). Thus, an increase in AMPK activity under various physiological and pathological conditions can lead to an increase in NO synthesis by eNOS. Indeed, the activation of AMPK in human skeletal muscle during exercise was associated with an increase in the phosphorylation and activation of eNOS (Chen *et al.* 2000). This will increase blood flow and nutrient uptake, thereby favoring the oxidation of both glucose and fatty acids by skeletal muscle. In addition to physiological stimuli, AMPK can be activated by pharmacological agents. For example, AMPK activator, 5-aminoimidazole-4-carboxamide-1-B-D-ribofuranoside (AICAR), enhances NOS activity and glucose transport in muscle cells (Fryer *et al.* 2000). It is noteworthy that both *in vivo* investigations (Shearer *et al.* 2004) and *in vitro* studies involving isolated skeletal (Fryer *et al.* 2000) or cardiac muscles (Li *et al.* 2004) demonstrated that NOS inhibitors diminished AICAR-stimulated glucose uptake, indicating an important role for NO in mediating the role of AMPK in promoting glucose uptake. Most recently, activation of AMPK has been shown to decrease iNOS

expression in myocytes, macrophages, and adipocytes (Pilon *et al.* 2004), suggesting an anti-inflammatory role for AMPK in cells.

While AMPK can regulate NO synthesis, NO can modulate AMPK activity through two mechanisms: a change in gene expression and AMPK activation via peroxynitrite. For example, dietary supplementation of arginine to ZDF rats increased AMPK mRNA levels in adipose tissue approximately 2-fold (Fu *et al.* 2005). Recently, a novel pathway to activate AMPK has been shown in animal cells without a change in [AMP]/[ATP] ratio (Zou *et al.* 2003). Peroxynitrite ($ONOO^-$), generated from the spontaneous reaction of NO with superoxide anion (O_2^-), activates AMPK through a c-Src-mediated and phosphatidylinositol 3-kinase (PI3K)-dependent pathway in cultured bovine aortic endothelial cells as well as mouse aorta and hearts (Zou *et al.* 2003). Although $ONOO^-$ at high levels (>100 μM) can cause oxidative stress, $ONOO^-$ at 1-10 μM activates AMPK (Zou *et al.* 2004). The anti-diabetic drug metformin enhances AMPK activity through this distinct pathway by increasing $ONOO^-$ production within a physiological range (Zou *et al.* 2004). Conversely, an inhibition of $ONOO^-$ formation either by scavenging O_2^- or by using NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) diminished metformin-induced AMPK activation. Importantly, in eNOS knock-out mice, metformin had no effect on AMPK activity in endothelial cells, which emphasizes the importance of endogenous NO synthesis in AMPK activation. The PI3K activation by $ONOO^-$ may specifically increase the association of AMPK with its upstream kinase LKB1 without changing the activity of LKB1 (Zou *et al.* 2004). These findings support

the conclusion that physiological levels of free radicals are signaling molecules involved in metabolic regulation (Wu *et al.* 2004c).

NO and mitochondrial function

NO and mitochondrial respiration

Recently, much attention has been directed towards the identification of mitochondrial NOS and the effect of NO on mitochondrial respiration (Ghafourifar & Richter, 1997; Tatoyan & Giulivi, 1998; Clementi & Nisoli, 2005). There is substantial evidence showing the presence of nNOS in the mitochondria (also known as mitochondrial NOS or mtNOS) of many mammalian tissues, such as brain, liver, muscle, heart, and kidney (Bates *et al.* 1996). Thus, it is reasonable to expect that NO plays a role in mitochondrial oxidation and oxygen sensitivity of respiration in tissues. NO regulates mitochondrial respiration through distinct mechanisms. First, physiological levels of NO increase blood flow, therefore increasing the supply of metabolic substrates and oxygen to mitochondria. Second, high levels of NO may directly modulate the activity of the mitochondrial electron transport system (Brown, 2001). For example, at concentrations that were likely above physiological levels of NO in mitochondria, exogenous NO was shown to reversibly bind the oxygen-binding site of cytochrome c oxidase (the terminal enzyme in the mitochondrial respiratory chain), thus inhibiting electron transport and oxygen consumption. The result was a reduced supply of ATP from substrate oxidation and enhanced glycolysis as a significant alternative ATP-producing pathway (Nisoli *et al.* 2003; Brown, 2001; Nisoli *et al.* 2004a). This mechanism may play an important role in acute oxygen sensing and in adaptation to low-oxygen conditions when NO levels are

high. However, under normal physiological conditions, acetyl-CoA produced from energy substrates is readily oxidized in mitochondria (Jungas *et al.* 1992). Thus, it is unlikely that endogenous NO generated by nNOS and eNOS inhibits mitochondrial respiration in animal cells when iNOS is expressed at a low level.

NO and mitochondrial biogenesis

NO has been shown to have a beneficial effect on mitochondrial biogenesis, i.e. mitochondrial proliferation and activation (Nisoli *et al.* 2003). NO-induced mitochondrial biogenesis is a key to long-term regulation of cellular metabolism (Clementi & Nisoli, 2005). In a variety of mammalian cell lines, such as brown adipocytes, 3T3-L1 cells, skeletal muscle L6 cells, and immortalized Hela cells, NO triggers mitochondrial biogenesis and enhances coupled respiration and ATP concentrations (Nisoli *et al.* 2003; Nisoli *et al.* 2004a). This process is mediated via a cGMP-dependent signaling pathway that activates the expression of PGC-1α, a master regulator of mitochondrial biogenesis and oxidative phosphorylation (Nisoli *et al.* 2003). As noted above, PGC-1α is a key transcriptional cofactor regulating the expression of PPARα, which in turns stimulates the expression of NRF-1 and mtTFA (Wu *et al.* 1999b). Collectively, these transcription factors regulate expression of nuclear and mitochondrial genes encoding proteins that are involved in the respiratory chain as well as mitochondrial DNA transcription and replication (Bossy-Wetzel & Lipton, 2003; Nisoli *et al.* 2004b). Thus, eNOS-knockout mice exhibited a reduced level of mitochondrial content in many tissues, such as brain, liver, and muscle, which is

accompanied by reduced O_2 consumption, reduced steady-state ATP levels, defective energy expenditure, and increased body- fat gain (Nisoli *et al.* 2003; Nisoli *et al.* 2004a).

The actions of NO on mitochondrial function are coordinated to meet the energy requirement of the cell. The mitochondrion is an important site for the oxidation of glucose, fatty acids, and amino acids, where the released chemical energy is either coupled with ATP synthesis or dissipated as heat. Therefore, mitochondria play a crucial role in regulating energy balance (Bossy-Wetzel & Lipton, 2003). Since NO stimulates the formation of metabolically active new mitochondria, the endogenous or exogenous provision of NO is of great interest regarding its effect on the treatment of obesity. In rat skeletal muscle, incubation with sodium nitroprusside (SNP; an NO donor) increased the oxidation of glucose, pyruvate, palmitate and leucine via a cGMP-dependent pathway (Young *et al.* 1997; Young & Leighton, 1998a). Likewise, dietary supplementation of arginine to ZDF rats increased expression of PGC-1α as well as the oxidation of glucose and fatty acids in abdominal fat tissue (Fu *et al.* 2005). These findings provide a promising solution to reducing fat mass through modulation of the arginine-NO pathway in animals and humans and emphasize the metabolic basis for the pharmacotherapy of arginine (Flynn *et al.* 2002).

The role for the arginine-NO pathway in glucose metabolism

NO and gluconeogenesis/glycogen synthesis

There is evidence suggesting a role for NO in regulating hepatic gluconeogenesis. The conversion of lactate and pyruvate into glucose in rat hepatocytes is inhibited by NO donors in a dose-dependent manner (Horton *et al.* 1994). The mechanism may involve

an inhibition of the activity and/or expression of phosphoenol-pyruvate carboxykinase (Horton *et al.* 1994), which is now known to be mediated by AMPK (Kahn *et al.* 2005a). On the other hand, glycogen synthesis from glucose is inhibited by NO in hepatocytes (Spranger *et al.* 1998) and 3T3-L1 cells (Egan *et al.* 1995) due to a decreased activity of glycogen synthase (Spranger *et al.* 1998). Thus, elevated levels of NO are expected to increase the release of glucose from the liver under conditions of iNOS induction. Interestingly, NO also reduces glycolysis in hepatocytes by decreasing the activities of key enzymes such as glucokinase (Monti *et al.* 2000) and glyceraldehyde-3-phosphate dehydrogenase (Stadler *et al.* 1995) in coordinate action to spare glucose utilization. However, the net effect of NO on hepatic glucose metabolism likely depends on the balance between the two opposing effects (synthesis and utilization). We recently found that dietary supplementation of arginine, which increased NO synthesis in various tissues within physiological ranges (Alderton *et al.* 2001), normalized plasma glucose levels in streptozotocin-induced diabetic rats (Kohli *et al.* 2004), and attenuated hyperglycemia in ZDF rats (Fu *et al.* 2005).

NO and glucose transport

Glucose transport across the cell membrane by glucose transporter 4 (GLUT-4) is a key regulatory step for glucose utilization in skeletal muscle, heart, and adipose tissue. Insulin is a major hormone regulating glucose uptake by these tissues. It stimulates the phosphorylation of an insulin receptor substrate (IRS) and the activation of PI3K. In skeletal muscle, in addition to insulin, contraction or exercise also enhances glucose uptake (Barnard & Youngren, 1992; Nesher *et al.* 1985). However, unlike the insulin

signaling pathway, exercise/contraction has no effect on insulin receptor phosphorylation or PI3K activity (Jessen & Goodyear, 2005). Two different intracellular pools of GLUT-4 may exist in skeletal muscle and respond to the two different signaling pathways (Jessen & Goodyear, 2005). When activated, GLUT-4 undergoes translocation from an intracellular tubulovesicular reservoir to the plasma membrane of skeletal muscle cells (Coderre *et al.* 1995; Kahn, 1992) or adipocytes (McPherson & Jones, 2003). Compelling evidence shows that NO mediates the stimulatory effect of exercise on glucose transport by skeletal muscle. For example, NO donors dose-dependently increased glucose transport in isolated skeletal muscle (Jessen & Goodyear, 2005; Balon & Nadler, 1997; Bradley *et al.* 1999). Exercise increases eNOS gene expression (Sessa *et al.* 1994), eNOS protein levels (Balon & Nadler, 1997), and eNOS activity (Roberts *et al.* 1999) in skeletal muscle, as well as vascular NO production (Sessa *et al.* 1994; Matsumoto *et al.* 1994; Patil *et al.* 1993). Conversely, an inhibition of NOS activity reduced both basal and exercise-stimulated glucose transport in muscle (Balon & Nadler, 1997). Further, Roberts *et al.* (1997) reported that exercise increased sarcolemma GLUT-4 content in rat skeletal muscle, and this effect was blunted by administration of L-NAME, a competitive NOS inhibitor. Thus, the infusion of an NOS inhibitor during cycling exercise reduced leg glucose uptake, which was overcome by co-infusion with L-arginine (Bradley *et al.* 1999). In addition to muscle, NO stimulates glucose transport in 3T3-L1 adipocytes through GLUT-4 translocation to the plasma membrane via a mechanism independent of the insulin-signaling pathway (Tanaka *et al.* 2003).

NO may mediate the effect of insulin in stimulating glucose transport in skeletal muscle and adipose tissue. Administration of L-NAME impaired insulin secretion and resulted in reduced glucose tolerance in rats (Roy *et al.* 1998; Balon *et al.* 1999; Baron *et al.* 1995b) and humans (Laakso *et al.* 1992). Another competitive NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), also attenuated the insulin-mediated increase in glucose uptake by muscles, including heart, soleus muscle, extensor digitorum longus (EDL) muscle, and gastrocnemius muscle (Roy *et al.* 1998). Infusion of L-NMMA to rats also inhibited glucose uptake in many adipose tissue depots, such as brown interscapular, retroperitoneal and gonadal adipose tissues (Roy *et al.* 1998). This is consistent with the recent observation that there is an inverse relationship between plasma levels of L-NMMA and glucose disposal in obese or aging subjects (Marliss *et al.* 2006). In cultured 3T3-L1 adipocytes, addition of SNP increased glucose uptake, while treatment with an NO scavenger or guanylate cylase inhibitor reduced the SNP-stimulated glucose uptake to basal levels (Tanaka *et al.* 2003). Of note, some studies indicated that NOS inhibitors had no effect on insulin-stimulated glucose transport in incubated skeletal muscle (Balon & Nadler, 1997; Roberts *et al.* 1997), but NO production was not directly measured in these short-term experiments. The disparity between these *in vitro* and *in vivo* findings may be explained by: 1) microvascular involvement, which occurs *in vivo* but is virtually absent from an incubated tissue (Vincent *et al.* 2003); and 2) NO concentrations in the target site. Thus, the roles of NO in enhancing glucose uptake by insulin-sensitive tissues appear to involve both blood flow-dependent and independent mechanisms. NO is a major vasodilator, and thus an

increase in physiological levels of NO is expected to increase the delivery of glucose to muscle cells and adipocytes (Baron, 1994; Baron *et al.* 1995a). Consequently, an inhibition of NO synthesis by L-NMMA reduced endothelium-dependent blood flow and inhibited insulin-stimulated glucose uptake in humans (Baron *et al.* 1995b).

NO and glycolysis/glucose oxidation

In addition to increasing glucose transport, NO also mediates glucose metabolism via glycolysis and the Krebs cycle in skeletal muscle. For example, incubation of rat soleus muscle with SNP significantly increased lactate release and glucose oxidation in a dose-dependent manner (1-25 mM of SNP) (Young *et al.* 1997; Young & Leighton, 1998a; Young & Leighton, 1998b). This finding is interesting in that high levels of NO donors did not inhibit mitochondrial respiration in skeletal muscle. It is noteworthy that dietary arginine supplementation enhanced glucose oxidation in both retroperitoneal and epididymal fat tissues of ZDF rats (Fu *et al.* 2005). These findings support the view that physiological levels of NO stimulate glucose oxidation in skeletal muscle and adipose tissue.

The mechanism for the action of NO on glucose metabolism in skeletal muscle and adipose tissue is mediated through cGMP production and PKG signaling. Soluble guanylate cyclase activity is present in skeletal muscle (Vesely, 1979; Kojima *et al.* 1995) and adipose tissue (Engeli *et al.* 2004). Indeed, incubation with NO donors rapidly increased the cGMP level in skeletal muscle, whereas the addition of a guanylate cyclase inhibitor blocked this effect (Young & Leighton, 1998a; Young & Leighton, 1998b). The inhibitor also blocked NO donor-stimulated glucose transport and utilization

(Young *et al.* 1997; Young & Leighton, 1998a). Likewise, a cGMP analogue stimulates glucose metabolism in skeletal muscle (Young *et al.* 1997). In ZDF rats, an increase in cGMP level in response to an inhibition of its hydrolysis was associated with elevated glucose transport and glucose oxidation in soleus muscle (Young & Leighton, 1998b). However, in insulin-resistant obese Zucker rats, this inhibitor failed to increase the cGMP level or improve glucose utilization (Young & Leighton, 1998b). These results suggest that the NO/cGMP pathway is altered in skeletal muscle of obese or diabetic subjects.

The role for the arginine-NO pathway in lipid metabolism

NO synthesis by iNOS and obesity

Obesity is associated with an increase in expression of iNOS and overproduction of NO. Gene expression of iNOS in subcutaneous adipose tissue and adipocytes was higher in obese compared with nonobese humans (Giordano *et al.* 2002). In adolescents, elevated serum NO_x levels (an indicator of systemic NO synthesis) were strongly correlated with increased body adiposity (Choi *et al.* 2001). Although eNOS expression is also enhanced in adipocytes of obese subjects, iNOS is primarily responsible for the increased production of NO in obesity-related metabolic diseases. In genetically obese and diabetic rodent models, such as ob/ob mice and ZDF rats, iNOS protein expression in both adipose tissue and skeletal muscle was higher in comparison with the lean, non-obese wild-types (Fujimoto *et al.* 2005; Perreault & Marette, 2001). Remarkably, treatment with an iNOS inhibitor (Fujimoto *et al.* 2005) or selectively disrupting the gene encoding iNOS (Perreault & Marette, 2001) improved insulin sensitivity in ob/ob

mice and ZDF rats, thereby preventing these animals from diet-induced obesity. In support of these *in vivo* findings, high levels of NO in culture medium promoted the differentiation of pre-adipocytes of white adipocyte- or brown adipocyte-lineage into mature adipocytes (Yan *et al.* 2002; Nisoli *et al.* 1998). We hypothesize that an inhibition of inducible NO synthesis may inhibit PPARγ expression, and, thus, adipocyte differentiation and proliferation.

NO and lipogenesis/lipolysis

The release of nonesterified fatty acids (NEFA) from adipose tissue plays an important role in the development of obesity and insulin resistance (Wu *et al.* 2004a). There is some evidence showing that NO affects lipid metabolism in adipose tissue, including both lipogenesis and lipolysis. However, the current literature is rather confusing. Whether exogenous NO stimulates or inhibits lipid metabolism appears to depend on the types and doses of NO donors, tissue site, and intracellular redox state (Gaudiot *et al.* 1998; Gaudiot *et al.* 2000; Fruhbeck & Gomez-Ambrosi, 2001). For example, we found that 1 to 5 μM SNP stimulated lipolysis, but 25 to 500 μM SNP inhibited lipolysis in the adipose tissue of obese humans in a concentration-dependent manner (Wu *et al.* 2007a). Of note, atrial natriuretic peptide (ANP) induced lipolysis in human adipose tissue via the NO-cGMP pathway, but had no effect in rat adipose tissue (Fain *et al.* 2003). Further, Gaudiot and coworkers demonstrated that different NO donors had differential effects on basal or stimulated lipolysis in isolated adipocytes (Gaudiot *et al.* 1998), and this modulation was mediated through an antioxidant-related effect (Gaudiot *et al.* 2000). Additionally, some studies showed that NO donors increased basal lipolysis

(Fruhbeck & Gomez-Ambrosi, 2001) but inhibited isoprenaline-induced lipolysis in adipocytes (Lincova *et al.* 2002). It should be borne in mind that these *in vitro* studies involved NO donors at concentrations ranging from 20 μM to 2 mM (e.g. 0.5-2 mM S-nitroso-N-acetyl-penicillamine (SNAP) or 20 μM – 2 mM SNP). Therefore, the physiological significance of the findings is not clear at present. High levels of NO may oxidize and inactivate catecholamines non-enzymatically, thereby reducing the rate of stimulated lipolysis in adipocytes.

Although the responses of fat metabolism to exogenous NO donors appear to vary greatly, *in vivo* studies involving an inhibition of NO synthesis have consistently demonstrated that physiological levels of NO increase lipolysis in rats. For example, NO mediates the stimulatory effect of leptin on lipolysis in adipose tissue of rats (Fruhbeck & Gomez-Ambrosi, 2001). In addition, dietary supplementation of arginine to adult ZDF rats, which increased NO synthesis, stimulated lipolysis and fatty acid oxidation in adipose tissue, contributing to a marked reduction in body fat mass (Fu *et al.* 2005).

Hypocholesterolemic effect of NO

Because hypercholesterolemia is a significant risk factor for endothelial dysfunction, there is increasing interest in its prevention through dietary and pharmacological means. As noted above, NO plays an important role in the regulation of fatty acid metabolism in liver. Compelling evidence shows that NO donors exhibit a hypocholesterolemic effect by mediating lipoprotein metabolism. For example, administration of an NO donor to rabbits caused a decrease in plasma LDL level (Kurowska & Carroll, 1998), suggesting a

role for NO in inhibiting LDL production. In support of this view, SNP reduced the release of apo B (the major protein component of LDL) from incubated hepatocytes (Kurowska & Carroll, 1998). Conversely, feeding the rats with NOS inhibitors (0.02% in the diet) resulted in marked increases in serum triglyceride, cholesterol, and VLDL, as well as body fat (Khedara *et al.* 1996). NO regulates hepatocyte lipid metabolism mainly by inhibiting *de novo* fatty acid synthesis and decreasing the activity of ACC (Garcia-Villafranca *et al.* 2003). As a result, malonyl-CoA level is decreased in response to NO, which activates CPT-I activity and stimulates fatty acid oxidation in liver.

The effects of competitive NOS inhibitors on elevating serum fatty acids, LDL and VLDL could be reversed by the co-administration of arginine (Khedara *et al.* 1999). Thus, in streptozotocin-induced diabetic rats, treatment with 2% arginine in their diet attenuated the elevated serum levels of TAG and LDL by about 20% (Miguez *et al.* 2004). Likewise, arginine treatment improved lipid metabolism in genetically diabetic and fatty rats (Kawano *et al.* 2003). Most recently, Fu *et al.* (2005) reported that a long-term arginine treatment reduced retroperitoneal fat mass by 45% and had no effect on the weight of other tissues in ZDF rats. Importantly, an increase in dietary arginine intake from 2.5 to 5-7.5 g/day is associated with a rise in the serum level of HDL and a decrease in systolic blood pressure in humans (Wells *et al.* 2005). Thus, in addition to its beneficial effect on endothelial function (Wu & Meininger, 2000), dietary arginine supplementation may reduce plasma levels of VLDL and triglycerides through NO production.

The role for the arginine-NO pathway in amino acid metabolism

NO and amino acid oxidation/ureagenesis

Although arginine is the nitrogenous substrate for NO synthesis, little is known about the effects of physiological levels of NO on the metabolism of amino acids in animal cells. Limited evidence suggests that high levels of exogenous NO may indirectly increase rates of amino acid oxidation in the liver on the basis of enhanced urea synthesis by rat hepatocytes perfused with 0.2 mM SNP (Farghali *et al.* 1997). Interestingly, Stadler *et al.* (1995) reported that 0.05 to 5 mM SNAP did not affect urea production by rat hepatocytes incubated with 10 mM NH_4Cl (~100 times the physiological level of plasma NH_4^+) as the major source of exogenous nitrogen. Such a high level of ammonia might have maximized the capacity of hepatic ureagenesis, and, thus, urea production could not be further increased in response to NO provision.

Recent studies indicate that PPARα is a negative regulator of the urea cycle via down-regulation of the expression of carbamoylphosphate synthase-I, ornithine carbamoyltransferase, argininosuccinate synthase, and argininosuccinate lyase in the liver (Desvergne *et al.* 2004). Thus, in contrast to a high level of NO that may occur under conditions such as sepsis and inflammation, physiological levels of NO may reduce the expression of urea-cycle enzymes and thus urea synthesis from ammonia indirectly through enhanced expression of PPARα. This action of NO is expected to inhibit amino acid oxidation in the liver, thereby increasing the entry of dietary amino acids into the systemic circulation. It is likely that NO exhibits differential effects on amino acid synthesis and catabolism, depending on target cells and tissues, its local

concentration in the reaction site, interactions among signaling pathways, and nutritional states.

NO and intracellular protein turnover

In animal cells, amino acids are utilized for the synthesis of proteins, and proteins are continuously degraded to produce amino acids. This constitutes an intracellular protein turnover. At present, little is known about the effect of physiological levels of NO on protein turnover in cells. However, exposure of rat hepatocytes to high levels of exogenous NO has been reported to decrease protein synthesis (Curran *et al.* 1991), thereby increasing the availability of amino acids for catabolism. Interestingly, an increase in NO synthesis by iNOS mediates the effect of tumor necrosis factor-α on inhibiting protein synthesis and stimulating protein degradation in skeletal muscle (Buck & Chojkier, 1996). A catabolic effect of high levels of NO on protein metabolism in liver and skeletal muscle is consistent with the enhanced hepatic ureagenesis under septic and inflammatory conditions. Whether physiological levels of NO regulate protein turnover warrants investigation. Of note, physiological levels of NO stimulate the release of anabolic hormones (e.g., insulin and growth hormone) in both birds and mammals (Flynn *et al.* 2002), and, thus, are expected to promote a positive protein balance, particularly in young and growing animals.

NO and polyamine synthesis

Polyamines play a critical role in regulating DNA and protein synthesis, as well as cell proliferation and differentiation (Flynn *et al.* 2002). In vascular smooth muscle cells, the presence of extracellular NO donors (30 to 300 μM) inactivated ornithine decarboxylase,

thereby inhibiting polyamine synthesis from arginine/ornithine and cell proliferation in a dose-dependent manner (Ignarro *et al.* 2001). This effect of NO is beneficial in preventing atherosclerosis in animals and humans. In contrast, culture of endothelial cells with a lower extracellular concentration of an NO donor (10 μM) or with sepiapterin (a precursor for BH4 synthesis) increased polyamine synthesis and cell proliferation (Marinos *et al.* 2001). Such an effect would play an important role in angiogenesis and wound healing under various physiological conditions.

NO and the metabolic syndrome

Available evidence shows that the oxidation of fatty acids, glucose, and amino acids involves the exquisite coordination of multiple organs in animals. Since its initial description by Reaven (1988), there has been considerable debate about the precise definition of the metabolic syndrome or Syndrome X (Kahn *et al.* 2005b). Nonetheless, a cluster of key characteristics of the metabolic syndrome includes obesity, hyperglycemia (fasting serum glucose > 6.1 mM), hyperinsulinemia, hyperlipidemia, hypertension, and insulin resistance (an impaired response of cells or tissues to physiological concentrations of insulin). These factors, independently or collectively, contribute to a high risk for cardiovascular disease, a major cause of death in developed nations. Glucosamine, an inhibitor of NO synthesis by constitutive NOS (Wu *et al.* 2001), may contribute to the development of insulin resistance in the vasculature and skeletal muscle of obese or diabetic subjects. As noted in the preceding sections, physiological levels of NO can ameliorate all of these adverse features of the metabolic syndrome in animal models of obesity. Thus, administration of arginine can reduce plasma levels of glucose,

homocysteine, fatty acids, and triglycerides in streptozotocin-induced diabetic rats (Kohli *et al.* 2004) and ZDF rats (Fu *et al.* 2005), and can also improve insulin sensitivity in obese humans (Hardie, 2004; Khedara *et al.* 1999; Miguez *et al.* 2004; Piatti *et al.* 2001; Mendez & Balderas, 2001). A distinct advantage of arginine over drugs (e.g., metformin and thiazolidinediones) is that dietary arginine supplementation will not increase body fat mass. Another advantage of the arginine solution is to reverse endothelial dysfunction associated with major cardiovascular risk factors (hypercholesterolemia, smoking, hypertension, diabetes, obesity, insulin resistance, and aging) (Wu & Meininger, 2000) and to prevent ammonia toxicity and organ dysfunction brought about by hypoargininemia (Flynn *et al.* 2002; Wu *et al.* 2004b). Arginine or its effective precursor (citrulline) may provide a potentially novel, effective treatment for the prevention and treatment of the metabolic syndrome in humans and animals.

Summary and objectives

Nitric oxide (NO) is synthesized from L-arginine by NO synthase in virtually all cell types. Emerging evidence shows that NO regulates the metabolism of glucose, fatty acids, and amino acids in mammals. As an oxidant, pathological levels of NO inhibit nearly all enzyme-catalyzed reactions through protein oxidation. However, as a signaling molecule, physiological levels of NO stimulate glucose uptake as well as glucose and fatty-acid oxidation in skeletal muscle, heart, liver, and adipose tissue, inhibit the synthesis of glucose, glycogen and fat in target tissues (e.g., liver and adipose), and enhance lipolysis in adipocytes. Previous studies demonstrated that an inhibition of NO

synthesis causes hyperlipidemia and fat accretion in rats, whereas our laboratory showed that dietary arginine supplementation reduces fat mass in diabetic fatty rats.

The overall objective was to evaluate the effect of dietary arginine supplementation on the growth rate, fat mass, and energy-substrate utilization of diet-induced-obese rats. Gene and protein levels in major insulin-sensitive tissues (liver, muscle and fat) were examined in an effort to explain, in part, the molecular mechanisms of the beneficial effect of dietary arginine on obesity. The findings from the current study will provide a scientific basis for designing a novel, safe and effective way to prevent and treat obesity and/or the metabolic syndrome in humans.

Hypothesis

Our hypothesis was that dietary arginine supplementation might increase NO production and activate AMPK in diet-induced obese (DIO) rats, thus improving glucose and fatty acid metabolism in insulin-sensitive tissues in these rats, thereby decreasing their body fat mass.

Specific aims

Specific aims were to

1. evaluate the effect of dietary arginine supplementation on fat mass in DIO rats;
2. determine the molecular mechanism for the effect of fat loss in response to arginine supplementation;
3. define a role of NO in regulation of glucose and fatty acid metabolism in liver cells, muscle cells and adipocytes.

CHAPTER II

DIETARY L-ARGININE SUPPLEMENTATION REDUCES FAT MASS AND IMPROVES INSULIN SENSITIVITY IN DIET-INDUCED OBESE RATS

This study was conducted to test the hypothesis that dietary arginine supplementation reduced fat mass in diet-induced obese rats. Male Sprague-Dawley rats were fed either low- or high-fat diets for 15 wks (16 rats/diet). Thereafter, lean or obese rats continued to be fed their same respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) (n=8/treatment group). Twelve weeks after the initiation of the arginine treatment, serum and tissue samples were collected for biochemical assays. Results were statistically analyzed as a 2x2 factorial experimental design using ANOVA. When compared with rats fed the low-fat diet, high-fat diet increased the mass of retroperitoneal (RP) adipose tissue, epididymal (EP) adipose tissue, subcutaneous (SC) adipose tissue, and mesenteric (MT) adipose tissue by 76%, 49%, 96% and 64%, respectively, resulting in a heavier body weight. Concentrations of cholesterol in serum as well as lipids in skeletal muscle and liver were higher in obese rats, in comparison with lean rats. Arginine supplementation reduced the mass of RP, EP, SQ and MT fat pads by 35%, 20%, 32%, and 40%, respectively, while increasing the mass of extensor digitorum longus and soleus muscles, compared with alanine-supplemented rats. Serum leptin was elevated in obese rats but reduced in arginine-supplemented rats, whereas serum levels of other hormones (insulin, adiponectin, growth hormone, corticosterone, total T_3, and total T_4) did not differ.

Notably, arginine treatment reduced serum levels of glucose and triglycerides, and improved glucose tolerance. Collectively, these findings indicate that through reducing excess fat and improving insulin sensitivity, dietary arginine supplementation may provide a novel means for the treatment of obesity.

Obesity is caused by a chronic imbalance in energy metabolism, namely a greater energy intake than energy expenditure (Bray *et al.* 2004). This metabolic disorder continues to increase worldwide with an alarming rate in the past decade. It is a major health problem that impacts both adults and children (Bray & Bellanger, 2006). Particularly, obesity is closely associated with many diseases and is a major risk factor for insulin resistance, type II diabetes, atherosclerosis, stroke, hypertension, and some types of cancer (Nisoli & Carruba, 2004). The prevalence of obesity and the tremendous costs of its treatment necessitate the search for new alternative nutritional means.

In our recent studies aimed at improving vascular function in the Zucker diabetic fatty (ZDF) rat, we found that dietary supplementation with arginine selectively reduced fat mass by stimulating nitric oxide synthesis (Fu *et al.* 2005). The ZDF rat has a defect in the leptin receptor and is a genetically obese animal model of type II diabetes (Clark *et al.* 1983). While the finding with this animal model is important, its relevance to diet-induced obesity is not known. Although obesity results from complex interactions between genetic and environmental factors, recent studies indicate that diet plays a more important role in the current obesity epidemic than genetic factors (Hill & Peter, 1998). Therefore, the objective of the present study was to determine if dietary arginine

supplementation could decrease fat tissue and improve glucose tolerance in diet-induced obese (DIO) rats.

Materials and methods

Chemicals

Hexokinase and glucose-6-phosphate dehydrogenase were obtained from Roche Diagnostics (Indianapolis, IN). HPLC-grade methanol and water were purchased from Fisher Scientific (Houston, TX). Unless indicated, all other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Animals and diets

Male Sprague-Dawley rats (23-d-old, 80-100 g) were purchased from Harlan Laboratories (Indianapolis, IN). Upon arrival at the Texas A&M University Kleberg animal facilities, all rats were housed in individual cages in a temperature- and humidity-controlled room on a 12-h light:12-h dark cycle. After a 5-d period of adaptation during which animals were fed a regular rodent chow, rats were randomly assigned to either a low- (LF) or high-fat (HF) diet (n=16/diet) obtained from Research Diets (New Brunswick, NJ) (Table 2.1). The low- and high-fat diets provided 10% and 40% of total calories as fat, respectively. Body weight and food intake were recorded on a weekly basis during this initial phase of the study. After 15 wks of feeding, each group of rats was divided randomly into two sub-groups, which continued to be fed their same respective diets and received drinking water containing either 1.51% L-arginine-HCl (Arg) or 2.55% alanine (Ala; isonitrogenous control) (n=8/treatment group). The dosage

of arginine, as well as alanine as isonitrogenous control, was chosen on the basis of our previous studies with non-diabetic and diabetic rats (Kohli *et al.* 2004; Fu *et al.* 2005). Arginine-supplemented rats were individually pair-fed with alanine-supplemented rats on a kg body weight basis to ensure similar intakes of all nutrients except for arginine and alanine between the two groups of animals. Body weight, food intake and water intake of each rat were recorded on a daily basis throughout this supplementation phase of the study. After 12 wks of arginine supplementation, rats were fasted for 5 h to obtain blood samples (100 μL) for analyses of serum glucose and amino acids. Rats were then immediately anesthetized with CO_2 and euthanized by cervical dislocation. Cardiac blood samples were collected and centrifuged immediately to obtain sera for analyses of lipids and hormones. Tissues were dissected and weighed. Small portions of each tissue were either used freshly for metabolic assays or snap-frozen rapidly in liquid nitrogen for storage at -80^{o}C. This study was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Oral glucose tolerance test (OGTT)

An OGTT was performed at 10 wks after the initiation of arginine supplementation, as described by Vital *et al*. (2006) with modifications. After a 5-h fast, glucose (2 g/kg body weight) in water was administrated orally into stomach by gavage. Blood samples (20 μL) were obtained from the tail vein into plain tubes using microhematocrit capillary tubes at 0, 30, 60, 90, 120, 150, and 180 min after gavage. Blood samples were centrifuged immediately at 10,000 g for 1 min to obtain sera, which were stored at -80^{o}C until analysis for glucose.

Table 2.1. Composition of low- and high-fat diets

Ingredient	Low-fat diet		High-fat diet	
	g	Kcal	g	Kcal
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1260	72.8	291
Maltodextrin-10	35	140	100	400
Sucrose	350	1400	172.8	691
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	177.5	1598
Mineral Mix S10026	10	0	10	0
Dicalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Yellow Dye	0.05	0	-	0
Red Dye	-	0	0.05	0
Total	1055.05	4057	858.05	4057

Biochemical analyses of serum

Glucose was determined enzymatically using a fluorometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Kohli *et al.* 2004). Amino acids

were quantified using fluorometric HPLC methods after a derivatization reaction with o-phthaldialdehyde (Kohli *et al.* 2004). Assay kits for rats were employed for the following analyses: triglycerides, total cholesterol (Thermo DMA, Louisville, CO), free fatty acid (FFA; Wako Chemicals, Richmond, VA), insulin, leptin, adiponectin, growth hormone (Linco, St. Louis, MO), T3, T4, and coricosterone (MP Biomedicals, Orangeburg, NY).

Biochemical analyses of liver, skeletal muscle, and adipose tissue

Lipids in liver, gastrocnemius muscle and retroperitoneal (RP) adipose tissue were determined using the Folch method, in which a chloroform and methanol mixture (2:1 v/v) was used to extract lipid from tissues (Folch *et al*. 1957). Glycogen in liver and gastrocnemius muscle were measured by an enzymatic method involving amyloglucosidase, as described previously (Passoneau & Lauderdale, 1974). Glutathione (GSH; an indicator of oxidative stress (Wu *et al.* 2004c)) in liver was determined by an HPLC method after derivatization with dansyl chloride (Jones *et al*. 1998). Tetrahydrobiopterin (BH4; an indicator of the capacity for nitric oxide synthesis (Shi *et al.* 2004)) in liver was analyzed using an HPLC method (Meininger & Wu, 2002).

Calculations and statistical analysis

Results were expressed as means ± SEM. Data on body weight were analyzed using the growth curve model, namely a mixed effect model that fits fixed effects and random effects. The fixed effects include diet, amino acid (AA) treatment, their interaction (diet x AA), a third order polynomial of age in weeks (slope by age effect, curvature by age x age, and third order effect by age x age x age), different slopes by diet (age x diet), and

different slopes by treatment (age x AA). The random effects allow different rats to have different slopes (random age). The SAS PROC MIXED procedure was used to fit the mixed effects model. The oral glucose tolerance test was assessed by calculating the area under curve (AUC). Data on tissue weights, biochemical metabolites, and OGTT were analyzed statistically by two-way ANOVA using a General Linear Model by SPSS software (Version 12.0, Chicago, IL). The Tukey multiple comparison test was used to determine the effects of two main factors (diet and AA), and their interaction (diet x AA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

Intakes of food, water and energy

The high-fat diet contained more energy, protein, vitamins, and minerals than the low-fat diet. However, rats fed the high-fat diet consumed ($P<0.01$) proportionally less food than those fed the low-fat diet. Food intake did not differ ($P>0.10$) between arginine- and alanine-supplemented rats. Neither high-fat diet nor arginine supplementation affected ($P>0.10$) water consumption by rats. Total energy intake or intakes of nutrients (including alanine and arginine) from enteral diet did not differ ($P>0.10$) among the four groups of rats (Tables 2.2 and 2.3).

Body weight

Rats fed the high-fat diet gained 13.3% more body weight than rats fed the low-fat diet between 4 and 19 wks of age prior to arginine treatment (Fig. 2.1). After 15 wks of age, the growth rates of all rats slowed down. At 31 wks of age when the study was

completed, the average body weight of rats fed the high-fat diet was 15.3% greater than that of rats fed the low-fat diet (P<0.0001). Arginine treatment for 12 wks tended (P = 0.067) to reduce body weight.

Table 2.2. Intakes of food, water and energy by rats

Intake	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Food (g/kg body wt·d)	33.1±0.5	32.9±0.6	29.7±0.3	29.5±0.3	0.006	0.78	0.95
Water (mL/kg body wt·d)	70.8±1.1	77.7±1.8	74.8±1.8	73.8±1.9	0.97	0.46	0.30
Protein (g/kg body wt·d)	5.94±0.18	5.88±0.17	6.03±0.18	5.90±0.16	0.71	0.64	0.78
Minerals (mg/kg body wt·d)	905±27	901±27	911±23	902±25	0.88	0.82	0.92
Vitamins (mg/kg body wt·d)	358±11	357±11	364±10	358±10	0.74	0.71	0.80
Energy* (KJ/kg body wt·d)	600±18	589±17	603±19	583±17	0.96	0.82	0.32

Rats were fed with either low- or high-fat diet. In each diet group, rats were randomly assigned to receive drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. Data are expressed as means ± SEM (n=8).
* Total energy intake from both diet and drinking water.

Table 2.3. Intakes of arginine and alanine by rats

Intake	Low fat		High fat		*P* value		
(mg/kg body wt·d)	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Alanine from food	153±4.6	152±4.6	155±4.5	152±4.3	0.79	0.69	0.79
Alanine from drinking water	2166±105	--	1814±178	--	0.11	--	--
Arginine from food	214±6.4	212±6.3	216±6.2	211±6.0	0.81	0.71	0.81
Arginine from drinking water	--	1037±52	--	994±60	0.64	--	--

Rats were fed with either low- or high-fat diet. In each diet group, rats were randomly assigned to receive drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. Data are expressed as means ± SEM (n=8).

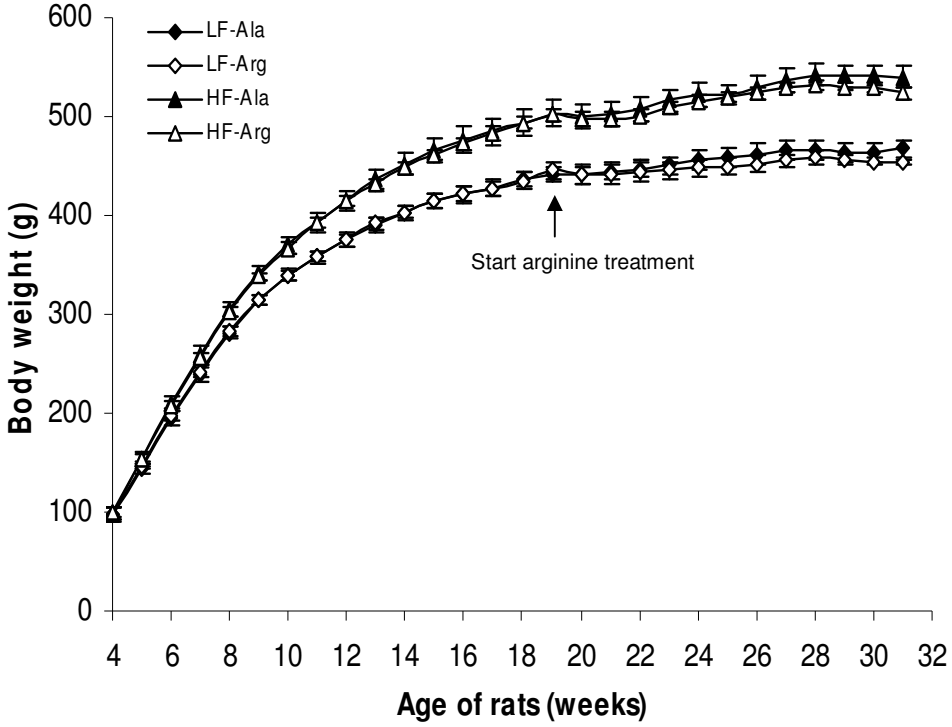


Fig. 2.1. Body weights of rats before and after the initiation of arginine treatment. Rats were fed either low- or high-fat diet between 4 and 19 wks of age. Thereafter, rats in each dietary group continued to be fed their respective diets and received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. Data are expressed as means ± SEM (n=8). *P* (diet) < 0.0001; *P* (AA) = 0.067; *P* (diet x AA) = 0.004; *P* (age) < 0.0001; *P* (AA x age) = 0.010; *P* (diet x age) < 0.0001; *P* (age x age) < 0.0001; *P* (age x age x age) < 0.0001.

Tissue weight

Effects of high-fat feeding and arginine supplementation on the absolute and relative weights of tissues are summarized in Tables 2.4 and 2.5, respectively. High-fat diet increased (P<0.01) the absolute weight of many tissues, including liver, heart, lungs, kidneys, adipose tissues, and skeletal muscles. Particularly, the absolute weights of RP, epididymal (EP), subcutaneous (SC), and mesenteric (MT) adipose tissues in rats fed the high-fat diet increased (P<0.01) by 56%, 48%, 96%, and 51%, respectively, when compared with rats fed the low-fat diet. However, the relative weights of these tissues, except for adipose tissue, testes and brain (expressed as a percentage of the body weight), did not differ between the rats fed low- and high-fat diets (Table 2.5). Notably, the relative weights of RP, EP, SC, and MT adipose tissues in rats fed the high-fat diet increased (P<0.01) by 37%, 38%, 69%, and 39%, respectively, in comparison with rats fed the low-fat diet.

Dietary arginine supplementation reduced (P<0.01) the absolute weights of RP, EP, SC, and MT fat pads by 36%, 20%, 33%, and 41%, respectively, when compared with alanine-supplemented rats. Similar effects of the arginine treatment on decreasing the relative weights of adipose tissue were also observed. Interestingly, the absolute and relative weights of extensor digitorum longus (EDL) muscle, soleus muscle, and brown fat increased (P<0.05) in response to dietary arginine supplementation, compared to alanine-treated rats.

Table 2.4. The absolute weights of tissues in rats

Tissues	Absolute weight (g)						
	LF-Ala	LF-Arg	HF-Ala	HF-Arg	Diet	AA	Diet x AA
Heart	1.67±0.07	1.71±0.07	2.05±0.10	1.94±0.10	0.002	0.66	0.36
Lungs	1.73±0.04	1.76±0.08	2.13±0.06	2.08±0.07	0.001	0.86	0.53
Liver	12.9±0.83	12.1±0.47	14.8±0.52	13.6±0.56	0.009	0.12	0.82
Spleen	0.84±0.04	0.80±0.03	0.89±0.02	0.88±0.04	0.064	0.47	0.62
Kidneys	2.85±0.10	2.85±0.08	3.13±0.06	3.04±0.10	0.011	0.62	0.61
RP adipose tissue	7.41±0.69	4.51±0.33	11.14±1.34	7.44±0.46	0.0001	0.0005	0.62
EP adipose tissue	7.45±0.63	5.89±0.33	10.96±0.86	8.79±0.60	0.001	0.01	0.64
SC adipose tissue	6.78±0.46	4.54±0.27	13.31±1.18	8.83±0.76	0.001	0.001	0.15
MT adipose tissue	3.16±0.35	1.75±0.16	4.57±0.63	2.84±0.18	0.003	0.001	0.68
Brown adipose tissue	0.57±0.02	0.73±0.03	0.62±0.04	0.81±0.04	0.083	0.001	0.72
Small intestine	6.41±0.45	5.85±0.29	7.64±0.33	6.41±0.40	0.022	0.022	0.37
Pancreas	0.96±0.09	1.06±0.04	1.04±0.04	1.08±0.12	0.53	0.43	0.71
Soleus muscle	0.16±0.01	0.18±0.004	0.19±0.01	0.20±0.01	0.001	0.015	0.67
EDL muscle	0.17±0.003	0.18±0.003	0.19±0.004	0.20±0.003	0.001	0.002	0.99
Testes	3.89±0.09	3.79±0.06	3.78±0.06	3.91±0.07	0.97	0.83	0.14
Brain	1.75±0.06	1.80±0.05	1.69±0.07	1.83±0.03	0.83	0.086	0.46

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues weights were measured. Data are expressed as means ± SEM (n=8/treatment group).

Abbreviations used: RP, retroperitoneal; EP, epididymal; SC, subcutaneous; MT, Mesenteric; EDL, extensor digitorum longus.

Table 2.5. The relative weights of tissues in rats

Tissues	Proportional weight (g tissue /kg body wt)						
	LF-Ala	LF-Arg	HF-Ala	HF-Arg	Diet	AA	Diet x AA
Heart	3.57±0.13	3.79±0.17	3.80±0.22	3.73±0.18	0.63	0.68	0.44
Lung	3.71±0.12	3.88±0.21	3.93±0.14	4.01±0.14	0.27	0.41	0.75
Liver	27.5±1.55	26.6±1.16	27.2±1.12	26.3±0.36	0.79	0.45	0.98
Spleen	1.79±0.08	1.76±0.06	1.64±0.04	1.70±0.08	0.12	0.89	0.48
Kidney	6.09±0.22	6.28±0.19	5.78±0.20	5.85±0.17	0.07	0.51	0.79
RP adipose tissue	14.8±1.2	9.76±0.62	20.8±2.1	14.3±0.8	0.0001	0.0005	0.60
EP adipose tissue	14.7±1.1	12.9±0.7	21.3±1.3	16.7±1.0	0.001	0.004	0.20
SC adipose tissue	14.6±1.1	10.0±0.6	24.5±2.1	17.0±1.4	0.001	0.001	0.31
MT adipose tissue	6.29±0.76	3.79±0.34	8.52±1.51	5.49±0.36	0.006	0.001	0.69
Brown adipose tissue	1.21±0.05	1.61±0.06	1.14±0.08	1.56±0.08	0.42	0.001	0.93
Small intestine	13.7±1.02	12.9±0.64	14.1±0.61	12.4±0.73	0.90	0.11	0.56
Pancreas	2.05±0.20	2.33±0.09	1.92±0.08	2.06±0.22	0.24	0.21	0.68
Soleus muscle	0.34±0.01	0.39±0.01	0.34±0.01	0.38±0.01	0.77	0.002	0.77
EDL muscle	0.36±0.01	0.40±0.01	0.35±0.01	0.39±0.01	0.08	0.001	0.87
Testes	8.11±0.16	8.30±0.18	7.13±0.16	7.48±0.16	0.001	0.11	0.64
Brain	3.74±0.11	3.97±0.10	3.12±0.13	3.54±0.07	0.001	0.004	0.37

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues weights were measured. Data are expressed as means ± SEM (n=8/treatment group).

Abbreviations used: RP, retroperitoneal; EP, epididymal; SC, subcutaneous; MT, Mesenteric; EDL, extensor digitorum longus.

Serum concentrations of amino acids

High-fat feeding increased (P<0.05) serum concentrations of most amino acids, including glutamine, citrulline, arginine, and branched-chain amino acids, in comparison with rats fed the low-fat diet (Table 2.6). Serum concentrations of arginine (+105%) and ornithine (+64%) increased (P<0.01) but serum concentrations of glutamate (-28%), glutamine (-15%) and branched-chain amino acids (-22%) decreased in arginine-supplemented rats, when compared with alanine-supplemented rats. Dietary arginine supplementation had no effect (P>0.05) on serum concentrations of other measured amino acids. Serum concentrations of alanine were 65% greater (P<0.01) in alanine- than in arginine-supplemented rats (Table 2.6).

Serum concentrations of glucose, lipids and hormones

High-fat feeding increased serum concentrations of cholesterol and leptin by 45% and 49%, respectively, and had no effect on serum concentrations of glucose, free fatty acids and triglycerides, when compared with rats fed the low-fat diet (Table 2.7). Dietary arginine supplementation reduced (P<0.05) serum concentrations of glucose, triglycerides, and leptin by 5%, 32%, and 37%, respectively, and did not affect (P>0.05) serum concentrations of free fatty acids and cholesterol, in comparison with alanine-supplemented rats. Serum concentrations of insulin, adiponectin, growth hormone, T_3, T_4, and corticosterone were not affected by either high-fat diet or dietary arginine supplementation.

Table 2.6. Serum concentrations of amino acids in rats

Amino Acids (nmol/mL)	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Aspartic acid	27±4.6	20±1.7	24±2.7	21±2.5	0.73	0.17	0.66
Glutamate	92±15	61±4.9	85±8.9	67±6.1	0.96	0.026	0.57
Asparagine	48±3.1	46±2.3	59±9.0	64±8.3	0.03	0.81	0.60
Serine	240±20	249±14	286±15	310±29	0.022	0.46	0.75
Glutamine	657±39	531±28	769±31	685±36	0.001	0.007	0.57
Histidine	53±4	49±3.3	84±8.4	77±9.0	0.000	0.42	0.84
Glycine	209±25	240±16	323±17	354±29	0.000	0.23	0.99
Threonine	188±12	203±11	247±13	214±18	0.024	0.53	0.11
Citrulline	60±4.9	56±4.0	77±7.7	74±11	0.03	0.69	0.99
Arginine	163±20	371±28	223±27	488±77	0.014	0.0005	0.20
β-Alanine	11±1.0	8.5±0.6	13±1.0	13±1.5	0.007	0.50	0.18
Taurine	385±41	369±25	427±20	417±38	0.23	0.72	0.93
Alanine	603±61	370±60	755±92	454±62	0.13	0.001	0.65
Tyrosine	58±4.2	62±4.7	87±8.5	69±8.2	0.011	0.33	0.14
Tryptophan	67±3.9	66±3.8	94±12	74±10	0.049	0.22	0.27
Methionine	34±2.4	31±2.5	64±7.7	54±6.3	0.0001	0.25	0.47
Valine	158±9.0	119±9.4	236±31	168±24	0.006	0.016	0.51
Phenylalanine	53±3.5	51±2.4	77±8.6	70±8.7	0.0001	0.17	0.29
Isoleucine	82±5	62±4.7	97±9.4	80±8.1	0.037	0.020	0.84
Leucine	131±6.8	104±7	161±9.3	138±8.4	0.002	0.006	0.85
Ornithine	31±2.2	58±6.2	36±2.2	52±6.5	0.92	0.0005	0.24
Lysine	284±20	307±36	439±36	394±37	0.0001	0.71	0.28

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, blood was obtained from tail vein and amino acids were measured in the serum. Data are expressed as means ± SEM (n=8/treatment group).

Table 2.7. Serum concentrations of glucose, lipids and hormones in rats

	Low fat		High fat		*P* value		
Variables	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Glucose (mmol/L)	5.99±0.09	5.76±0.15	6.08±0.15	5.74±0.13	0.81	0.039	0.69
Triglycerides (mmol/L)	0.93±0.11	0.68±0.07	1.05±0.08	0.66±0.07	0.57	0.001	0.45
Cholesterol (mg/dL)	177±10	164±6.8	231±20	262±15	0.0005	0.54	0.10
FFA (mmol/L)	0.81±0.12	0.62±0.07	0.80±0.13	0.72±0.09	0.68	0.23	0.61
Insulin (pmol/L)	95.9±20	116±27	113±25	105±13	0.62	0.56	0.20
Leptin (μg/L)	7.73±1.22	3.35±0.36	9.22±1.41	7.28±0.94	0.017	0.006	0.27
Adiponectin (μg/L)	4.95±0.30	5.64±0.33	4.98±0.23	5.03±0.13	0.26	0.16	0.22
Growth Hormone (μg/L)	4.82±1.18	4.84±1.37	4.62±1.45	5.06±0.96	0.99	0.88	0.89
Corticosterone (μg/L)	64.9±6.9	64.0±16.2	62.2±12.0	60.9±17.2	0.84	0.94	0.99
Total T_3 (ng/dL)	72.2±4.8	69.1±4.5	70.1±5.9	68.7±4.8	0.83	0.68	0.88
Total T_4 (ng/dL)	3.39±0.22	3.12±0.09	3.22±0.10	3.20±0.18	0.77	0.36	0.43

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tail blood was obtained for the measurement of serum glucose and cardiac blood was collected for the measurement of serum lipid and hormones. Data are expressed as means ± SEM (n=8/treatment group).

OGTT

The basal serum concentrations of glucose in rats were approximately 5 mmol/L, and did not differ among the 4 treatment groups (Fig. 2.2). In response to oral gavage with glucose, serum concentrations of glucose increased to peak values (8 to 9 mmol/L) at 30 min in all the rats. Between 60 and 180 min post administration of glucose, serum concentrations of glucose were the highest in alanine-supplemented obese rats among

the 4 groups of rats, and were the lowest in arginine-supplemented rats fed the low-fat diet. At 180 min, serum concentrations of glucose in rats fed the low-fat diet decreased to approximately 6.5 mmol/L, whereas those in alanine-supplemented rats fed the high-fat diet remained elevated (8.5 mmol/L). The glucose tolerance was lower (P<0.05) in rats fed the high-fat diet than in rats fed the low-fat diet. Dietary arginine supplementation increased (P<0.05) glucose tolerance in rats, compared with alanine supplementation.

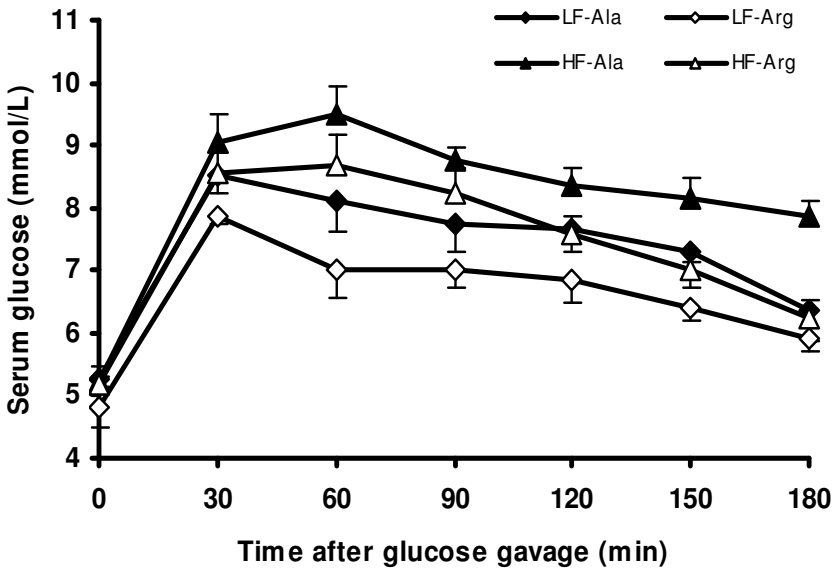


Fig. 2.2 Oral glucose tolerance test in rats. Rats were fed with either control diet or high-fat diet. In each diet group, rats were randomly assigned to receive drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control). At 10 wks after initiation of the arginine treatment, oral glucose tolerance test was performed in rats after a 5-h fast. Data are expressed as means ± SEM (n=8). P (diet)=0.0005, P (AA)=0.024, P (diet x AA)=0.20.

Lipid, glycogen, GSH and BH4 in tissues

Concentrations of lipids in liver and gastrocnemius muscle were greater (P<0.05) but concentrations of BH4 and GSH in liver were lower (P<0.05) in rats fed the high-fat diet, when compared with rats fed the low-fat diet (Table 2.8). Concentrations of oxidized glutathione (GS-SG) in liver were not detectable (< 0.05 μmol/g tissue) in all groups of rats. Dietary arginine supplementation increased (P<0.05) concentrations of BH4 in liver, compared with alanine supplementation. Neither high-fat diet nor arginine supplementation affected concentrations of lipids (81-83%) in adipose tissue or concentrations of glycogen in liver and gastrocnemius muscle.

Table 2.8. Concentrations of lipids, BH4, glycogen, and GSH in tissues of rats

Variables	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Lipids in liver (%)	3.85±0.11	3.64±0.06	4.59±0.24	4.66±0.17	0.001	0.67	0.40
Lipids in muscle (%)	1.60±0.11	1.64±0.12	1.94±0.14	1.92±0.15	0.049	0.42	0.52
Lipids in RP adipose tissue (%)	80.6±1.6	82.0±1.8	83.1±1.6	82.9±0.6	0.27	0.72	0.60
BH4 in liver (nmol/g tissue)	4.02±0.38	5.06±0.43	3.13±0.15	4.35±0.13	0.02	0.002	0.79
Glycogen in liver (mg/g)	21.8±1.7	18.6±0.9	19.7±0.5	19.1±1.8	0.55	0.16	0.31
Glycogen in muscle (mg/g)	1.26±0.19	1.13±0.20	1.01±0.13	1.05±0.11	0.36	0.80	0.65
GSH in liver (μmol/g)	6.09±0.27	5.59±0.33	4.41±0.43	4.24±0.33	0.001	0.41	0.67

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and metabolites in tissues were measured. Data are expressed as means ± SEM (n=8/treatment group).

Discussion

The DIO rat is a widely used animal model with which to develop new nutritional means to treat human obesity (Bray *et al.* 2004). In response to a high-fat diet, Sprague-Dawley rats usually gain more body weight and fat mass, compared with those fed a low-fat diet, therefore exhibiting complex metabolic abnormalities (Chalkley *et al.* 2002; Naderali *et al.* 2004; Barnard *et al.* 1998). Some studies suggested that the excess fat accretion in rats fed the high-fat diet resulted from an increased energy intake (Ghibaudi *et al.* 2002). However, when fed isocaloric high-energy diets, rats still accreted a greater amount of white adipose tissues in response to a high-fat diet than a high-carbohydrate diet (Boozer *et al.* 1995). These results indicate that dietary fat is more efficient in promoting energy storage in white adipose tissue, compared to carbohydrates. Consistent with this view, we found that obesity developed in rats on a high-fat diet in comparison with rats fed a low-fat diet (Tables 2.4 and 2.5), even though dietary energy intakes did not differ between the two groups of rats (Table 2.2). Likewise, serum concentrations of leptin, a hormone released predominantly by white adipose tissue (Fruhbeck, 2006), were greater in rats fed the high-fat diet than in rats fed the low-fat diet (Table 2.7). Additionally, results of the present study demonstrate that the DIO rats exhibited oxidative stress (indicated by a reduced concentration of GSH in liver), an attenuated capacity for nitric oxide synthesis (indicated by a reduced availability of BH4), and impaired insulin action (indicated by a reduced disposal of oral glucose) (Table 2.8 and Fig. 2.2). Collectively, these findings established that the DIO rat provideds a useful model to define an effect

of dietary arginine supplementation on reducing fat mass in environmentally-induced obesity.

Results of the present study demonstrate for the first time that dietary arginine supplementation was highly effective in reducing fat mass in DIO rats (Tables 2.3 and 2.4), as reported for genetically obese ZDF rats (Fu *et al.* 2006) and obese patients with type-II diabetes mellitus (Lucotti *et al*. 2006). Accordingly, serum concentrations of leptin were lower in arginine- than in alanine-supplemented rats (Table 2.7). Arginine exerted a fat-reducing effect in rats fed either the low- and high-fat diet that provided similar amounts of energy, protein, vitamins and minerals (Table 2.2). The percentage loss of MT adipose tissue was the greatest in response to the arginine treatment, followed by RP, SC, and EP fat pads in decreasing order. The free fatty acids released from fat hydrolysis in the white adipose tissue of arginine-supplemented rats likely undergo increased oxidation in liver, skeletal muscle, and brown fat. In support of this view, serum concentrations of triglycerides were lower, in arginine- than in alanine-supplemented rats (Table 2.7), as reported for chemically-induced diabetic rats (Kohli *et al.* 2004; Miguez *et al.* 2004) and ZDF rats (Fu *et al.* 2005). Further, the arginine treatment markedly increased the mass of brown fat in rats by 29% (Table 2.3) likely due to mitochondrial biogenesis induced by nitric oxide (Nisoli *et al.* 2003). Brown adipose tissue is rich in mitochondria, where fatty acid and glucose oxidation results in the production of heat rather than ATP because of the presence of uncoupling protein-1 (Cannon & Nedergaard, 2004). An increase in brown-fat mass brought about by the arginine treatment is expected to augment the oxidation of fatty acids and glucose in this

tissue, thereby reducing their use for fat synthesis in white adipose tissue and their plasma concentrations. Therefore, our novel observation on the increase in brown adipose tissue may provide an explanation for the increased oxidation of energy substrates in arginine-supplemented rats.

Elevated plasma concentrations of triglycerides are known to impair insulin sensitivity and reduce glucose utilization in skeletal muscle (Jobgen *et al.* 2006). In response to an oral glucose load, serum concentrations of glucose remained elevated for a longer period of time in obese than in lean rats (Fig. 2.2). With a reduction in serum levels of triglycerides, dietary arginine supplementation is expected to ameliorate insulin resistance in obese animals (Jobgen *et al.* 2006). Consistent with this notion are the following lines of evidence. First, serum levels of glucose were lower in arginine- than in alanine-supplemented rats (Table 2.7), as reported for chemically-induced diabetic rats (Kohli *et al.* 2004; Miguez *et al.* 2004) and ZDF rats (Fu *et al.* 2005). Second, the glucose tolerance in obese rats was improved in response to the arginine treatment (Fig. 2.2). These beneficial effects of dietary arginine supplementation can result from both improved insulin sensitivity in skeletal muscle and an increase in its mass (Table 2.3).

Little is known about an effect of high-fat feeding on plasma concentrations of amino acids. Interestingly, serum concentrations of most amino acids increased in rats fed the high-fat diet in comparison with rats fed the low-fat diet (Table 2.6), despite similar intakes of dietary protein (Table 2.2). This result can be explained by an inhibition of hepatic amino acid oxidation in response to high-fat feeding (Estornell *et al.* 1994). Additionally, increased serum concentrations of glutamine in DIO rats, which

results partly from its elevated production by adipose tissue (Kowalski *et al.* 1997), may have important implications for obesity-related metabolic defects. Glutamine is a substrate for the synthesis of glucosamine, which impairs the generation of nitric oxide by endothelial cells (Wu *et al.* 2001) and contributes to insulin resistance in skeletal muscle (Buse, 2006).

As reported for streptozotocin-induced diabetic rats (Kolhi *et al.* 2004) and ZDF rats (Fu *et al.* 2005), the administration of arginine via drinking water effectively increased serum concentrations of arginine and ornithine (Table 2.6). Arginase activity is widespread in animal tissues (Wu & Morris, 1998) and, therefore, the supplemental arginine is rapidly catabolized to yield ornithine in rats and other animals (Wu *et al.* 2007b). Importantly, dietary arginine supplementation reduced serum concentrations of glutamine in lean and DIO rats (Table 2.6), as reported for ZDF rats (Fu *et al.* 2005) as well as neonatal pigs (Kim *et al.* 2004) and gilts (Mateo *et al.* 2007). This consistent observation from studies with different animal models indicates a regulatory role for arginine in whole-body glutamine metabolism. As an allosteric activator of N-acetylglutamate synthase, arginine maintains the urea cycle in an active state to convert ammonia into urea (Wu & Morris, 1998). In addition, through the generation of nitric oxide, arginine may inhibit protein degradation (Jobgen *et al.* 2006) and, therefore, ammonia production. A limited availability of ammonia is expected to reduce *de novo* synthesis of glutamine in tissues (including skeletal muscle and adipose tissue). Another interesting finding is that serum concentrations of branched-chain amino acids were lower in arginine- than in alanine-supplemented rats (Table 2.6), which may be

explained by a reduction in their release from skeletal muscle due to an anabolic effect of arginine on protein accretion (Table 2.3).

In contrast to ZDF rats (Fu *et al.* 2005), dietary arginine supplementation did not substantially reduce the body weight of DIO rats (Fig. 2.1). This result can be explained by our observation that the mass of skeletal muscle was greater (both absolute and relative increases) in lean and DIO rats in response to the arginine treatment (Tables 2.3 and 2.4). Importantly, such an anabolic effect of arginine was achieved independent of changes in serum concentrations of insulin and growth hormone (Table 2.7). These findings suggest that, through yet unknown signaling pathways, arginine may regulate intracellular protein turnover, favoring net protein deposition in skeletal muscle, as recently reported for neonatal pigs (Frank *et al.* 2007). Alternatively, through nitric oxide generation, dietary arginine supplementation may increase insulin sensitivity and amplify its signaling mechanisms on net protein synthesis (Jobgen *et al.* 2006).

In summary, results of the present study demonstrated for the first time that dietary arginine supplementation reduced excess fat mass, increased muscle mass, decreased serum concentrations of glucose and triglycerides, and improved insulin sensitivity in diet-induced obesity. Arginine supplementation may represent a safe and an efficient nutritional treatment of obesity, a growing health problem worldwide. Future studies are warranted to define the cellular and molecular mechanisms responsible for the beneficial effects of arginine in ameliorating the metabolic syndrome in obese subjects.

CHAPTER III

DIETARY L-ARGININE SUPPLEMENTATION REGULATES GLUCOSE AND FATTY ACID METABOLISM IN RATS

Dietary arginine supplementation reduced fat mass in diet-induced obese rats (Chapter II). This study was conducted to test the hypothesis that the arginine treatment regulates glucose and fatty acid metabolism in insulin-sensitive tissues. Male Sprague-Dawley rats (4-week-old) were fed either low- or high-fat diets for 15 wks (n=16/diet). Thereafter, lean or obese rats rats were fed their respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) (n = 8/treatment group). After 12 wks of treatment, rats were euthanized and tissue samples were collected for biochemical assays. High-fat feeding increased the size of adipocytes isolated from retroperitoneal (RP) adipose tissue, while arginine treatment reduced their size. The total numbers of adipocytes did not differ among the four groups of rats. Glucose oxidation in extensor digitorum longus (EDL) muscle, soleus muscle and RP adipose tissue were reduced in response to high-fat feeding. On the contrary, oleic acid oxidation in RP adipose tissue was enhanced in rats fed the high-fat diet. Arginine treatment stimulated both glucose and oleic acid oxidation in EDL and soleus muscles, while having no effect on the glucose oxidation, oleic acid oxidation or basal lipolysis in RP adipose tissue. Collectively, these results indicate that dietary supplementation with arginine to diet-induced obese rats promoted the oxidation of energy substrates in skeletal muscle, thereby reducing body fat and improving insulin-sensitivity.

L-Arginine is a physiological precursor of nitric oxide (NO), a free radical that is synthesized in almost all cell types by NO synthase (NOS). Through cGMP-dependent and independent pathways, NO plays an important role in regulating vascular tone, neurotransmission, host immunity, and whole-body homeostasis (Alderton *et al.* 2001; Shi *et al.* 2004). There is growing evidence that the arginine-NO pathway is also involved in regulating energy-substrate metabolism, such as glycolysis, glucose transport and oxidation, gluconeogenesis, lipolysis, fatty acid synthesis and fatty acid oxidation (Jobgen *et al.* 2006). In our previous study, we found that dietary arginine supplementation reduced body fat mass, increased muscle weight, and improved whole-body insulin sensitivity in both lean and diet-induced obese (DIO) rats (Chapter II). This anti-obesity effect of arginine has also been reported for Zucker diabetic fatty (ZDF) rats (Fu *et al.* 2005) and obese humans with type II diabetes (Lucotti *et al.* 2006). Additionally, short-term oral administration of arginine improved insulin sensitivity in obese humans with type-II diabetes mellitus (Wascher *et al.* 1997).

Based on the previous findings that implicate an important role for NO in regulating the metabolism of energy substrates (Jobgen *et al.* 2006), we hypothesized that dietary arginine supplementation to DIO rats reduced fat mass through increasing the oxidation of glucose and fatty acids in insulin-sensitive tissues. This hypothesis was tested with the use of ^{14}C-labeled glucose and oleic acid as well as the measurement of lactate and glycerol release from adipose tissue.

Materials and methods

Chemicals

D-[U-^{14}C]glucose and [1-^{14}C]oleic acid were purchased from American Radiolabeled Chemicals (St Louis, MO). Collagenase (type I) was obtained from Worthington Biochemical Corporation (Lakewood, NJ). Bovine serum albumin (BSA) was procured from Intergen Company (Purchase, NY). HPLC-grade methanol and water were obtained from Fisher Scientific (Houston, TX). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Animals

Male Sprague-Dawley rats from Harlan Labs were used in this study. The experimental design was detailed in Chapter II. Briefly, 4-wk-old male Sprague-Dawley rats were fed either a low- or high-fat diet for 15 wks (n=16/diet). Thereafter, rats were fed their same respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) (n=8/treatment group). After 12 wks of treatment, rats were euthanized and tissue samples were collected for biochemical assays.

Determination of NO synthesis in freshly isolated tissues

Retroperitoneal (RP) adipose tissue and gastrocnemius muscle (~100 mg) were minced and rinsed with oxygenated (95%O_2/5%CO_2) Krebs bicarbonate buffer (pH 7.4) containing 5 mM of D-glucose and 4% BSA (KRB). Tissues were then incubated with gentle shaking (70 oscillations/min) at 37^{o}C in 1 mL of fresh KRB buffer containing 1 mM arginine. At the end of a 3-h incubation period, incubation medium was collected

and analyzed for nitrite (a major stable end product of NO oxidation) by an HPLC method (Li *et al.* 2000). In all experiments, the incubation medum without tissues were included as blanks.

Determination of lipolysis and lactate release from RP adipose tissue

RP adipose tissue (~100 mg) was minced and rinsed with KRB, as described by Fried *et al.* (1993b), and was then incubated at 37^{o}C in KRB containing 0 or 10 μM norepinephrine (NE) to determine basal and NE-stimulated lipolysis, respectively. After a 2-h incubation period, all tubes were placed on ice immediately, and the incubation media were rapidly transferred into clean tubes for determination of glycerol (Laurell & Tibling, 1966) and lactate (Wu *et al.* 1995).

Isolation of adipocytes and determination of RP adipose tissue cellularity

Adipocytes from RP adipose tissue were isolated according to the method described by Rodbell (1964). Briefly, RP adipose tissue (~1 g) was minced and rinsed with KRB, and then incubated with KRB containing 1 mg/mL collagenase for 45 min at 37^{o}C with gentle shaking. After incubation, the cell suspensions were filtered through a 250-μm nylon grid, and washed 3 times with KRB. After the cell suspension stood at room temperature for 3 min, the floating layer of adipocytes was collected into clean tubes and diluted with KRB to obtain a cell concentration of 10% (v/v). An aliquot of the cell suspension was spotted onto a slide and the sizes of adipocytes were determined under a microscope. For each slide, at least 120 cells were measured for their sizes, and the mean volume of adipocytes was calculated according to the method described by Goldrick (1967): $V = \pi/6 \times d (d^2 + 3\sigma^2)$, where d is the mean diameter and σ is the variance of the

diameter of cells. The lipid content in RP adipose tissue on per gram basis was determined using the Folch method (Folch *et al.* 1957). Based on the assumption that adipocytes are primarily triolein droplets and the density of adipocytes is 0.915 (Mandenoff *et al.* 1982), the lipid content per adipocyte and the number of adipocytes in the whole RP adipose tissue pad was calculated.

Determination of glucose and fatty acid oxidation

RP adipose tissue, extensor digitorum longus (EDL) muscle, and soleus muscle were minced and rinsed with KRB. The tissues were then placed in polystyrene tubes and incubated at 37^{o}C for 2 h in 1 mL oxygenated KRB containing 3 nM insulin and either 0.5 μCi D-[U-^{14}C] glucose or 0.2 mM of oleic acid plus [1-^{14}C] oleic acid (0.5 μCi). The tubes were filled with 95%O_2/5%CO_2, sealed with rubber stoppers and fitted with hanging center-wells. At the end of a 2-h incubation period, 0.2 mL NCS-II (Amersham, Piscataway, NJ) was added through the rubber stopper into a 500-μL microcentrifuge tube placed in the center-well, followed by addition of 0.2 mL of 1.5 M $HClO_4$ to the incubation medium. After an additional 1-h incubation period, the microcentrifuge tubes were carefully transferred to scintillation vials. Fifteen milliliters of counting cocktail was added into the vials and ^{14}C radioactivity was determined using a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL). The specific activities of D-[U-^{14}C] glucose and [1-^{14}C] oleic acid in the incubation media were used to calculate the rate of $^{14}CO_2$ production.

Data analysis

Results were given as means ± SEM. Data for muscle tissues were expressed per gram tissue weight, and data for adipose tissue were expressed on the basis of both gram tissue and 10^6 adipocytes. Statistical analysis was performed using two-way ANOVA with the Tukey multiple comparison to determine the effects of diet, amino acid (AA), and the interaction between diet and AA (diet x AA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

The size and number of adipocytes in RP adipose tissue

The high-fat diet enhanced ($P<0.005$) the mean diameter of adipocytes isolated from RP adipose tissue by 11% and 20% in alanine- and arginine-supplemented rats, respectively (Table 3.1). Adipocytes isolated from arginine-supplemented rats fed the low-fat diet exhibited the smallest size (mean diameter 77±3 μm), when compared to other three groups of rats (mean diameter > 90 μm) (Fig. 3.1). Dietary arginine supplementation reduced ($P<0.005$) the mean diameter of adipocytes by 15% and 7% in rats fed the low-fat and high-fat diets, respectively (Table 3.1). Arginine treatment resulted in a shift of adipocyte size toward a smaller diameter, compared with alanine supplementation (Fig. 3.2). The total numbers of adipocytes in the entire RP adipose tissue did not differ among the four groups of rats. However, high-fat feeding reduced ($P<0.005$) the density of adipocytes per gram of RP adipose tissue due to the increased lipid filling, compared with rats fed the low-fat diet (Table 3.1). In contrast, dietary arginine supplementation

increased (P<0.005) the density of adipocytes, in comparison with alanine-supplemented rats (Table 3.1).

Table 3.1. Cellularity of RP adipose tissue

Variables	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Adipocyte size (μm)	90.4±1.9	77.1±3.1	99.9±2.8	92.7±2.5	0.001	0.001	0.26
Total adipocytes (10^6 cells)	15.6±1.6	14.4±0.7	17.2±0.9	15.1±1.1	0.33	0.14	0.70
Adipocyte density (10^6 cells/g tissue)	2.13±0.16	3.27±0.27	1.62±0.11	2.05±0.14	0.001	0.001	0.22

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, RP adipose tissue was dissected and adipocytes were isolated using an enzymatic method. Data are expressed as means ± SEM (n=8/treatment group).

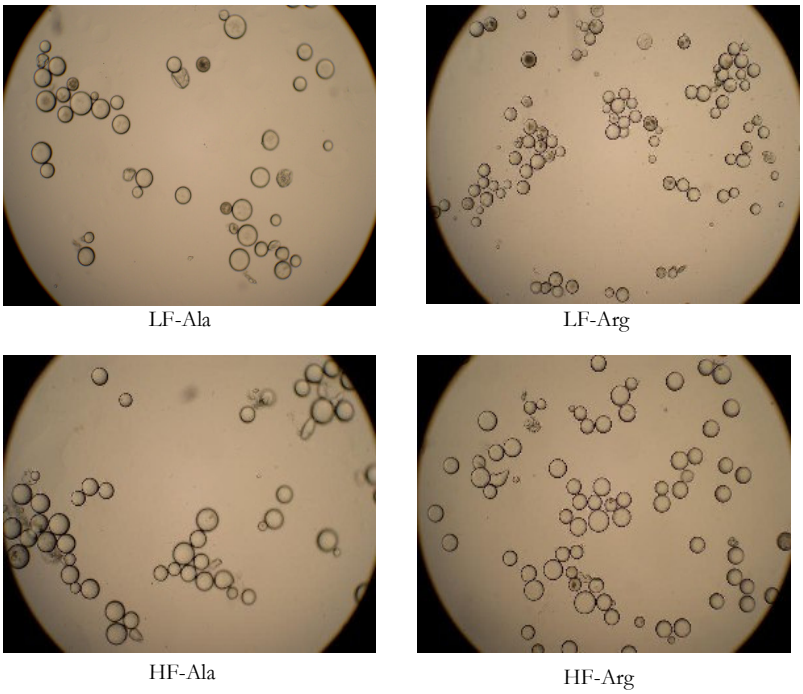


Fig 3.1. Morphology of adipocytes isolated from rat RP adipose tissue. Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, RP adipose tissue was dissected and adipocytes were isolated. Data are expressed as means ± SEM (n=8/treatment group). Magnification scale was set at x10.

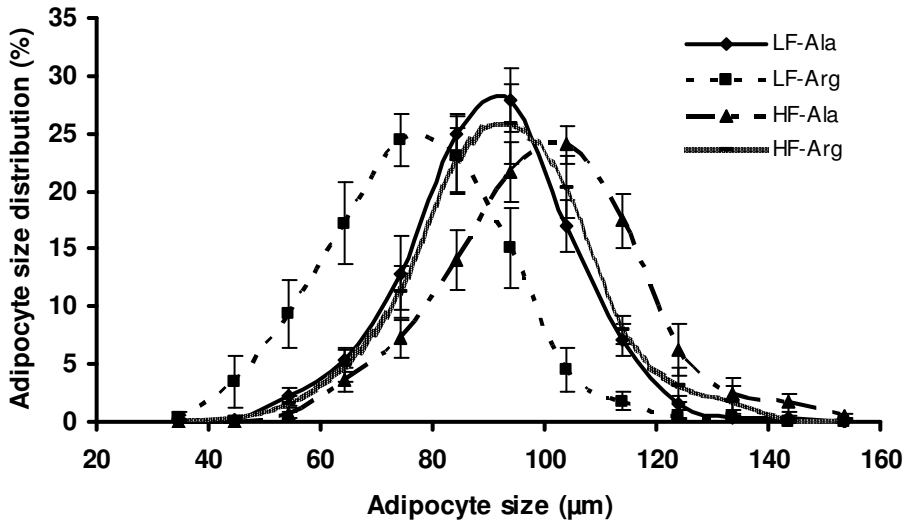


Fig 3.2. Size distribution of adipocyte from RP adipose tissue of rats. Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, RP adipose tissue was dissected, adipocytes were isolated using an enzymatic method, and their sizes were determined under a microscope. Data are expressed as means ± SEM (n=8/treatment group). *P* values are summarized below:

	Diameter of adipocytes (µm)										
P value	45	55	65	75	85	95	105	115	125	135	145
Diet	0.13	0.005	0.002	0.001	0.052	0.51	0.001	0.0005	0.015	0.086	0.13
AA	0.13	0.020	0.003	0.004	0.23	0.16	0.001	0.0005	0.14	0.62	0.021
Diet x AA	0.19	0.056	0.016	0.14	0.052	0.011	0.050	0.17	0.60	0.80	0.21

Lactate release and lipolysis in RP adipose tissue

Table 3.2 summarizes the effects of high-fat feeding and arginine treatment on lactate release (an indicator of glycolysis) and lipolysis in RP adipose tissue. Lactate release from the RP adipose tissue per gram tissue was reduced ($P<0.001$) in response to high-fat feeding, and dietary arginine supplementation increased lactate production ($P<0.005$). When data are expressed per 10^6 adipocytes, lactate release was unaltered by either high-fat feeding or dietary arginine supplementation. Both basal and NE-stimulated rates of lipolysis per gram tissue or per 100 mg lipid were lower ($P<0.001$) in rats fed a high-fat diet than in rats fed a low-fat diet. Dietary arginine supplementation increased ($P<0.05$) basal lipolysis and had no effect on NE-stimulated lipolysis. However, when data are expressed per 10^6 adipocytes, basal lipolysis did not differ either between rats fed the low- and high-fat diets or between alanine- and arginine-supplemented rats, whereas NE-stimulated lipolysis was lower in response to the arginine treatment due to the increased density of adipocytes ($P<0.05$).

NO production gastrocnemius muscle and fat

High-fat feeding did not affect NO synthesis in either gastrocnemius muscle or RP adipose tissue (Table 3.3). Dietary arginine supplementation increased ($P<0.05$) NO production in gastrocnemius muscle by 25%, but had no effect on RP adipose tissue, compared with alanine-supplemented rats.

Table 3.2. Lactate release and lipolysis in RPl adipose tissue

Variables	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Lactate release							
µmol lactate/g tissue · h	1.10±0.06	1.35±0.14	0.84±0.07	1.02±0.04	0.001	0.004	0.67
µmol lactate /10^6 adipocytes · h	0.52±0.05	0.42±0.04	0.54±0.06	0.52±0.05	0.23	0.23	0.39
Lipolysis							
µmol glycerol/g tissue · h							
Basal	1.36±0.07	1.54±0.10	0.90±0.33	1.20±0.15	0.001	0.035	0.60
NE-stimulated	7.95±0.66	7.27±0.74	5.11±0.33	5.93±0.70	0.001	0.78	0.47
µmol glycerol/100 mg lipid · h							
Basal	0.17±0.01	0.19±0.01	0.11±0.01	0.15±0.02	0.002	0.03	0.46
NE-stimulated	0.98±0.08	0.89±0.09	0.62±0.04	0.65±0.09	0.001	0.75	0.46
µmol glycerol/10^6 adipocytes · h							
Basal	0.68±0.06	0.51±0.03	0.57±0.07	0.59±0.07	0.92	0.23	0.13
NE-stimulated	3.97±0.46	2.35±0.14	3.20±0.19	2.83±0.55	0.71	0.016	0.11

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, RP adipose tissue were collected and lactate and glycerol were measured in tissue incubation media. Data are expressed as means ± SEM (n=8/treatment group).

Table 3.3. Nitrite production in gastrocnemius muscle and RP adipose tissue of rats

Nitrite production	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Gastrocnemius muscle							
nmol/g tissue · h	0.23±0.02	0.29±0.02	0.21±0.03	0.26±0.02	0.23	0.032	0.83
RP adipose tissue							
nmol/g tissue · h	0.17±0.02	0.19±0.02	0.11±0.02	0.16±0.02	0.61	0.081	0.59
pmol/10^6 adipocytes · h	78±6	63±9	72±9	81±9	0.53	0.79	0.23

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and nitrite/nitrate was measured in tissue incubation media. Data are expressed as means ± SEM (n=8/treatment group).

Oxidation of glucose and fatty acids in muscle and RP adipose tissue

The effects of high-fat diet and arginine treatment on glucose and oleic acid oxidation in EDL muscle, soleus muscle, and RP adipose tissue are summarized in Table 3.4. Glucose oxidation per gram tissue in all three tissues was 18%, 28%, and 76% lower ($P<0.05$), respectively, in rats fed the high-fat diet than in rats fed the low-fat diet. Glucose oxidation in RP adipose tissue per 10^6 adipocytes was approximately 64% lower in high-fat-fed rats, compared with low-fat-fed rats. Oleic acid oxidation in skeletal muscles did not differ between rats fed the low- and high-fat diets ($P>0.05$). In RP adipose tissue, when data were expressed per gram tissue or 10^6 adipocytes, oleic acid oxidation increased ($P<0.001$) by 33% and 79%, respectively, in high-fat-fed rats,

in comparison with low-fat-fed rats. Dietary arginine supplementation increased (P<0.05) glucose and oleic acid oxidation per gram tissue in skeletal muscles and RP adipose tissue. However, when data were expressed per 10^6 adipocytes, neither glucose nor oleic acid oxidation in RP adipose tissue differed between alanine- and arginine-supplemented rats.

Table 3.4. Glucose and oleic acid oxidation in EDL muscle, soleus muscle and RP adipose tissue

Tissues	Low fat		High fat		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
Per g tissue							
Glucose oxidation (nmol glucose/g tissue · h)							
EDL muscle	34.4±4.0	44.1±2.0	28.5±2.7	35.9±2.5	0.039	0.015	0.73
Soleus muscle	38.1±3.8	49.0±5.6	31.0±2.4	31.4±5.1	0.009	0.04	0.40
RP adipose tissue	346±59	595±51	104±10	120±14	0.001	0.014	0.098
Oleic acid oxidation (nmol oleic acid/g tissue · h)							
EDL muscle	1.51±0.28	2.77±0.49	1.70±0.22	1.94±0.15	0.34	0.03	0.13
Soleus muscle	3.46±0.45	4.64±0.41	3.45±0.36	3.97±0.22	0.35	0.027	0.38
RP adipose tissue	0.80±0.06	0.93±0.07	1.05±0.06	1.25±0.08	0.001	0.036	0.63
Per 10^6 adipocytes							
Glucose oxidation (nmol glucose/10^6 adipocytes · h)							
RP adipose tissue	171±32	192±15	67±9	62±7	0.001	0.72	0.55
Oleic acid oxidation (nmol oleic acid/10^6 adipocytes·h)							
RP adipose tissue	0.41±0.05	0.31±0.03	0.67±0.06	0.62±0.05	0.001	0.18	0.63

Lean and diet-induced obese rats were fed low- and high-fat diets, respectively. Rats in each dietary group received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were measured glucose and oleic acid oxidation. Data are expressed as means ± SEM (n=8/treatment group).

Discussion

Oxidation of energy substrates in skeletal muscle, liver and adipose tissue plays an important role in fuel homeostasis and fat accretion of animals (Jobgen *et al.* 2006). Therefore, to provide a metabolic basis for fat loss in arginine-supplemented DIO rats (Chapter II), we chose to study glucose and oleic acid oxidation in EDL muscle (primarily glycolytic fiber), soleus muscle (primarily oxidative fiber), and RP adipose tissue. In addition, the production of lactate and glycerol from adipose tissue was measured as indicators of glycolysis and lipolysis, respectively (Fried *et al.* 1993b).

Berger and Barnard (1999) reported that two months of high-fat feeding increased the size of adipocytes and reduced their number in rats per gram tissue. Consistent with this finding, results of our study demonstrated a 15% increase in the size of adipocytes and a 32% reduction in their density in the RP adipose tissue of rats fed the high-fat diet, compared with rats fed the low-fat diet (Table 3.1). Because high-fat feeding did not affect lipolysis per 10^6 adipocytes (Table 3.2), the increased synthesis of triglycerides may be the major factor for the hypertrophy of the RP adipose tissue in DIO rats (Table 3.1), which is consistent with the report that high-fat diet increased the activity of lipoprotein lipase (LPL) in white adipose tissue (Robert *et al.* 2002). A novel finding from the present study is that dietary arginine supplementation reduced the size of adipocytes by enhancing the loss of their stored fat, without affecting the total number of adipocytes (Table 3.1). This outcome likely resulted from an increase in the basal hydrolysis of triglycerides and the subsequent oxidation of fatty acids per gram tissue in the fat depots (Tables 3.2 and 3.4). In support of this notion, physiological levels of

nitric oxide, whose production in adipose tissue increased in response to arginine treatment (Table 3.3), stimulated basal lipolysis in human adipose tissue (Wu *et al.* 2007a) and rat adipocytes (Canova *et al.* 2006).

Dietary fat intake is known to regulate muscle glucose oxidation (Oakes *et al.* 1997). Accordingly, glucose oxidation was lower in both EDL and soleus muscles of rats fed the high-fat diet, when compared with rats fed the low-fat diet (Table 3.4). This result can be explained by reduced glucose uptake (Todd *et al.* 2005) and increased concentrations of intramuscular lipids for oxidation (Chapter II) in DIO rats. The acetyl-CoA derived from fatty acids can potently inhibit pyruvate dehydrogenase activity and results in the formation of citrate (an inhibitor of fructose-6-phosphate kinase-I), thereby decreasing glucose oxidation via glycolysis and the tricarboxylic-acid cycle (Newsholme *et al.* 1993). In support of this view, there is an inverse relationship between intramuscular concentrations of triglycerides and the whole-body glucose oxidation in humans (Krssak *et al.* 1999; Pan *et al.* 1997).

Another important finding from the present study is that the fat tissue of adult rats exhibited a much higher rate of glucose oxidation than skeletal muscle per gram tissue (Table 3.4). Considering its greater mass in obese animals (Chapter II), white adipose tissue may play a quantitatively important role in regulating glucose homeostasis in DIO rats. Interestingly, glucose oxidation decreased, but oleic acid oxidation increased, in the RP adipose tissue of rats fed the high-fat diet, in comparison with rats fed the low-fat diet (Tables 3.2 and 3.4). These results indicate that high-fat feeding reduced glucose oxidation in adipose tissue, shifting its utilization of energy substrates from glucose to

fatty acids. These findings are similar to those previously reported by other investigators (Lichtenstein & Schwab, 2000).

A novel and important finding from the present study is that dietary arginine supplementation increased the oxidation of both glucose and fatty acids in skeletal muscle. An increase in muscular NO production within the physiological range may play a role in mediating this beneficial action of the arginine treatment (Table 3.3). Emerging evidence shows that NO regulates the metabolism of energy substrates in insulin-sensitive tissues (Jobgen *et al.* 2006). For example, sodium nitroprusside (SNP), an NO donor, increased glucose transport and oxidation in isolated muscle (Balon & Nadler, 1997; Young *et al.* 1997). Consequently, an inhibition of NO synthesis reduced both basal and exercise-stimulated glucose transport in skeletal muscle in both *in vitro* and *in vivo* studies (Bradley *et al.* 1999; Balon & Nadler, 1997; Roberts *et al.* 1997; Kingwell *et al.* 2002). The underlying mechanisms may involve the cellular signaling cascade, which includes soluble guanylyl cyclase (the target of NO) and cGMP-dependent protein kinase (Young & Leighton, 1998a). In support of this view, Garcia-Villafranca *et al.* (2003) found that NO and cGMP increased fatty acid oxidation and reduce fatty acid synthesis in rat hepatocytes. Additionally, the NO/cGMP pathway has been reported to trigger mitochondrial biogenesis in different mammalian cell types and tissues through the activation of peroxisome proliferators-activator receptor γ coactivator 1α (PGC-1α) (Nisoli *et al.* 2003). The outcome is to increase the mitochondrial oxidation of energy substrates, thereby reducing their availability for fat accretion in the body (Nisoli *et al.* 2004a). Indeed, our previous study with ZDF rats showed that dietary

arginine supplementation increased mRNA levels for PGC-1α in RP adipose tissue (Fu *et al.* 2005). At present, it is unknown whether arginine treatment also increases PGC-1α expression in other insulin-sensitive tissues of animals.

A role for NO in regulating adipose tissue metabolism remains elusive, primarily due to the conflicting reports of *in vitro* studies involving extremely high doses of NO donors (up to 2 mM). NO may act to stimulate the oxidation of energy substrates at physiological levels but to suppress this event at excessive levels (Lincova *et al.* 2002; McGrowder *et al.* 2006). In addition, the function of NO in adipose tissue may also be dependent on the location and types of tissues (Canova *et al.* 2005). On the basis of per 10^6 adipocytes, we found that arginine supplementation did not affect NO production, basal lipolysis or substrate oxidation in the RP adipose tissue, suggesting that arginine may have only a modest effect on glucose or fatty acid metabolism in the adipose tissue of DIO rats after a 12-wk period of its dietary supplementation. Thus, we suggest that liver and skeletal muscle may play a quantitatively more important role than white adipose tissue in the anti-obesity action of arginine.

In sum, results of our study indicate that dietary arginine supplementation decreased the size of adipocytes and increased the oxidation of glucose and fatty acids in skeletal muscle. These findings provide a metabolic basis for explaining an anti-obesity effect of arginine in DIO rats. Further research is necessary to elucidate the underlying molecular mechanisms.

CHAPTER IV

DIETARY L-ARGININE SUPPLEMENTATION INCREASES HEPATIC EXPRESSION OF AMP-ACTIVATED PROTEIN KINASE IN RATS

The goal of this study was to elucidate the molecular mechanisms responsible for the anti-obesity effect of arginine supplementation in diet-induced obese rats. Male Sprague-Dawley rats were fed either a low-fat or high-fat diet for 15 wks. Thereafter, lean or obese rats were pair-fed their same respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) for 12 wks. Gene and protein expression of key enzymes in the metabolism of energy substrates were determined using real-time PCR and western blotting techniques. The mRNA levels of hepatic fatty acid synthase and stearoyl-CoA desaturase were reduced but those of AMP-activated protein kinase (AMPK), peroxisome proliferator activator receptor γ coactivator-1 α and carnitine palmitoyltransferase I (CPT-I) as well as skeletal muscle CPT-I were increased by arginine treatment. The protein expression and activity of hepatic AMPK markedly increased but the activity of hepatic acetyl-CoA carboxylase (ACC) decreased in response to dietary arginine supplementation. Collectively, our results indicate that liver is the major target for the action of dietary arginine supplementation on reducing fat mass in DIO rats by inhibiting fatty acid synthesis and increasing fatty acid oxidation via the AMPK-ACC signaling pathway. Additionally, increased CPT-I expression in skeletal muscle may also contribute to the enhanced oxidation of long-chain fatty acids in arginine-supplemented rats.

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme consisting of 3 subunits: a catalytic α subunit and two regulatory units β and γ (Cheung *et al.* 2000; Hardie & Carling, 1997). AMPK (a sensor for cellular energy) is expressed in a variety of tissues (including liver, heart, muscles, adipose tissues) and plays an important role in regulating glucose and fatty acid metabolism in animals (Kahn *et al.* 2005a). The overall effect of AMPK activation is to switch off ATP-consuming pathways (including lipogenesis and gluconeogenesis), while turning on ATP-producing pathways (including fatty acid and glucose oxidation) (Kahn *et al.* 2005a; Carling, 2005; Steinberg *et al.* 2006).

Liver and adipose tissue are the primary and secondary sites, respectively, for *de novo* fatty acid synthesis in both human and rodents (Bergen & Mersmann, 2005). Acetyl-CoA carboxylase (ACC) is the rate-controlling enzyme for the synthesis of malonyl-CoA, a precursor for *de novo* fatty acid synthesis. There are two major isoforms of ACC, ACC1 (or ACCα) and ACC2 (or ACCβ), which are encoded by two different genes. The malonyl-CoA synthesized by ACC1 is used for fatty acid synthesis, whereas the malonyl-CoA produced by ACC2 is an allosteric inhibitor of carnitine palmitoyltransferase I (CPT-I), a rate-controlling enzyme for fatty acid oxidation (Viollet *et al.* 2006). Malonyl-CoA decarboxylase (MCD) is an enzyme that converts malonyl-CoA back to acetyl-CoA. Both ACC and MCD are downstream targets of AMPK. In addition, peroxisome proliferator activator receptor γ coactivator-1 α (PGC-1α), which is an important transcription coactivator and a master regulator of

mitochondrial biogenesis and oxidative phosphorylation (Puigserver, 2005), may regulate the expression of AMPK and the oxidation of energy substrates (Nisoli *et al.* 2003; Jobgen *et al.* 2006). Therefore, the AMPK-ACC system has emerged as a key pharmacological target for treating obesity and the metabolic syndrome (Ruderman & Prentki, 2004).

We recently found that dietary arginine supplementation increased the oxidation of glucose and fatty acids in skeletal muscle, reduced body fat and serum concentrations of triglycerides, enhanced the mass of skeletal muscle, and improved insulin sensitivity in diet-induced obese rats (Chapters II and III). The present study was conducted to test the hypothesis that dietary arginine supplementation regulates expression of AMPK, ACC, and PGC-1α, and possibly other key enzymes involved in the metabolism of energy substrates in insulin-sensitive tissues.

Materials and methods

Animals

Male Sprague-Dawley rats from Harlan Labs (Indianapolis, IN) were used in the present study. The experimental design was detailed in Chapter II. Briefly, 4- wk-old male Sprague-Dawley rats were fed either low- or high-fat diets for 15 wks (n=16/diet). Thereafter, lean or obese rats were then fed their respective diets and received drinking water containing either 1.51% L-arginine-HCl or 2.55% alanine (isonitrogenous control) (n = 8/treatment group). After 12 wks of treatment, rats were euthanized and tissues were isolated. Tissues for RNA analysis were immediately placed in RNA*later* solution

(Ambion/Applied Biosystems, Austin, TX) and stored at -80^{o}C until analysis. Tissues for western blot analysis were snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis.

RNA extraction and real-time polymerase chain reaction analysis (RT-PCR)

Tissues were minced on ice and homogenized in TRIzol (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacture's recommendation. Briefly, RNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in DEPC-treated water. The quantity and quality of RNA were assessed by measuring the absorbance at 280 and 260 nm, as well as by electrophoresis on 1.25% agarose gels. First-strand cDNAs were synthesized from 1 μg of total RNA using oligo (deoxythymidine) primers, random hexamer primers and SuperScript II Reverse Transcriptase as described previously (Hayashi *et al*. 2004)**.** RT-PCR analysis was performed using the SYBR Green method and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal cycling parameters were as follows: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. Primers were designed using Primer Express Software Version 1.5 (Applied Biosystems, Foster City, CA). The information on primer pairs for the selected genes, their specific locations in cDNA sequences and the product sizes are summarized in Table 4.1. All the data were normalized with the GAPDH gene in the same samples (Barber *et al.* 2005), and data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

Table 4.1. Primers of genes for real-time PCR analysis

Gene	Accession number	Forward primer	Reverse primer	cDNA location	Size	CDs
AMPKα	NM_023991	ACACCTCAGCGCTCCTGTTC	CTGTGCTGGAATCGACACT	1451–1527	67	8-1666
PGC-1α	NM_031347	CAACGCGGACAGAACTGAGA	CCGCAGATTTACGGTGCAT	2057–2127	71	42-2432
FAS	NM_017332	GGCCCTACCTTAACACGACTCA	GAACACAGTGATGGAACCTTCAAT	6745-6834	90	82-7599
SCD-1	NM_139192	CCTCCTGCTCATGTGCTTCA	GGCTGTGCAGGAAAGTTTCG	762-835	74	103-1179
ACC1	NM_022193	TGGGCACCCCAGAGCTAAG	AACTCCTCCCGCTCCTTCAA	6449-6518	70	1-7038
ACC2	NM_053922	GCGCCTACATCGTGGACAGT	GGCACCGGGAGGGATATAGA	6518-6585	68	7-7371
MCD	NM_053477	CACGGGCGACCCTGTTC	TCTCAGACTTCGCCCACTCA	1095-1162	68	1-1476
CPT-I a (liver)	NM_031559	GCACTATGGAGTCCTGCAACTTT	CCTCTGCTCTGCCGTTGAC	1925-1989	65	103-2424
CPT-I b (muscle)	NM_013200	GCGGATGCAGTGGGACAT	GCCTTGGCTACTTGGTACGAA	1587-1655	69	40-2358
UCP-3	NM_013167	GGATTCATGCCCTCCTTTCTG	CGTTTCAGCTGCTCGTAGGTT	939-1012	74	129-1055
GLUT-4	NM_012751	TGTGGCCTTCTTTGAGATTGG	CTGAAGAGCTCGGCCACAA	1313-1375	69	141-1670
HSL	NM_012859	CCTCTACTCGTCACCCATAGTCAA	GGCAGGGTCTTCAGCATGA	3094-3171	78	194-3400
LPL	NM_012598	CCCAGCTTTGTCATCGAGAAG	CCTGGCACAGAAGATGACCTTT	1444-1515	72	175-1599
Perilipin	NM_013094	GCTGTCTCCTCCACCAAAGG	ACCACAGTGTCTACCACGTTATCC	1210-1292	83	88-1641
NOS-1	NM_052799	CGCACCCTTCCGAAGCT	GCACGGATTCATTCCTTTGTG	3861–3927	67	102-4493
NOS-3	NM_021838	GGACATTTTCGGACTCACATTG	GCTGTCGCTCCTGCAAAGA	3474-3556	83	7-3615
GAPDH	NM_017008	GGGCAGCCCAGAACATCAT	CCAGTGAGCTTCCCGTTCAG	1445-1532	88	850-1851

Abbreviations used: AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator activator receptor γ coactivator-1 α; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase-1; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase; CPT-1, carnitine palmitoyltransferase I; UCP; uncoupling protein; GLUT-4, glucose transporter-4; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; NOS-1, nitric oxide synthase 1; NOS-3, nitric oxide synthase 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Isolation of mitochondria

Mitochondria were isolated from brown adipose tissue (BAT) followed the procedure of Xiao *et al.* (2004) with modifications. Briefly, BAT was minced and homogenized in an ice-cold buffer containing 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM ATP and 1x protease inhibitor cocktail (Calbiochem La Jolla, CA).

After incubation on ice for 10 min, the homogenate was centrifuged at 600 x g for 10 min at 4^{o}C. The supernatant fluid was collected and then centrifuged at 14,000 x g to obtain mitochondria. The mitochondria-enriched pellet was resuspended in a lysis buffer that consisted of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM of EDTA, 1% Triton X-100 and 1x protease inhibitor cocktail. The protein content was determined using the bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL).

Western blot analysis

Frozen tissues of liver, gastrocnemius muscle, extensor digitorum longus (EDL) muscle, and retroperitoneal (RP) adipose tissue were ground to powder under liquid nitrogen and then homogenized in the lysis buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM of EDTA, 1% Triton X-100, 1x protease inhibitor cocktail and 1x phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). For fat tissues, the homogenate was incubated at 37^{o}C for 1 h, during which it was mixed thoroughly by vortexing every 5-10 min. The samples were then centrifuged for 5 min at 10,000 x *g* at room temperature. The infranatants below fat was used for protein assay and western blot. For liver and muscle samples, homogenates were incubated on ice for 30 min, and then were centrifuged at 10,000 g for 10 min. The supernatant was used for protein assay and western blot. Proteins in homogenates were analyzed using the BCA method, and the samples were subsequently diluted with 2x Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% w/v SDS, 10% 2-mercaptoethanol, 12% glycerol, and 0.004% w/v bromphenol blue) and heated in boiling water for 5 min. Aliquots of samples were loaded onto SDS-polyacrylamide gels. After separation on 4-12% gels, proteins were transferred to a

nitrocellulose membrane (Bio-Rad, Hercules, CA) under 12V overnight, using the Bio-Rad Transblot apparatus (Hercules, CA). Membranes were blocked in 5% fat-free dry milk in TTBS (20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then were incubated with the following primary antibodies overnight at 4^{o}C with gentle rocking: AMPKα (Cell Signaling, 1:1000), phosphorylated AMPKα (Thr^{172}) (Cell Signaling, 1:1000), ACC (Cell Signaling, 1:1000), phosphorylated ACC (Ser^{79}) (Cell Signaling, 1:1000), or UCP-1 (Santa Cruz, 1:200). After washing three times with TTBS, the membranes were incubated at room temperature for 2-3 h with secondary antibodies at 1:50,000 (peroxidase-labeled donkey anti-goat or anti-rabbit IgG, Jackson Immuno Research). Finally, the membranes were washed with TTBS, followed by development using Supersignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL). The signals were detected on Fujifilm LAS-3000 (Tokyo, Japan).

Protein kinase B (PKB) and ribosomal protein S6 kinase (S6K1) in EDL muscle were analyzed as described by Suyawan *et al.* (2004). Briefly, proteins were separated on 10% SDS-polyacrylamide gels, and transferred to polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Membranes were then incubated with antibodies against phosphorylated PKB (Thr^{473}) (Cell Signaling, 1:1000) or phorsphorylated S6K1 (Thr^{389}) (Cell Signaling, 1:1000) to assess the phosphorylation of PKB and S6K1. To assess the total PKB and total S6K1, membranes were then stripped and reprobed with antibodies against PKB (Cell Signaling, 1:1000) or S6K1 (Santa Cruz, 1:1000). After incubation with secondary antibodies (goat-anti-rabbit IgG, Bio-Rad, 1:5000), the blots

were developed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL), and visualized by a ChemiDoc-It imaging system (UVP, Upland, CA). All data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

Data analysis

Results were expressed as means ± SEM. Statistical analysis were performed using two-way ANOVA with Tukey multiple comparison test to determine the effects of diet, amino acid (AA), and the interaction between diet and AA (diet x AA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

mRNA levels for genes involved in metabolism of energy substrates in liver, gastrocnemius muscle and RP adipose tissue

The mRNA levels for major genes involved in the oxidation of energy substrates increased ($P<0.05$) in the liver of arginine-supplemented rats: CPT-I (1.9-fold), AMPK (1.5-fold), and PGC-1α (1.6-fold), when compared with alanine-supplemented rats (Table 4.2). Hepatic mRNA levels for key lipogenic enzymes, including stearoyl-CoA desaturase 1 (SCD-1) and ACC1, were lower in rats fed the high-fat diet ($P<0.05$) than in rats fed the low-fat diet. In contrast, the arginine treatment reduced ($P<0.05$) hepatic expression of two key lipogenic genes: fatty acid synthase (FAS; -60%) and SCD-1 (-40%). Hepatic mRNA levels for MCD decreased ($P<0.01$) in rats fed the high-fat diet, but increased ($P<0.05$) in response to dietary arginine supplementation.

Gastrocnemius muscle contains both fast-twitch white and fast-twitch red fiber types (Raja *et al*. 2003). Among all the genes studied in muscle, only CPT-1 exhibited an increase ($P<0.05$) in its mRNA levels in response to dietary arginine supplementation, while mRNA levels for AMPK, PGC-1α, glucose transporter-4 (GLUT-4), uncoupling protein-3 (UCP-3), and two NOS isoforms (NOS-1 and NOS-3) did not differ between alanine- and arginine-supplemented rats (Table 4.3). mRNA levels for all these genes did not differ between rats fed the low- and high-fat diets.

Table 4.2. Relative mRNA levels for key genes involved in energy metabolism in rat liver

Genes	Low-fat diet		High-fat diet		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
CPT-I	1.00±0.21	2.11±0.15	1.02±0.13	1.72±0.46	0.53	0.04	0.46
AMPKα	1.00±0.15	1.59±0.12	1.00±0.07	1.29±0.30	0.44	0.03	0.45
PGC-1α	1.00±0.15	1.67±0.11	0.97±0.13	1.39±0.27	0.41	0.006	0.51
FAS	1.00±0.26	0.39±0.08	0.49±0.11	0.35±0.09	0.12	0.021	0.11
SCD-1	1.00±0.09	0.62±0.19	0.44±0.16	0.24±0.07	0.001	0.043	0.51
ACC1	1.00±0.17	0.90±0.17	0.63±0.13	0.48±0.07	0.012	0.39	0.89
ACC2	1.00±0.07	0.82±0.11	0.90±0.12	0.83±0.18	0.70	0.35	0.68
MCD	1.00±0.21	1.76±0.26	0.63±0.08	0.77±0.19	0.002	0.031	0.50

Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and mRNA levels were measured using RT-PCR. Data are expressed as means ± SEM (n=6-8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

Table 4.3. Relative mRNA levels for key genes involved in energy metabolism in rat gastrocnemius muscle

Genes	Low-fat diet		High-fat diet		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
AMPKα	1.00±0.13	1.12±0.12	0.81±0.12	0.88±0.11	0.14	0.51	0.86
PGC-1α	1.00±0.25	1.16±0.22	1.02±0.23	0.92±0.16	0.67	0.91	0.61
CPT-I	1.00±0.16	1.48±0.47	1.05±0.25	1.65±0.30	0.73	0.032	0.63
GLUT-4	1.00±0.12	1.25±0.27	1.12±0.13	1.29±0.27	0.74	0.39	0.87
UCP-3	1.00±0.17	1.11±0.23	0.87±0.15	1.12±0.40	0.82	0.52	0.80
NOS-3	1.00±0.25	1.20±0.23	1.53±0.21	1.26±0.20	0.22	0.89	0.33
NOS-1	1.00±0.20	0.97±0.21	1.03±0.11	0.79±0.06	0.68	0.44	0.55

Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and mRNA levels were measured by RT-PCR. Data are expressed as means ± SEM (n=6-8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

In the RP adipose tissue, high-fat feeding decreased (P<0.05) the expression of key lipogenic genes, including FAS (-72%) and SCD-1 (-81%), whereas the expression of AMPK and GLUT-4 were reduced by 24% and 82% respectively in response to high-fat feeding (Table 4.4). Dietary arginine supplementation had no effect on expression of the genes for AMPK, hormone-sensitive lipase (HSL), perilipin, and lipoprotein lipase (LPL) in the adipose tissue. However, the arginine treatment reduced (P<0.05) expression of PGC-1α, while increasing (P<0.05) expression of PPAR-γ (Table 4.4), an important transcription factor involved in lipogenesis (Rosen *et al.* 2000).

Table 4.4. Relative mRNA levels for key genes involved in energy metabolism in rat RP adipose tissue

Genes	Low-fat diet		High-fat diet		*P* value		
	Alanine	Arginine	Alanine	Arginine	Diet	AA	Diet x AA
AMPKα	1.00±0.08	1.11±0.09	0.76±0.08	0.79±0.13	0.042	0.65	0.82
PGC-1α	1.00±0.13	0.79±0.12	1.34±0.69	0.46±0.07	0.14	0.012	0.37
GLUT-4	1.00±0.07	1.20±0.19	0.69±0.12	0.52±0.06	0.036	0.55	0.20
FAS	1.00±0.19	1.48±0.16	0.39±0.05	0.31±0.06	0.001	0.14	0.044
SCD-1	1.00±0.12	1.29±0.28	0.23±0.04	0.20±0.04	0.001	0.56	0.48
PPAR-g	1.00±0.22	1.59±0.24	1.07±0.22	1.51±0.25	0.98	0.045	0.76
LPL	1.00±0.14	0.73±0.19	0.80±0.21	0.90±0.29	0.97	0.69	0.39
HSL	1.00±0.09	0.88±0.16	1.13±0.26	1.04±0.28	0.22	0.67	0.42
Perilipin	1.00±0.19	0.98±0.25	1.08±0.26	1.16±0.11	0.41	0.63	0.46
NOS-1	1.00±0.13	1.31±0.19	1.05±0.32	1.27±0.18	0.99	0.25	0.82

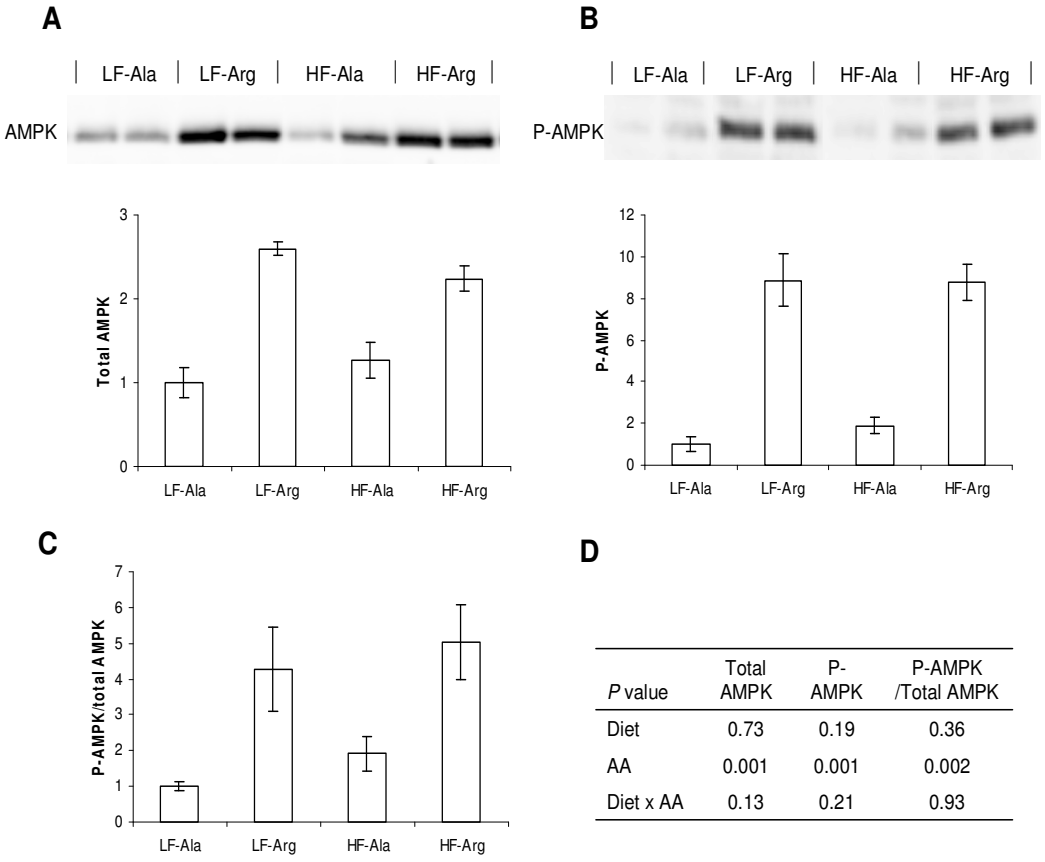
Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and mRNA levels were measured by RT-PCR. Data are expressed as means ± SEM (n=6-8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

Protein levels for AMPK and ACC in liver, gastrocnemius muscle and RP adipose tissue

Hepatic protein levels for total AMPK (Fig. 4.1A) or phosphorylated AMPK (Fig. 4.1B) were not affected by high-fat diet, but increased (P<0.001) by 1.2- and 6.0-fold, respectively, in response to dietary arginine supplementation. The ratio of phosphorylated AMPK to total AMPK in the liver was 2.2-fold greater in arginine- than in alanine-supplemented rats (Fig. 4.1C). Hepatic protein levels for total ACC (both ACC1 and ACC2) did not differ among the four groups of rats (Fig. 4.2A), but the levels

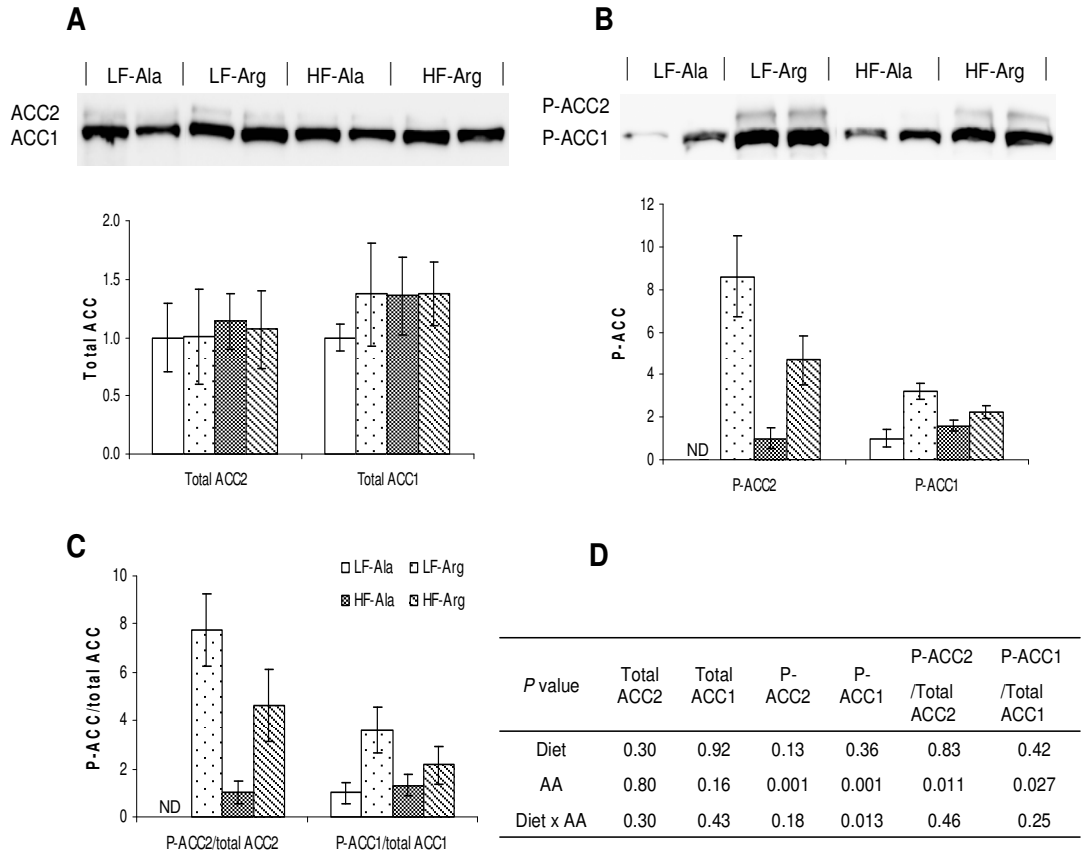
for phosphorylated ACC were altered by both diet and arginine treatment (Fig. 4.2B). Particularly, in the liver of alanine-supplemented rats, ACC2 phosphorylation was undetectable or barely detectable in response to a low-fat or high-fat diet, respectively. In contrast, dietary arginine supplementation markedly increased ($P<0.001$) hepatic protein levels for both phosphorylated ACC1 and phosphorylated ACC2. There was an interaction ($P<0.05$) between diet and arginine treatment regarding the phosphorylation of ACC1, in that the effect of the arginine treatment was greater in lean than in obese rats (Fig. 4.2B). The ratio of phosphorylated ACC to total ACC (for both ACC1 and ACC2) also increased ($P<0.05$) in response to dietary arginine supplementation (Fig. 4.2C).

In contrast to the liver, protein levels for AMPK and ACC (both total and phosphorylated forms) in gastrocnemius muscle were not affected by either high-fat diet or arginine treatment (Fig 4.3 and 4.4). Similar results were observed for AMPK expression in RP adipose tissue (Fig. 4.5). However, high-fat diet decreased ($P<0.05$) protein levels for ACC2 (both total and phosphorylated forms) by approximately 80%, and reduced the amounts of ACC1 (both total and phosphorylated forms) below a detectable level (Fig. 4.6A, B). Dietary arginine supplementation had no effect in rats fed high-fat diet, but increased protein levels for phosphorylated ACC1 and ACC2 in rats fed the low-fat diet. Also, the ratios of phosphorylated ACC1/total ACC1 and phosphorylated ACC2/ACC2 increased ($P<0.05$) by approximately 1.2- and 2.7-fold, respectively, in response to arginine supplementation.



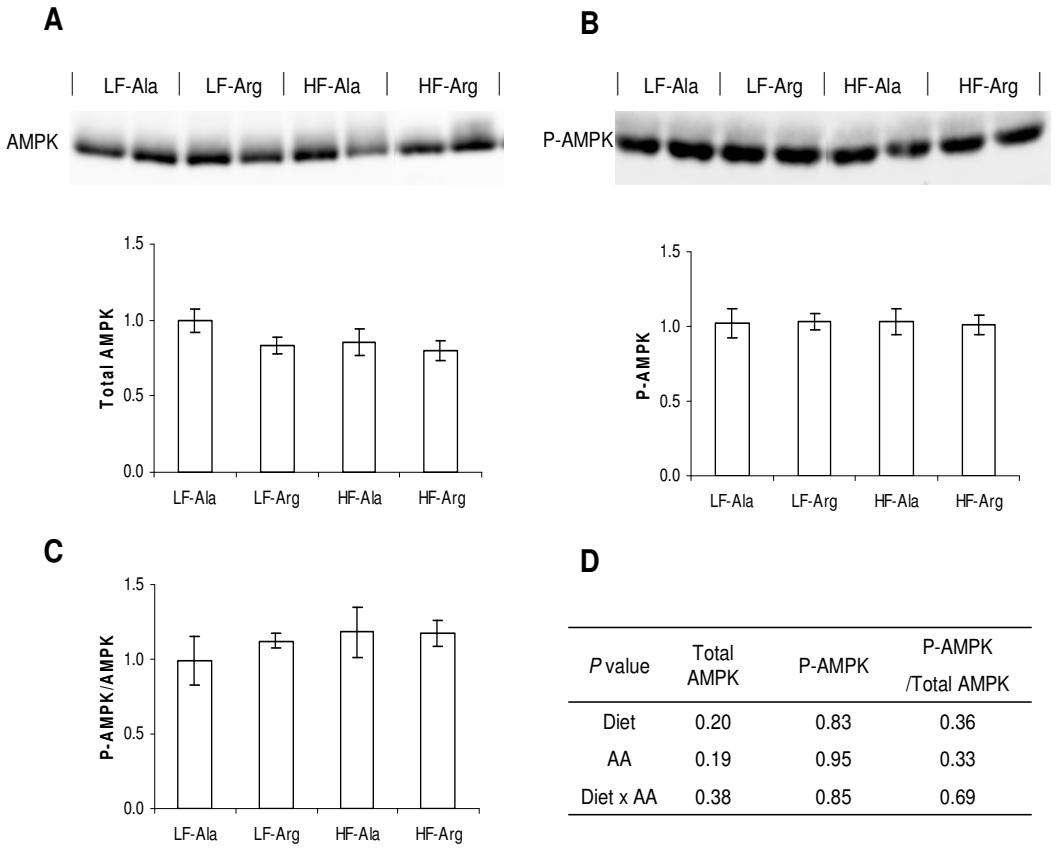
P value	Total AMPK	P-AMPK	P-AMPK /Total AMPK
Diet	0.73	0.19	0.36
AA	0.001	0.001	0.002
Diet x AA	0.13	0.21	0.93

Fig. 4.1. Relative protein levels for total AMPK (A) and phosphorylated AMPK (P-AMPK; B) as well as the ratio of P-AMPK to total AMPK (C) in rat liver. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 µg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.



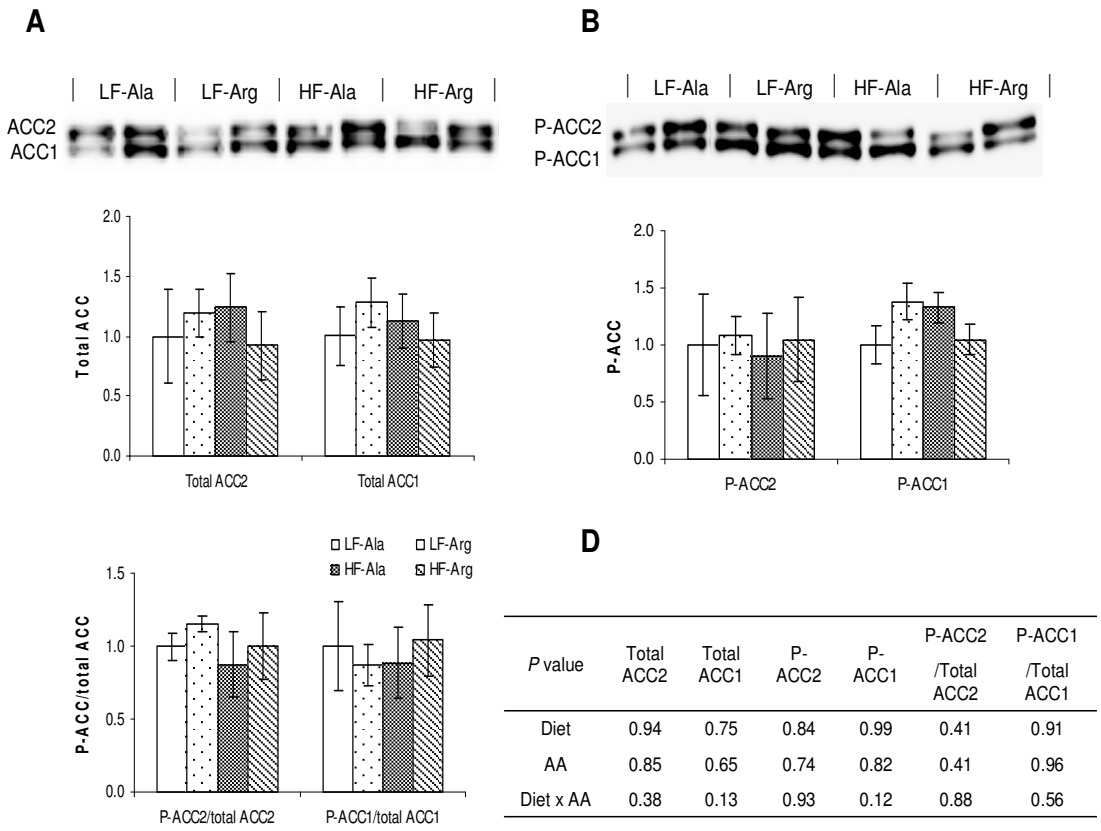
P value	Total ACC2	Total ACC1	P-ACC2	P-ACC1	P-ACC2 /Total ACC2	P-ACC1 /Total ACC1
Diet	0.30	0.92	0.13	0.36	0.83	0.42
AA	0.80	0.16	0.001	0.001	0.011	0.027
Diet x AA	0.30	0.43	0.18	0.013	0.46	0.25

Fig. 4.2. Relative protein levels for total ACC (A) and phosphorylated ACC (P-ACC; B) as well as the ratios of P-ACC to total ACC (C) in rat liver. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 μg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet. ND: not detectable.



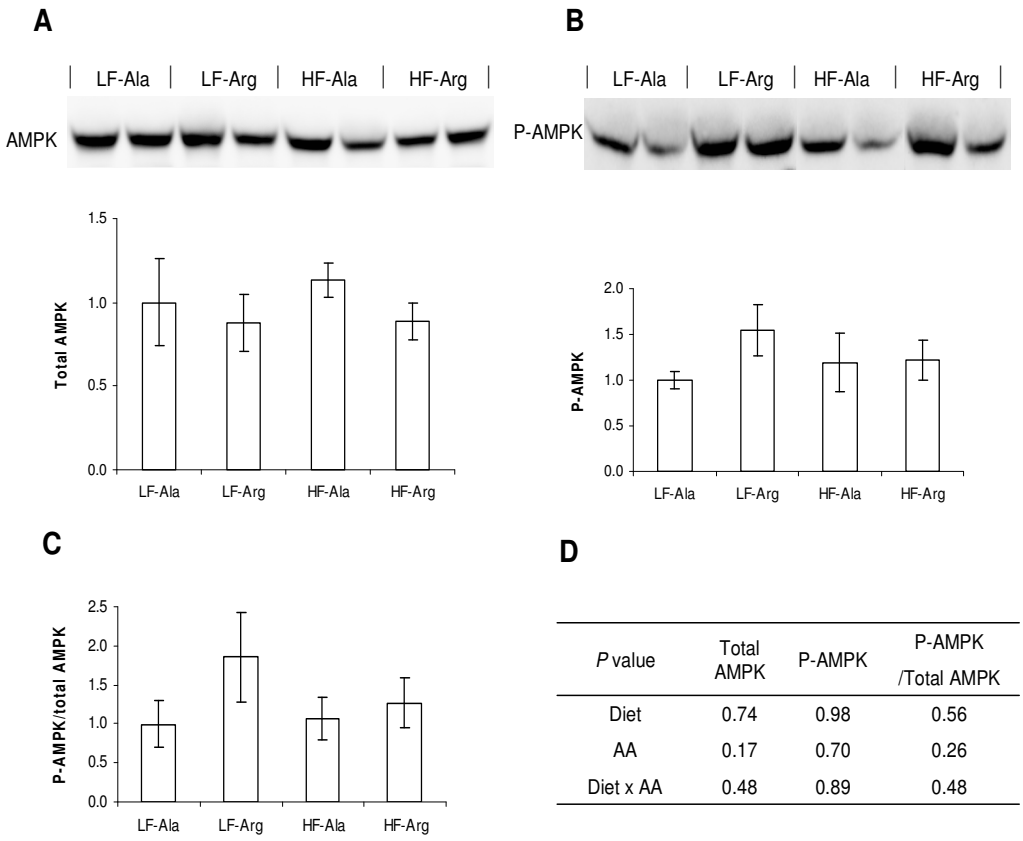
P value	Total AMPK	P-AMPK	P-AMPK /Total AMPK
Diet	0.20	0.83	0.36
AA	0.19	0.95	0.33
Diet x AA	0.38	0.85	0.69

Fig. 4.3. Relative protein levels for total AMPK (A) and phosphorylated AMPK (P-AMPK; B) as well as the ratio of P-AMPK to total AMPK (C) in rat gastrocnemius muscle. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 μg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.



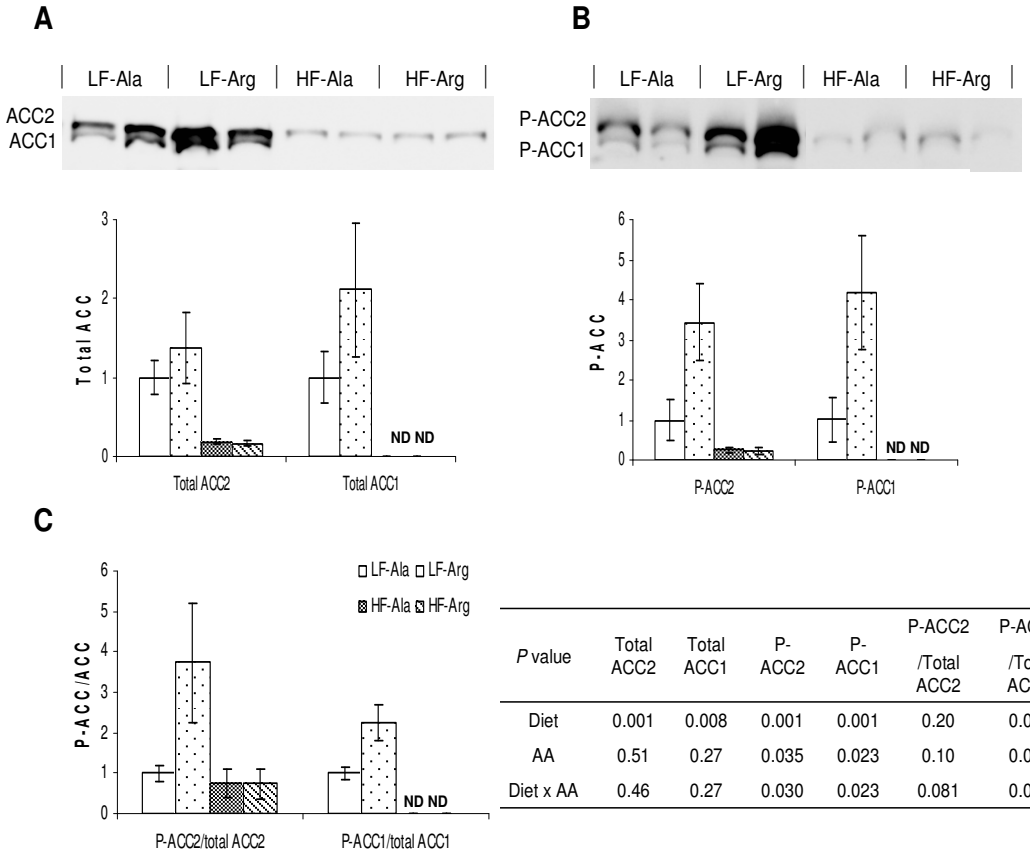
P value	Total ACC2	Total ACC1	P-ACC2	P-ACC1	P-ACC2 /Total ACC2	P-ACC1 /Total ACC1
Diet	0.94	0.75	0.84	0.99	0.41	0.91
AA	0.85	0.65	0.74	0.82	0.41	0.96
Diet x AA	0.38	0.13	0.93	0.12	0.88	0.56

Fig. 4.4. Relative protein levels for total ACC (A) and phosphorylated ACC (P-ACC; B) as well as the ratios of P-ACC to total ACC (C) in rat gastrocnemius muscle. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 µg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.



P value	Total AMPK	P-AMPK	P-AMPK /Total AMPK
Diet	0.74	0.98	0.56
AA	0.17	0.70	0.26
Diet x AA	0.48	0.89	0.48

Fig. 4.5. Relative protein levels for total AMPK (A) and phosphorylated AMPK (P-AMPK; B) as well as the ratio of P-AMPK to total AMPK (C) in rat RP adipose tissue. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 µg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.



P value	Total ACC2	Total ACC1	P-ACC2	P-ACC1	P-ACC2 /Total ACC2	P-ACC1 /Total ACC1
Diet	0.001	0.008	0.001	0.001	0.20	0.001
AA	0.51	0.27	0.035	0.023	0.10	0.006
Diet x AA	0.46	0.27	0.030	0.023	0.081	0.006

Fig. 4.6. Relative protein levels for total ACC (A) and phosphorylated ACC (P-ACC; B) as well as the ratios of P-ACC to total ACC (C) in rat RP adipose tissue. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (30 µg) were separated on 4-12% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet. ND: not detectable.

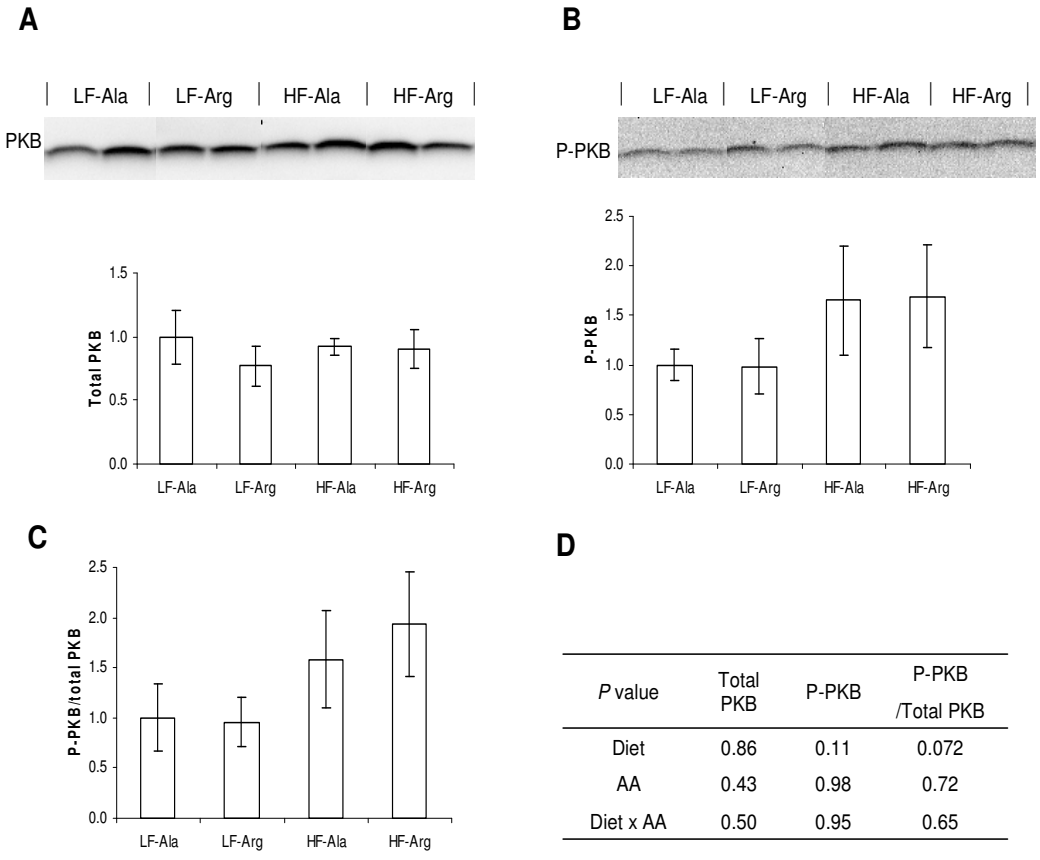
Protein levels for PKB and S6K1 in EDL muscle and uncoupling protein 1 (UCP-1) in brown adipose tissue

Neither high-fat diet nor dietary arginine supplementation affected protein levels for total PKB or phosphorylated PKB in EDL muscle (Fig. 4.7). Similar results were obtained for S6K1 (Fig. 4.8). Protein concentrations for UCP-1 in brown adipose tissue did not differ among the four groups of rats (Fig. 4.9).

Discussion

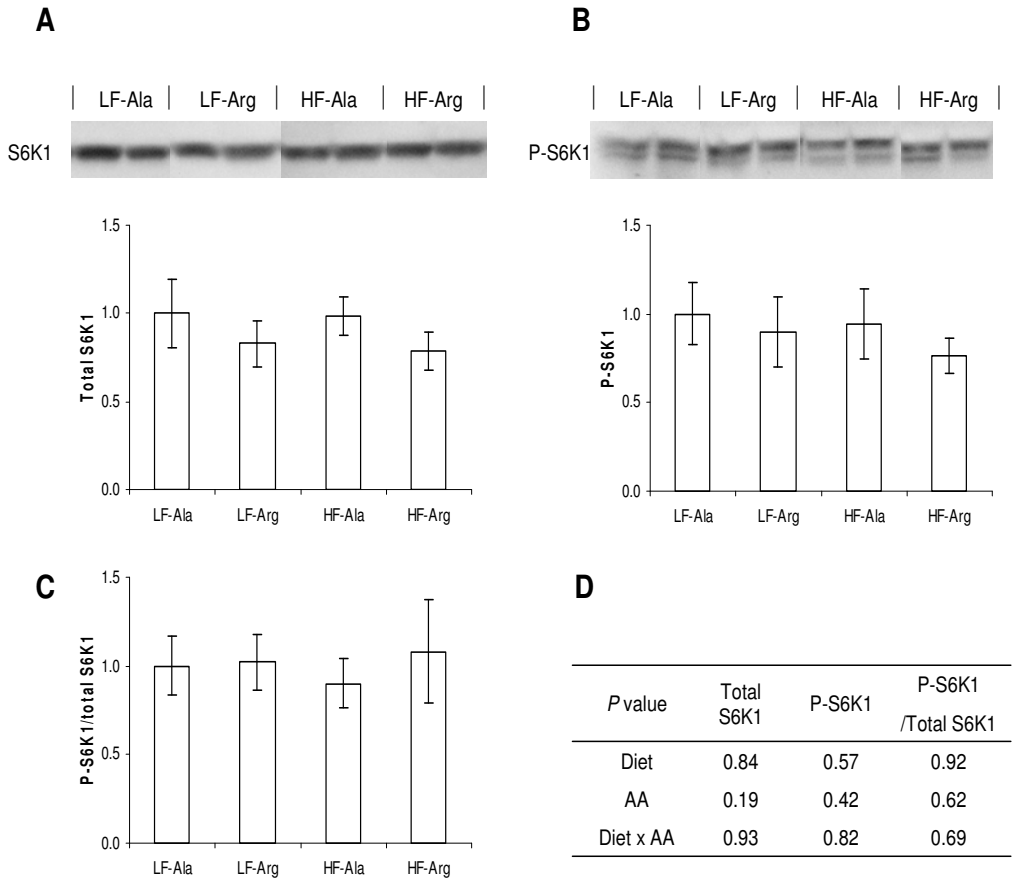
A novel and important finding from the present study is that dietary arginine supplementation enhanced hepatic expression of AMPK (indicated by increases in both mRNA and protein levels of AMPK) as well as hepatic AMPK phosphorylation. These results demonstrate for the first time that the arginine treatment increases AMPK expression and activity in the liver at transcriptional, translational, and post-translational levels. Phosphorylated AMPK is the active form of AMPK, and previous studies have established that the phosphorylation of AMPK is positively correlated to AMPK activity (Park *et al.* 2002b). Along with the activation of AMPK, protein levels for phosphorylated ACC, which is negatively related to its activity (Park *et al.* 2002b), also increased in response to the arginine treatment. Moreover, there was an increase in hepatic mRNA expression of MCD in arginine-supplemented rats (Table 4.2). Further, PGC-1α mRNA expression was elevated in the liver of arginine-supplemented rats. These changes in hepatic expression and activity of AMPK, ACC-2, MCD and PGC-1α

in response to dietary arginine supplementation favor the hepatic oxidation of long-chain fatty acids (Stanley *et al.* 2005; Viollet *et al.* 2006).



P value	Total PKB	P-PKB	P-PKB /Total PKB
Diet	0.86	0.11	0.072
AA	0.43	0.98	0.72
Diet x AA	0.50	0.95	0.65

Fig. 4.7. Relative protein levels for PKB (A) and phosphorylated PKB (P-PKB; B) as well as the ratio of P-PKB to total PKB (C) in rat EDL muscle. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (50 µg) were separated on 10% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.



P value	Total S6K1	P-S6K1	P-S6K1 /Total S6K1
Diet	0.84	0.57	0.92
AA	0.19	0.42	0.62
Diet x AA	0.93	0.82	0.69

Fig. 4.8. Relative protein levels for S6K1 (A) and phosphorylated S6K1 (P-S6K1; B) as well as the ratio of P-S6K1 to total S6K1 (C) in rat EDL muscle. Lean and obese rats, which were fed low (LF)- and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, tissues were collected and protein levels were measured. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

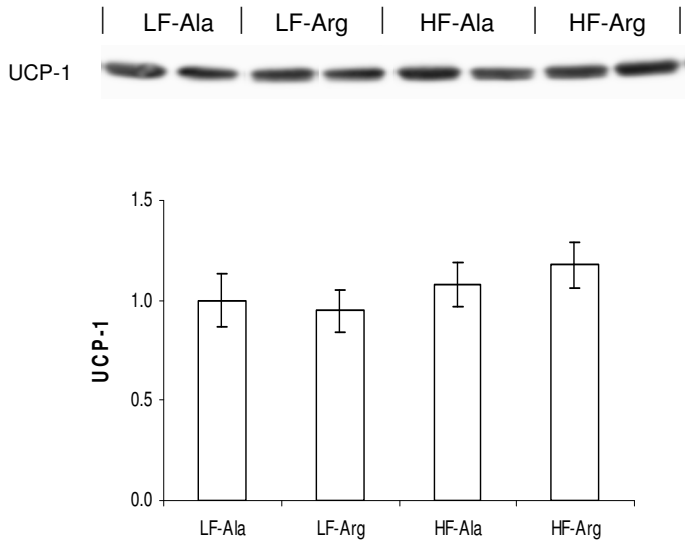


Fig. 4.9. Relative protein levels for UCP-1 in rat brown adipose tissue. Lean and obese rats, which were fed low (LF) and high-fat (HF) diets, respectively, received drinking water containing either 1.51% of L-arginine·HCl or 2.55% L-alanine (isonitrogenous control) for 12 wks. At the end of the experiment, brown adipose tissues were collected and mitochondria were isolated. Mitochondrial proteins (10 μg) were separated on 10% SDS- polyacrylamide gels. Data are expressed as means ± SEM (n=8/treatment group). Data are expressed as the relative values to those of alanine-supplemented rats fed the low-fat diet.

Liver is the major site for the *de novo* synthesis of fatty acids in rats (Jobgen *et al.* 2006). In addition to the activation of the hepatic AMPK-ACC system, dietary arginine supplementation reduced hepatic expression of key genes that are involved in fatty acid synthesis, including FAS and SCD-1 (Table 4.2). In arginine-supplemented rats, the reduced expression of hepatic lipogenic genes, coupled with the decreased activity of ACC1, is expected to decrease the synthesis of fatty acids and triglycerides in the liver. Indeed, it has been shown that the deficiency of SCD-1, a key lipogenic enzyme in monounsaturated fatty acid synthsis, resulted in a reduced synthesis of triglycerides and

an increased oxidation of fatty acids (Dobrzyn & Ntambi, 2005). It is not known whether arginine directly regulates hepatic gene expression or whether its effect is mediated by NO or other metabolites. However, there is growing evidence that physiological levels of NO reduce fatty acid synthesis and increase fatty oxidation in rat hepatocytes (Garcia-Villafranca *et al.* 2003).

Skeletal muscle also plays a crucial role in the homeostasis of glucose and fatty acids (Jobgen *et al.* 2006). Results from this dissertation research indicate that the oxidation of these substrates in skeletal muscle was decreased by high-fat feeding but increased in response to dietary arginine supplementation (Chapter III). Physiological levels of NO, synthesized from arginine, may stimulate glucose transport and oxidation by muscle cells (Jobgen *et al.* 2006). Additionally, the higher expression of CPT-I in gastrocnemius muscle of arginine-supplemented rats may explain an increase in the oxidation of long-chain fatty acids. In contrast, the expression of AMPK or ACC in gastrocnemius muscle was not affected by high-fat diet or arginine treatment (Fig. 4.6). This result is consistent with the recent report that total AMPK and phosphorylated AMPK in soleus muscle did not differ between mice fed low- and high-fat diets (Martin *et al.* 2006). These findings, however, are not consistent with the observation of Liu *et al.* (2006) that feeding male Wistar rats a high-fat diet for 5 months reduced both mRNA and protein levels for AMPK in skeletal muscle. This discrepancy may result from differences in the length of high-fat feeding and the fiber type of skeletal muscle studied.

Dietary arginine supplementation increased skeletal muscle mass in rats fed low- and high-fat diets (Chapter II). This anabolic effect may occur through the nutrient-signaling

pathway, which includes the activation of PKB/Akt and mammalian target of rapamycin (mTOR) (Dann & Thomas, 2006). mTOR mediates mRNA translation mainly through phosphorylation of its two downstream targets, key translation regulators: ribosomal S6K1 and eIF4E-binding protein-I (4E-BP1) (Holz *et al.* 2005). Interestingly, neither high-fat diet nor dietary arginine supplementation affected protein levels for PKB or S6K1 (both total and phosphorylated forms) in EDL muscle (Fig. 4.7 and 4.8). It is possible that the arginine treatment can inhibit protein degradation in skeletal muscle. Further studies are necessary to test this hypothesis.

In contrast to the liver, dietary arginine supplementation increased expression of some key lipogenic genes in rat adipose tissue, as reported by Yan *et al.* (2002) for preadipocytes during their differentiation. In addition, PGC-1α was reduced by the arginine treatment (Table 4.4). The major function of white adipose tissue is to store lipid and release free fatty acids as an energy source for other tissues, such as liver and skeletal muscles (Frayn *et al.* 2003; Gaidhu *et al.* 2006). Therefore, dietary arginine supplementation may be capable of increasing the formation of triglycerides from the existing fatty acids and the glucose-derived glycerol. However, there is little *de novo* synthesis of fatty acids in the white adipose tissue of adult rats (Jobgen *et al.* 2006), and therefore, conversion of glucose into fatty acids is virtually absent from this tissue in 7- to 8-month-old rats.

Brown adipose tissue is rich in mitochondria, where fatty acid oxidation results in the production of heat rather than ATP, because of the presence of UCP-1 (Himms-Hagen, 2001). Although dietary arginine supplementation did not affect UCP-1

concentrations in brown adipose tissue (Fig. 4.9), an increase in its mass brought about by the arginine treatment (Chapter II) is expected to enhance fatty acid oxidation in this tissue. This may provide another important mechanism for the loss of fat mass in arginine-supplemented DIO rats (Chapter II).

In summary, dietary arginine supplementation increased expression of key enzymes for glucose and fatty acid oxidation in the liver and expression of CPT-1 in skeletal muscle, while decreasing hepatic expression of key enzymes for lipogenesis. These coordinate changes in gene expression among insulin-sensitive tissues provide a molecular mechanism for the loss of excess fat mass in DIO rats in response to dietary arginine supplementation.

CHAPTER V

L-ARGININE INCREASES AMPK PHOSPHORYLATION AND OXIDATION OF ENERGY SUBSTRATES IN HEPATOCYTES, SKELETAL MUSCLE CELLS AND ADIPOCYTES

Previous work has shown that dietary arginine supplementation reduced fat mass in obese rats. The present study was conducted with cell models to define direct effects of arginine on energy-substrate oxidation in hepatocytes, skeletal muscle cells, and adipocytes. BNL CL2 mouse hepatocytes, C2C12 mouse myotubes and 3T3-L1 mouse adipocytes were treated with different extracellular concentrations of L-arginine (0, 15, 50, 100 and 400 μM) or 400 μM arginine + 0.5 mM N^G-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) for 48 h. Increasing arginine concentrations dose-dependently increased glucose and oleic acid oxidation in all three cell types, lactate release from C2C12 cells, and oleic acid incorporation into lipids in BNL CL.2 and 3T3-L1 cells. Arginine at 400 μM also stimulated the phosphorylation of AMP-activated protein kinase (AMPK) in all three cell types and increased NO production in C2C12 and BNL CL.2 cells. The inhibition of NOS by L-NAME moderately reduced glucose and oleic acid oxidation, lactate release, and the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) in BNL CL.2 cells, but had no effect on C2C12 or 3T3-L1 cells. Collectively, these results indicate that arginine increased AMPK activity and energy-substrate oxidation in BNL CL.2, C2C12 and 3T3-L1 cells through both NO-dependent and independent mechanisms.

Nitric oxide (NO) is a free radical produced from L-arginine (Arg) by NO synthases (NOS) (Wu & Morris, 1998). Three isoforms of the NOS are expressed in various tissues, including insulin-sensitive tissues (liver, muscle and adipose tissue) that play an important role in whole body homeostasis of energy substrates (Jobgen *et al.* 2006). In cell culture models, NO production has been detected for hepatocytes (Nicholls-Grzemski *et al.* 1999), skeletal muscle cells (Williams *et al.* 1994) and adipocytes (Yan *et al.* 2002) under both basal and stimulated conditions. The actions of NO are mediated primarily by the activation of guanylyl cyclase and the resultant production of guanosine 3',5'-cyclic monophosphate (cGMP) (Denninger & Marletta, 1999).

Our previous studies using Zucker diabetic fatty rats (Fu *et al.* 2005) and diet-induced obese rats (Chapter III) demonstrated that dietary L-arginine supplementation increased glucose and fatty acid oxidation in muscle and adipose tissue, therefore reducing body fat mass. Further, we found that this beneficial effect of arginine was mediated partially through the activation of hepatic AMP-activated protein kinase (AMPK) (Chapter IV). There is growing evidence that NO regulates energy metabolism in cells and animals (Jobgen *et al.* 2006). Therefore, we hypothesized that L-arginine increased energy metabolism through enhancing NO production and activating AMPK. This hypothesis was tested using established cell models for hepatocytes, skeletal muscle cells and adipocytes (Wang & Watford, 2007).

Materials and methods

Materials

BNL CL.2 mouse hepatocytes, C2C12 mouse skeletal muscle cells and 3T3-L1 mouse preadipocytes were obtained from ATCC (Manassa, VA). Dulbecco's modified Eagle medium (DMEM) with or without arginine, horse serum, trypsin-EDTA solution, penicillin (10,000 U/mL), streptomycin (10,000 µg/mL), and 4-12% NuPAGE Bis-Tris gels were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS), NCS-II, and the bicinchoninic acid kit were procured from Hyclone (Logan, UT), Amersham (Piscataway, NJ), and Pierce (Rockford, IL), respectively. HPLC-grade methanol and water were purchased from Fisher Scientific (Houston, TX). D-[U-^{14}C]glucose and [1-^{14}C]oleic acid were products of American Radiolabeled Chemicals (St Louis, MO). Protease inhibitor and phosphatase inhibitor cocktails were obtained from Calbiochem (La Jolla, CA). Unless indicated, all other chemicals were procured from Sigma-Aldrich (St Louis, MO).

Cell culture

BNL CL.2 cells, C2C12 cells, and 3T3-L1 cells were cultured in 100-mm dishes with DMEM containing 10% FBS and 100 U/mL penicillin plus 100 µg/mL streptomycin until 75-80% confluent. Cells were trypsinized and split into 25 cm^2 Falcon flasks with approximately 2 x 10^5 cells per flask and maintained at 37^{o}C in a 5% CO_2 incubator. The basal DMEM contained 25 mM glucose, 4 mM glutamine, and 0.4 mM arginine. For the experiment, BNL CL.2 cells at confluence were used directly, whereas differentiated C2C12 and 3T3-L1 cells were employed. After C2C12 cells reached

confluence, differentiation was initiated by substituting 10% FBS with 2% horse serum in the culture medium. The medium was changed every two days. Four days after the initiation of differentiation, myotubes were formed and then used for the experiment. For differentiating 3T3-L1 cells, they were cultured in DMEM with 10% FBS until confluence. Two days post-confluence, differentiation was initiated by switching the cells to DMEM containing 10% FBS, 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μM dexamethasone. Two days later, the medium was changed to DMEM containing 10% FBS and 10 μg/mL insulin. After an additional 2 d, cells were switched to DMEM containing 10% FBS. The medium was then changed every other day. Eight days after the initiation of cell differentiation, mature adipocytes were obtained and then used for the experiment.

The day before performing an experiment, 3T3-L1 adipocytes and BNL CL.2 hepatocytes were switched to DMEM containing 0.5% FBS for overnight, whereas C2C12 cells were maintained in DMEM containing 2% horse serum. The concentrations of serum were chosen on the basis of the consideration that a high level might mask the effect of arginine, while cell viability might be compromised in serum-free medium. On the day of the experiment, all cells were switched to arginine-free DMEM containing 0.5% FBS, and this culture medium contained 0.7 μM arginine. L-Arginine was then added to the medium to provide a concentration of 0, 15, 50, 100, or 400 μM. In some experiments, culture media contained 400 μM arginine plus 500 μM N^G-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor). To ensure optimal inhibition of NO production in cells, L-NAME was added to the media 1 h earlier than arginine. All the

cells were cultured at 37°C for 48 h, with the medium changed every 24 h. At the end of the 48-h culture period, cells were used for biochemical studies and the media were analyzed for nitrite (an indicator of NO synthesis).

Glucose and oleic acid oxidation

Cells were rinsed with PBS three times, and were then incubated in the same flasks at 37°C in 2 mL of oxygenated (95%O_2/5%CO_2) Krebs bicarbonate buffer (KRB, pH 7.4) containing 5 mM of D-glucose and either 0.5 μCi D-[U-^{14}C] glucose or 0.2 mM oleic acid plus 0.5 μCi [1-^{14}C]oleic acid. Arginine or L-NAME was added to incubation medium to obtain the same respective concentration as in the previous 48-h culture medium. The flasks were sealed tightly with rubber stoppers fitted with hanging center-wells. At the end of a 2-h incubation period, flasks were placed in the straight-up position, and 0.2 mL NCS-II was added through the stopper into a 500-μl microcentrifuge tube placed in the center-well, followed by addition of 0.2 mL of 1.5 M $HClO_4$ to the incubation medium. After an additional 1-h incubation, the microcentrifuge tubes with NCS-II were carefully transferred to scintillation vials. Fifteen milliliters of counting cocktail were added to the vials and ^{14}C radioactivity was determined by a Packard liquid scintillation counter. The specific activities of D-[U-^{14}C] glucose and [1-^{14}C] oleic acid in the incubation media were used to calculate the rate of $^{14}CO_2$ production. Lactate in the acidified medium was analyzed using an enzymatic method (Wu *et al.* 1995). The cells were lysed and collected for the determination of protein concentration using the bicinchoninic acid kit.

Glucose and oleic acid incorporation into lipids

The determination of D-[U-^{14}C] glucose incorporated into lipids was performed as described by Ceddia *et al.* (2000). Briefly, the acidified incubation medium was collected and mixed with 5 mL of Doles's reagent (isopropanol/*n*-heptane/H_2SO_4; 4:1:0.25, v/v/v) (Dole & Meinertz, 1960). The heptane layer, which contained lipid, was carefully transferred to scintillation counting vials and dried under a fumehood. Then, 15 mL of counting cocktail was added to the vials for the measurement of ^{14}C radioactivity. The incorporation of [1-^{14}C] oleic acid into lipids was determined as described by Oliveira and Vaughan (1964) with modifications. Briefly, the acidified incubation medium was mixed with 15 mL of a chloroform-methanol solution (2:1, v/v). After shaking for 20 min, 9 mL of 4% Na_2CO_3 (w/v in water) was added, followed by 20 minutes of mixing. The tubes were then centrifuged to allow the separation of aqueous and chloroform layers. The chloroform layer was transferred into clean tubes and subjected to two more washes with 4% Na_2CO_3. After the chloroform layer was transferred to a scintillation vial and dried under a fumehood, 15 mL of counting cocktail was added to the vials for the measurement of ^{14}C radioactivity.

Nitrite analysis

Nitrite in cell culture medium was analyzed using an HPLC method as described by Li *et al.* (2000). This technique offers the advantages of high sensitivity and specificity for the determination of nitrite as an oxidation product of nitric oxide (Jobgen *et al*. 2007). The medium without cells was included as blanks.

Western blot analysis

BNL CL.2 cells, C2C12 cells, and 3T3-L1 cells were cultured for 48 h in the presence of 0, 50, 400 μM L-arginine, or 400 μM L-arginine plus 500 μM L-NAME, as described above. After the 48-h culture period, cells were washed with PBS three times, followed by lysis in a buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM of EDTA, 1% Triton X-100, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail. Cells were then scraped off into tubes and centrifuged for 10 min at 10,000 x *g*. Protein content in the supernatant fraction was analyzed using the bicinchoninic acid kit. For western blot analysis, 30 μg of protein in a sample was separated on 4-12% polyacrylamide gradient gels, transferred to nitrocellulose membranes, blocked with 5% fat-free dry milk in TTBS, and incubated with the antibodies to AMPK, phosphor-Thr^{172}-AMPK, ACC and phosphor-Ser^{79}-ACC. The detailed procedures were described in Chapter IV.

Statistical analysis

Data, expressed as means ± SEM, were analyzed using one-way ANOVA. The Tukey multiple comparison method was used to determine differences among the means of the treatment groups for 0-400 μM arginine. A t-test was employed to analyze the data for cells treated with either 400 μM arginine or 400 μM arginine plus 0.5 mM L-NAME. Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

BNL CL.2 mouse hepatocytes

Table 5.1 summarizes data on glucose and oleic acid oxidation, lactate production, and the incorporation of glucose and oleic acid into lipids in BNL CL.2 cells. There were no differences in glucose oxidation, oleic acid oxidation, or the incorporation of oleic acid into lipids between 0 and 50 μM arginine. However, increasing medium concentrations of arginine from 50 to 400 μM increased (P<0.05) glucose and oleic acid oxidation, as well as the incorporation of oleic acid into lipids. Exposure of the cells up to 400 μM arginine had no effect on lactate production or the incorporation of glucose into lipids. In the presence of various concentrations of arginine, 70-80% of metabolized glucose carbons appeared as lactate, whereas 85-90% of metabolized oleic acid was incorporated into lipids in BNL CL.2 cells.

Total and phosphorylated levels of AMPK and ACC in BNL CL.2 cells are presented in Figs. 5.1 and 5.2, respectively. There were no differences in total or phosphorylated AMPK or ACC between 0 and 50 μM arginine. Increasing arginine concentrations from 50 to 400 μM had no effect on total AMPK levels but increased (P<0.05) phosphorylated AMPK levels by approximately 110%, and therefore, the ratio of phosphorylated AMPK to total AMPK by 80%. Compared with 0 or 50 μM arginine, exposure of the cells to 400 μM arginine did not affect total or phosphorylated levels of ACC, or the ratio of phosphorylated ACC to total ACC.

Table 5.1. Glucose and oleic acid metabolism in BNL CL.2 cells

Variables	Medium arginine level (μM)					*P* value
	0	15	50	100	400	
Glucose oxidation (nmol glucose/mg protein·h)	24.5±2.2^{c}	31.6±0.84^{c}	33.6±3.2^{bc}	42.9±2.3^{ab}	53.2±2.5^{a}	0.0005
Oleic acid oxidation (nmol oleic acid/mg protein·h)	0.99±0.05^{b}	1.01±0.07^{b}	1.10±0.05^{b}	1.74±0.11^{a}	2.08±0.12^{a}	0.0005
Lactate release (nmol lactate/mg protein·h)	218±16	218±34	207±10	262±29	250±19	0.49
Glucose incorporation into lipids (nmol glucose/mg protein·h)	0.85±0.04	1.01±0.13	0.96±0.06	1.08±0.09	0.82±0.09	0.23
Oleic acid incorporation into lipids (nmol oleic acid/mg protein·h)	7.73±0.81^{b}	6.18±0.81^{b}	8.39±1.53^{b}	15.92±1.31^{a}	18.89±2.51^{a}	0.0005

Cells were incubated for 48 h in DMEM containing 0, 15, 50, 100 or 400 μM arginine. Data are expressed as means ± SEM, n=5. Means with different superscript letters in a row are different (P<0.05).

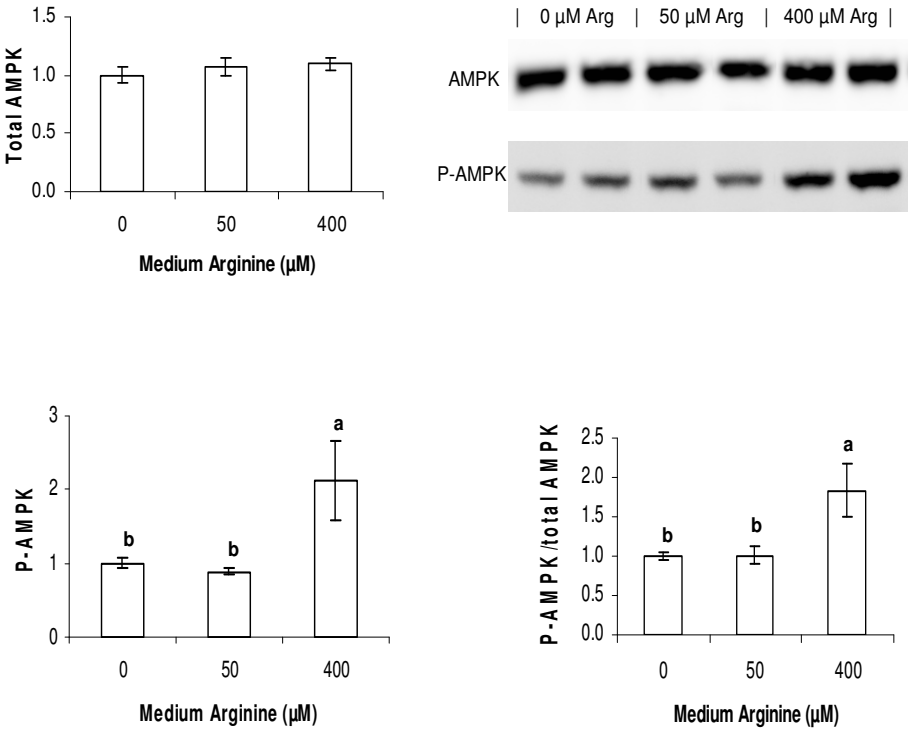


Fig. 5.1. Relative protein levels for total AMPK, phosphorylated AMPK (P-AMPK) and the ratio of P-AMPK to total AMPK in BNL CL.2 cells. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 μM arginine. Data are expressed as means ±SEM, n=4. Means with different letters are different (P<0.05).

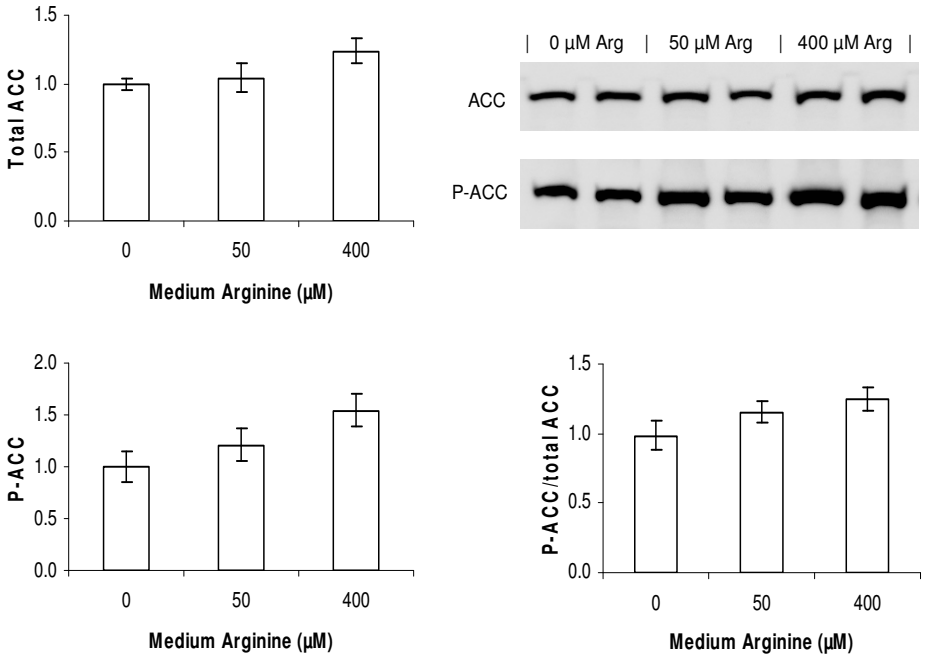


Fig. 5.2. Relative protein levels for total ACC, phosphorylated ACC (P-ACC) and the ratio of P-ACC to total ACC in BNL CL.2 cells. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 μM arginine. Data are expressed as means ±SEM, n=4.

C2C12 skeletal muscle cells

In C2C12 myotubes, increasing extracellular concentrations of arginine from 0 to 50 μM increased (P<0.05) oleic acid oxidation but had no effect on glucose oxidation or lactate production (Table 5.2). Increasing arginine concentration from 50 to 400 μM increased (P<0.05) glucose oxidation and lactate production, but had no effect on oleic acid oxidation. The rate of oleic acid oxidation was 23% greater (P<0.05) in the presence of

400 μM arginine than in the absence of its addition. In the presence of various levels of arginine, 88-91% of metabolized glucose carbons appeared as lactate.

Increasing extracellular concentrations of arginine from 0 to 50 and 400 μM had no effect on total AMPK levels in C2C12 myotubes but progressively increased (P<0.05) phosphorylated AMPK levels by 117% and 256%, respectively, and thus the ratio of phosphorylated AMPK to total AMPK by 92% and 231 %, respectively (Fig. 5.3). Arginine at 50 μM increased (P<0.05) both total ACC and phosphorylated ACC levels approximately by 100%, and arginine at 400 μM further increased (P<0.05) total ACC and phosphorylated ACC levels by 174% and 236%, respectively, in comparison with the values obtained at 0 μM arginine. As a result, the ratio of phosphorylated ACC to ACC was not changed by 50 μM arginine, but was increased by 400 μM arginine (P<0.05) (Fig. 5.4).

Table 5.2. Glucose and oleic acid metabolism in C2C12 myotubes

Variables	Medium arginine level (μM)					*P* value
	0	15	50	100	400	
Glucose oxidation						
(nmol glucose/mg protein·h)	11.5±0.4^{c}	11.3±0.4^{c}	13.4±1.1^{c}	18.6±1.0^{b}	22.3±0.9^{a}	0.0005
Oleic acid oxidation						
(nmol oleic acid/mg protein·h)	1.46±0.08^{b}	1.66±0.03^{ab}	1.75±0.08^{a}	1.72±0.08^{a}	1.80±0.03^{a}	0.030
Lactate release						
(nmol lactate/mg protein·h)	176±10^{b}	196±7.8^{b}	251±11^{b}	436±29^{a}	453±26^{a}	0.0005

Cells were incubated for 48 h in DMEM containing 0, 15, 50, 100 or 400 μM arginine. Data are expressed as means ± SEM, n=6. Means with different superscript letters in a row are different (P<0.05).

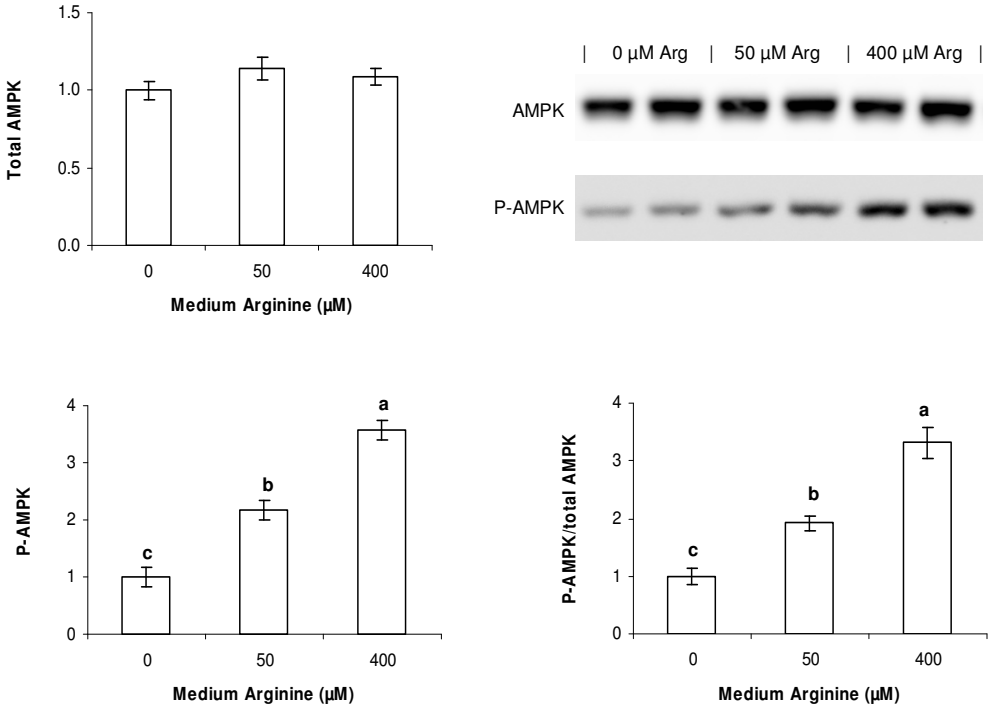


Fig. 5.3. Relative protein levels for total AMPK, phosphorylated AMPK (P-AMPK) and the ratio of P-AMPK to total AMPK in C2C12 myotubes. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 μM arginine. Data are expressed as means ±SEM, n=4. Means with different letters are different (P<0.05).

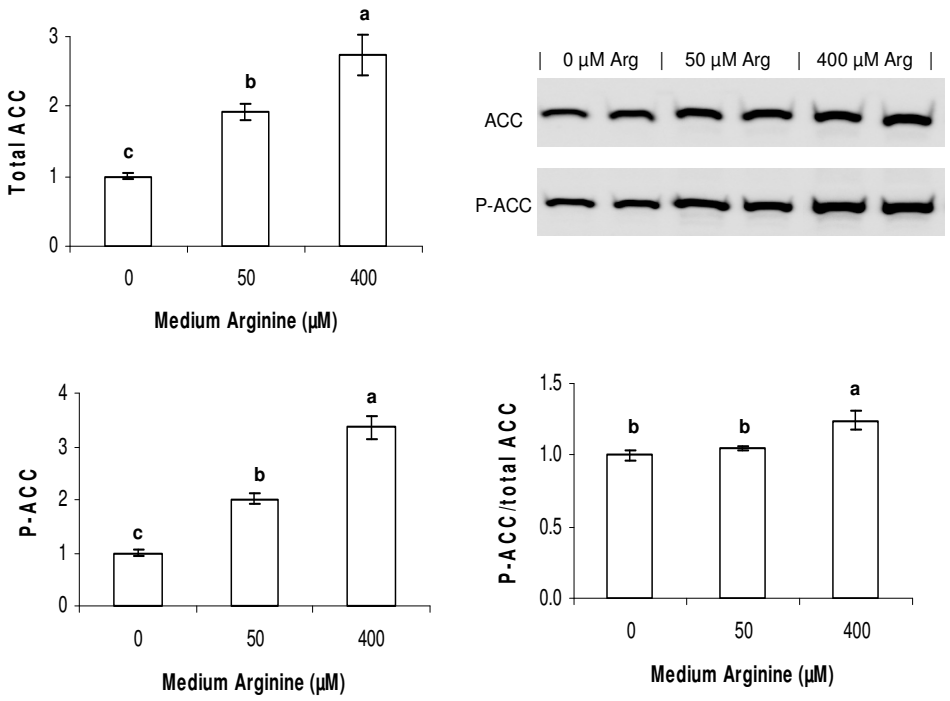


Fig. 5.4. Relative protein levels for total ACC, phosphorylated ACC (P-ACC) and the ratio of P-ACC to total ACC in C2C12 myotubes. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 μM arginine. Data are expressed as means ±SEM, n=4. Means with different letters are different (P<0.05).

3T3-L1 adipocytes

Table 5.3 summarizes the effect of arginine on glucose and oleic acid metabolism in 3T3-L1 cells. Lactate was the major product of glucose metabolism in adipocytes, representing 80-82% of the metabolized glucose carbons. Increasing extracellular concentrations of arginine from 0 up to 400 μM did not affect lactate production or the incorporation of glucose into lipids. The rates of glucose oxidation did not differ

between 0 and 100 μM arginine, but were 45% greater (P<0.05) in the presence of 400 μM arginine than in the absence of its addition. Incorporation into lipids was the major fate (80-90%) of oleic acid utilization by 3T3-L1 cells. Increasing extracellular concentrations of arginine from 0 to 50 μM had no effect on the oxidation of oleic acid or its incorporation into lipids. However, the rates of oleic acid oxidation were approximately 40% greater (P<0.05) in the presence of 100-400 μM arginine than in its absence. Also, increasing arginine concentration from 50 to 100 and 400 μM increased (P<0.05) the incorporation of oleic acid into lipids by 136% and 190%, respectively.

Table 5.3. Glucose and oleic acid metabolism in 3T3-L1 adipocytes

Variables	Medium arginine level (μM)					*P* value
	0	15	50	100	400	
Glucose oxidation (nmol glucose/mg protein·h)	12.1±0.4^{b}	14.4±1.2^{ab}	15.5±1.3^{ab}	16.1±0.9^{ab}	17.6±1.6^{a}	0.037
Oleic acid oxidation (nmol oleic acid/mg protein·h)	1.77±0.07^{c}	2.04±0.08^{bc}	2.12±0.10^{abc}	2.40±0.09^{ab}	2.48±0.16^{a}	0.001
Lactate release (nmol lactate/mg protein·h)	197±17	184±5	204±17	200±5	203±10	0.78
Glucose incorporation into lipids (nmol glucose/mg protein·h)	9.54±0.53	11.1±0.74	9.35±0.43	11.0±0.44	9.73±1.05	0.25
Oleic acid incorporation into lipids (nmol oleic acid/mg protein·h)	6.40±1.12^{b}	6.88±1.98^{b}	10.9±1.38^{b}	25.7±3.64^{a}	31.6±3.05^{a}	0.0005

Cells were incubated for 48 h in DMEM containing 0, 15, 50, 100 or 400 μM of arginine. Data are expressed as means ± SEM, n=5. Means with different superscript letters in a row are different (P<0.05).

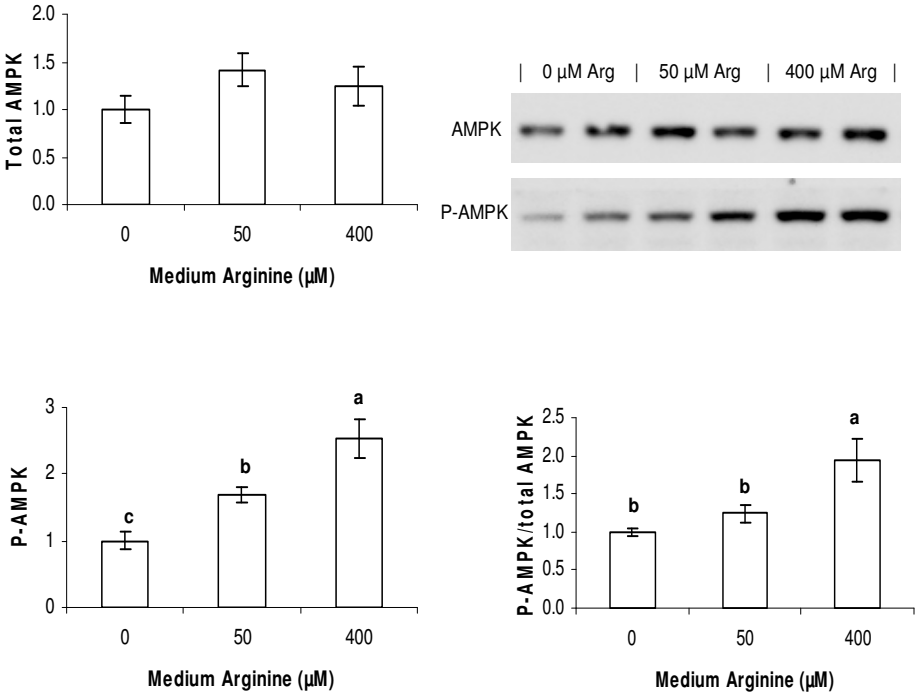


Fig. 5.5. Relative protein levels for total AMPK, phosphorylated AMPK (P-AMPK) and the ratio of P-AMPK to total AMPK in 3T3-L1 adipocytes. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 μM arginine. Data are expressed as means ±SEM, n=4. Means with different letters are different (P<0.05).

Increasing extracellular concentrations of arginine from 0 to 400 μM did not affect total AMPK but increased (P<0.05) phosphorylated AMPK levels. Arginine at 400 μM increased (P<0.05) the ratio of phosphorylated AMPK to total AMPK by approximately 90% compared to 0 μM arginine (Fig. 5.5). In contrast, increasing arginine

concentrations from 0 to 400 µM had no effect on total or phosphorylated ACC levels, or the ratio of phosphorylated AMPK to total AMPK (Fig. 5.6).

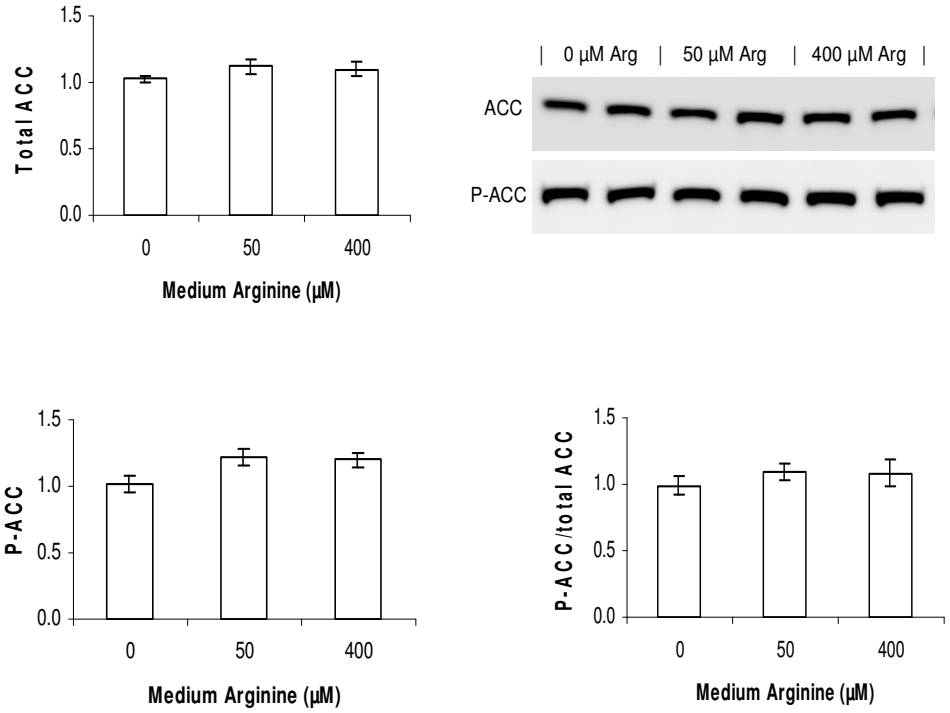


Fig. 5.6. Relative protein levels for total ACC, phosphorylated ACC (P-ACC) and the ratio of P-ACC to total ACC in 3T3-L1 adipocytes. Cells were cultured for 48 h in DMEM containing 0, 50 or 400 µM arginine. Data are expressed as means ±SEM, n=4. Means with different letters are different (P<0.05).

NO synthesis

Increasing extracellular concentrations of arginine from 0 to 400 µM did not affect nitrite production in 3T3-L1 cells (Table 5.4). In both BNL CL.2 and C2C12 cells,

nitrite production did not differ between 0 and 100 μM arginine (Table 5.4). However, rates of NO synthesis were 42% and 47% greater (P<0.05) in the presence of 400 μM arginine than in its absence in BNL CL.2 and C2C12 cells, respectively.

Table 5.4. The effect of arginine on nitrite production in BNL CL.2, C2C12 and 3T3-L1 cells

Nitrite	Medium arginine level (μM)				*P* value
(pmol/mg protein·24h)	0	50	100	400	
BNL CL.2	112±12^{b}	119±10^{ab}	125±13^{ab}	159±4^{a}	0.020
C2C12	351±19^{b}	372±61^{ab}	363±15^{ab}	516±41^{a}	0.025
3T3-L1	200±18	207±16	232±28	264±27	0.45

Cells were incubated for 48 h in DMEM containing 0, 15, 50, 100 or 400 μM of arginine. Data are expressed as means ± SEM, n=5. Means with different superscript letters in a row are different (P<0.05).

Effect of L-NAME on BNL CL.2, C2C12 and 3T3-L1 cells

Addition of 0.5 mM L-NAME to culture media reduced (P<0.05) glucose oxidation, oleic acid oxidation and lactate production by approximately 20% in BNL CL.2 cells but had no effect on the incorporation of glucose or oleic acid into lipids, compared with the absence of the NOS inhibitor (Fig. 5.7A). L-NAME treatment did not affect total or phosphorylated AMPK levels, but reduced the ratio of phosphorylated AMPK to total AMPK (P<0.05). Total ACC levels were not altered by 0.5 mM L-NAME, but both phosphorylated ACC levels and the ratio of phosphorylated ACC to total ACC in

hepatocytes were reduced (P<0.05) by L-NAME treatment (Fig. 5.7B). In C2C12 myotubes (Fig. 5.8) or 3T3-L1 adipocytes (Fig. 5.9), 500 µM L-NAME did not affect glucose oxidation, oleic acid oxidation, lactate release, or protein levels for AMPK and ACC.

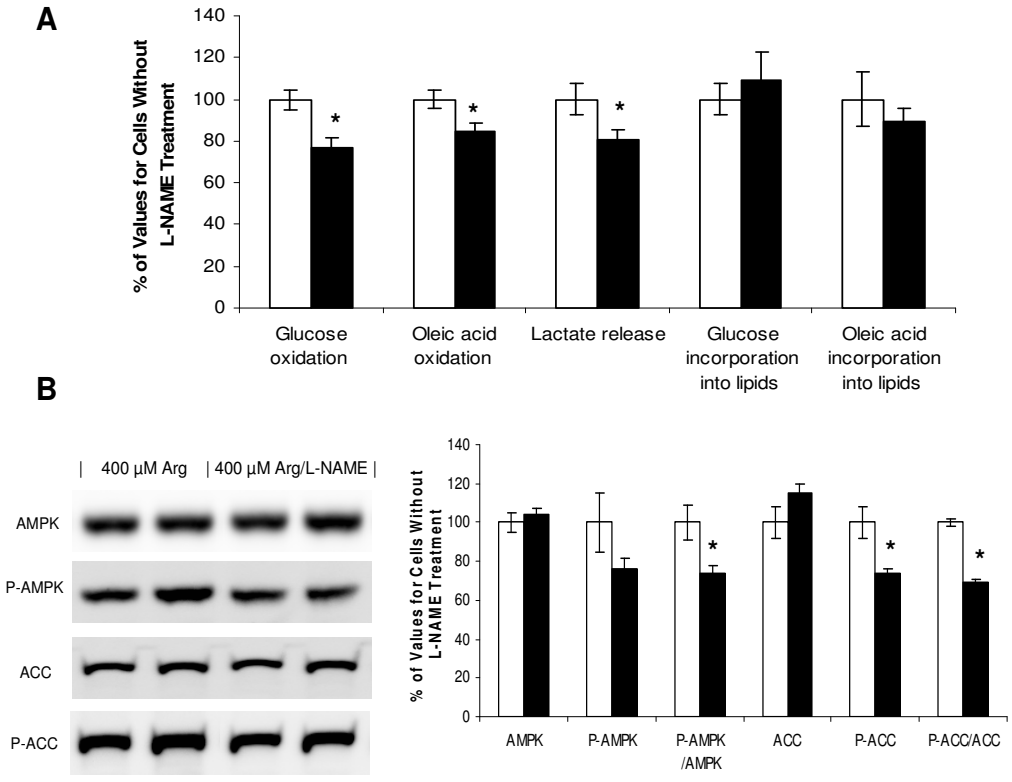


Fig. 5.7. The effect of L-NAME on glucose and oleic acid oxidation, lactate release, glucose and oleic acid incorporation into lipids (A) as well as total and phosphorylated levels of AMPK and ACC (B) in BNL CL.2 cells. Cells were cultured for 48 h in DMEM containing either 400 µM arginine (open bars) or 400 µM arginine plus 0.5 mM L-NAME (black bars). Data are expressed as means ±SEM, n=5 for biochemical assays and n=4 for western blots. * P< 0.05: Different from the group without L-NAME.

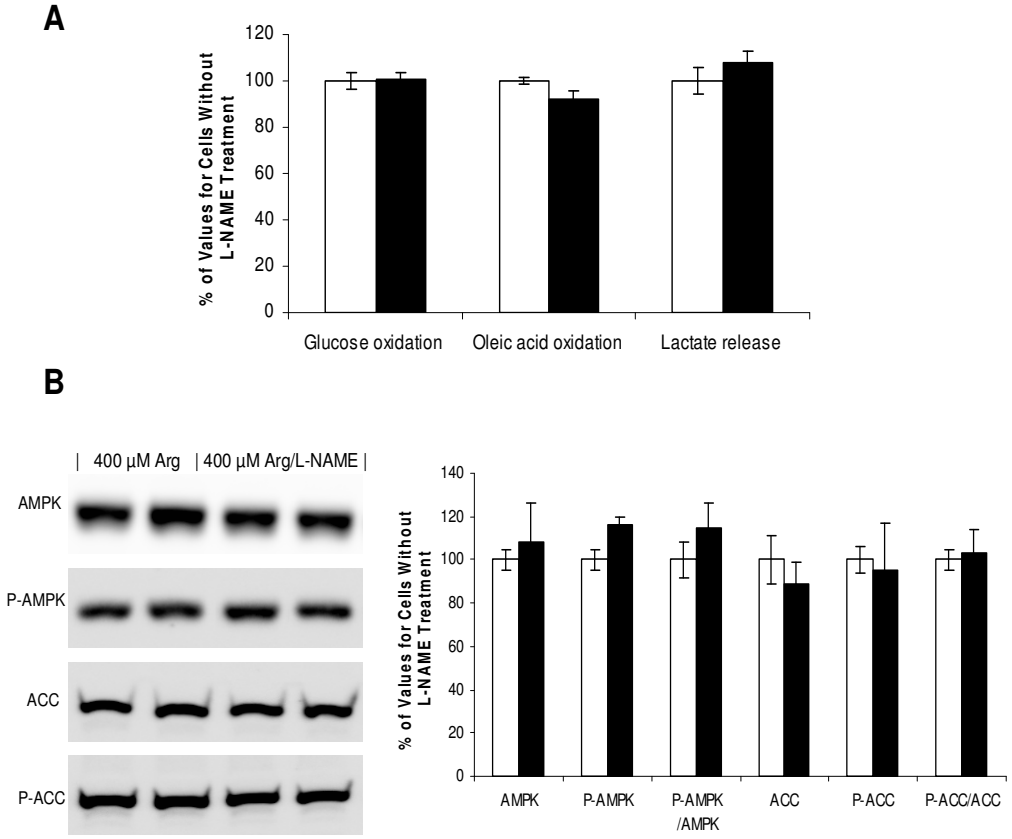


Fig. 5.8. The effect of L-NAME on glucose and oleic acid oxidation, lactate release, glucose and oleic acid incorporation into lipids (A) as well as total and phosphorylated levels of AMPK and ACC (B) in C2C12 myotubes. Cells were cultured for 48 h in DMEM containing either 400 µM arginine (open bars) or 400 µM arginine plus 0.5 mM L-NAME (black bars). Data are expressed as means ±SEM, n=6 for biochemical assays and n=4 for western blots.

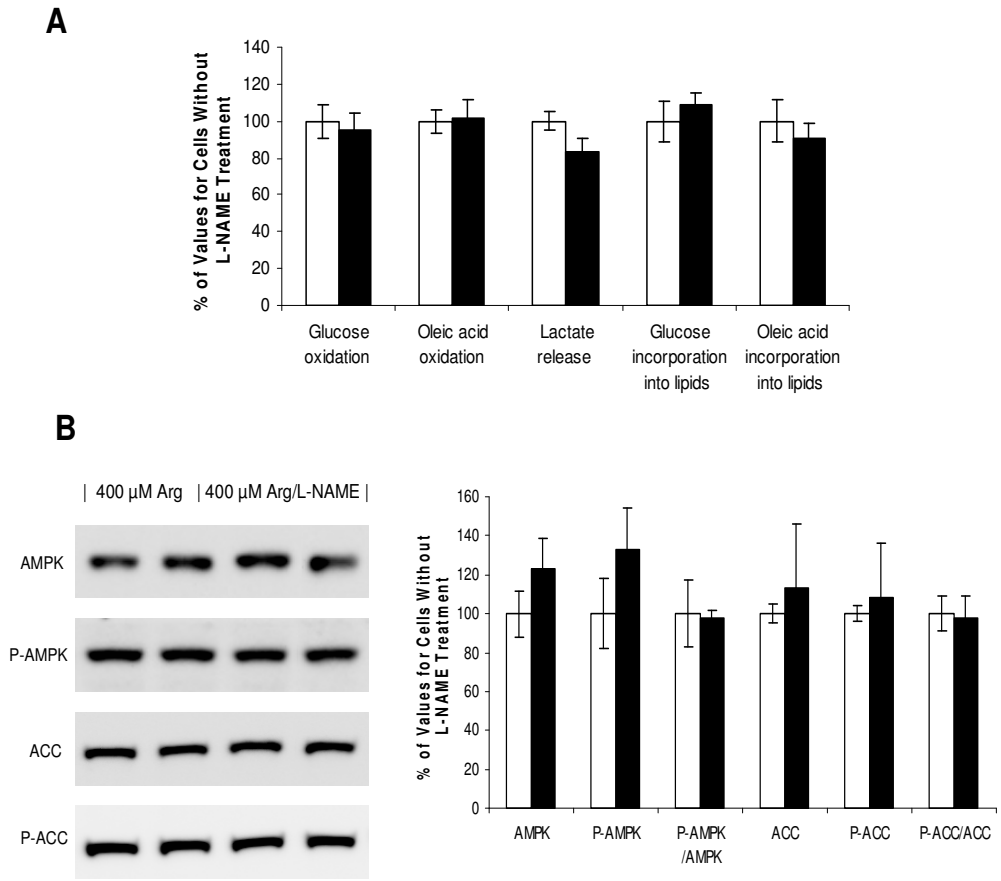


Fig. 5.9. The effect of L-NAME on glucose and oleic acid oxidation, lactate release, glucose and oleic acid incorporation into lipids (A) as well as total and phosphorylated levels of AMPK and ACC (B) in 3T3-L1 adipocytes. Cells were cultured for 48 h in DMEM containing either 400 µM arginine (open bars) or 400 µM arginine plus 0.5 mM L-NAME (black bars). Data are expressed as means ±SEM, n=5 for biochemical assays and n=4 for western blots.

Discussion

Plasma concentrations of arginine in healthy overnight-fasted and fed adults are approximately 100 and 200 µM, respectively (Wu & Morris, 1998). Dietary supplementation with 1% arginine to animals can increase plasma arginine

concentrations to 500 μM (Wu *et al.* 2007b). Interestingly, as low as 15 and 30 μM arginine are present in the plasma of liver-transplant recipients (Reid *et al.* 2007) and preterm infants (Wu *et al.* 2004b), due to the release of arginase from liver and the underdevelopment of endogenous arginine synthesis pathways, respectively. In contrast, porcine allantoic fluid contains 4 to 6 mM arginine during early gestation (Wu *et al.* 1996; 1998). Thus, the concentrations of arginine used in the present study (15 to 400 μM) are within the physiological and pathological ranges found in mammals, and the findings are of nutritional and clinical relevance.

In BNL CL.2 hepatocytes, C2C12 myotubes and 3T3-L1 adipocytes, glucose and oleic acid oxidation increased in response to elevated extracellular concentrations of arginine from 0 to 400 μM (Tables 5.1, 5.2 and 5.3), indicating a role for arginine in stimulating energy substrate oxidation in these cells. In addition, the arginine treatment enhanced glycolysis in muscle cells, as indicated by the increased production of lactate (Table 5.2). These results were consistent with the findings of the previous *in vivo* studies that dietary arginine supplementation increased the metabolism of energy substrates in skeletal muscle and adipose tissue (Chapter III; Fu *et al.* 2005).

We found that phosphorylated AMPK levels in all the three cell types used in the present study were increased in response to an increase in extracellular concentrations of arginine from 0 to 400 μM (Fig. 5.1, 5.3 and 5.5). This result may provide a mechanism for explaining the increased oxidation of glucose and fatty acid in arginine-treated hepatocytes, muscle cells and adipocytes, on the basis of the previous reports that AMPK phosphorylation promotes substrate oxidation in insulin-sensitive cells (Kahn *et*

al. 2005a). Also, in C2C12 cells, the levels of phosphorylated ACC, a downstream target of AMPK, increased in response to the arginine supplementation. Malonyl-CoA, a product of ACC, is a potent inhibitor of carnitine palmitoyltransferase I (CPT-I). Thus, the inactivation of ACC via its phosphorylation will lead to an increase in the activity of CPT-I (Viollet *et al.* 2006), thereby facilitating the transport of long-chain fatty acids from the cytosol to mitochondria for oxidation in arginine-treated C2C12 cells.

Arginine is catabolized by multiple pathways to form NO, ornithine and proline in cultured cells (Wu & Morris, 1998). The addition of 0.1 mM ornithine plus proline (the major products of arginine catabolism via the arginase pathway) to culture medium had no effect on glucose or oleic acid oxidation in endothelial cells or enterocytes (G. Wu, unpublished data). These results suggest that ornithine or proline do not mediate the effect of arginine on metabolism in these cell types. Whether ornithine and proline affect substrate metabolism in BNL CL.2 hepatocytes, C2C12 myotubes and 3T3-L1 adipocytes remains to be determined.

Interestingly, increasing extracellular concentrations of arginine from 0 to 400 μM increased NO synthesis in BNL CL.2 cells, as reported for arginine-treated rat hepatocytes (Morita *et al.* 1996). However, an inhibition of NO synthesis only moderately attenuated the effect of arginine on the phosphorylation of AMPK and ACC as well as glucose oxidation, glycolysis, and oleic acid oxidation in BNL CL.2 cells (Fig. 5.4). This result is consistent with the report that NO or cGMP can stimulate fatty acid oxidation through reducing ACC activity and enhancing CPT-I activity in rat hepatocytes (Garcia-Villafranca *et al.* 2005). However, an inhibition of NO synthesis

had no effect on AMPK and ACC phosphorylation or glucose and oleic acid metabolism in C2C12 cells and 3T3-L1 cells (Fig. 5.5 and 5.6). These findings suggest that, under the experimental conditions used in the present study where culture medium contained 4 mM glutamine to reduce NO synthesis (Meininger & Wu 1997; Houdijk *et al.* 1998; Wu *et al.* 2001), NO was not a major regulator of AMPK and ACC activity or the oxidation of energy substrates in these three cell types. Consistent with this view are the following observations. First, NO synthesis in 3T3-L1 cells did not differ between 0 and 400 μM arginine (Table 5.4). Second, increasing extracellular concentrations of arginine from 0 to 100 μM increased glucose and oleic acid oxidation in BNL CL.2 cells (Table 5.1) and C2C12 cells (Table 5.2) but did not affect NO production in these two cell types (Table 5.4). It is possible that arginine catabolism is not necessary for exerting its stimulatory effect on substrate oxidation in hepatocytes, adipocytes and muscle cells and that this amino acid may directly activate AMPK in a cell-specific manner. In support of this notion, previous studies have shown that arginine can directly increase expression of GTP cyclohydrolase-I in endothelial cells (Shi *et al*. 2004) and stimulate mTOR phosphorylation in intestinal cells (Rhoads *et al.* 2007).

In both BNL CL.2 cells and 3T3-L1 cells, arginine increased oleic acid incorporation into lipids. Liver and adipose tissue are the two major sites for *de novo* fatty acid synthesis in rodents (Bergen & Mersmann, 2005). Because free fatty acids are toxic to cells, an increase in their packaging into lipids may have important implications for cell integrity and the regulation of plasma concentrations of free fatty acids. This may help explain the finding that dietary arginine supplementation reduced the circulating levels

of free fatty acids in ZDF rats (Fu *et al*. 2005) and serum concentrations of triglycerides in diet-induced obese rats (Chapter II).

In sum, results of this study demonstrate that L-arginine enhances glucose and oleic acid oxidation in BNL CL2 hepatocytes, C2C12 myotubes and 3T3-L1 adipocytes in association with increased AMPK phosphorylation. This effect of arginine was mediated only to a small extent by an NO-dependent mechanism in BNL CL2 cells and was independent of NO production in C2C12 and 3T3-L1 cells. We suggest that arginine itself may directly activate AMPK, thereby stimulating the oxidation of energy substrates in insulin-sensitive cells.

CHAPTER VI

SUMMARY AND DIRECTION OF FUTURE RESEARCH

There is a substantial body of evidence showing the beneficial effect of L-arginine on ameliorating metabolic abnormalities, including hyperglycemia, dyslipidemia, and impaired NO synthesis in endothelial cells (Jobgen *et al.* 2006). Most recently, dietary arginine supplementation has been suggested to play a role in reducing fat mass in obese animals (Fu *et al.*, 2005) and humans with type-II diabetes mellitus (Lucotti *et al.* 2006). The major purpose of this dissertation research is to determine the effect of dietary arginine supplementation on the oxidation of energy substrates in insulin-sensitive tissues of diet-induced obese (DIO) rats and to elucidate the underlying mechanisms.

The first part of this study was designed to evaluate the effect of arginine on whole body metabolism in DIO versus lean rats (Chapter II). High-fat feeding increased body weight, white adipose tissue mass, lipid concentrations in liver and muscle, serum cholesterol and leptin concentrations, and resulted in impaired glucose tolerance. Importantly, dietary arginine supplementation for 12 wks reduced white adipose tissue mass in different anatomical locations, while increasing brown fat and skeletal muscle masses. In addition, the arginine treatment reduced serum levels of glucose, triglycerides and leptin, as well as improved whole-body insulin sensitivity. Collectively, these findings indicate a potentially novel means of using arginine to treat the metabolic syndrome in obese subjects.

The second part of the work was conducted to investigate the metabolic basis for the anti-obesity effect of arginine (Chapter III). Particularly, we focused on the oxidation of energy substrates in skeletal muscle and adipose tissue. Because of a concern about the biochemical viability of liver slices in incubation, the hepatic metabolism of glucose or oleic acid was not studied. Our results indicated that arginine supplementation did not affect total adipocyte numbers in RP adipose tissue, but reduced adipocyte size, therefore shifting the cell size distribution to a smaller diameter. Notably, dietary arginine supplementation increased the oxidation of both glucose and oleic acid in skeletal muscle, the major site for the utilization of energy substrates in the body. Increases in both skeletal muscle mass and its oxidative capacity greatly facilitate the oxidation of glucose and fatty acids (including the fatty acids derived from adipose tissue), contributing to the loss of fat mass in DIO rats.

Subsequent experiments were performed to define the molecular basis of the anti-obesity property of arginine (Chapter IV). Dietary arginine supplementation greatly enhanced AMPK and ACC phosphorylation in liver but had no effect in gastrocnemius muscle or adipose tissues. In addition, the arginine treatment increased expression of the hepatic genes (CPT-1, AMPK, and PGC-1α) for glucose and fatty acid oxidation, while decreased expression of the hepatic genes (FAS and SCD-1) for fatty acid synthesis. Because the liver is the principal organ for both *de novo* fatty acid synthesis and fatty acid oxidation in rats and humans (Bergen & Mersmann, 2005), an inhibition of fatty acid synthesis and an increase in fatty acid oxidation in the liver provides a key molecular basis for the anti-obesity effect of arginine. These findings indicate that

dietary arginine supplementation coordinately regulates expression of key genes and activity of proteins that are involved in the metabolism of energy substrates, thereby reducing excessive fat and improving insulin sensitivity in diet-induced obese rats.

Because dietary arginine supplementation can result in changes in the endocrine status and circulating levels of hormones and metabolites, it is not clear whether arginine itself can induce changes in expression of key genes responsible for nutrient metabolism in insulin-sensitive tissues and cells. Thus, we used cell culture models to define an effect of arginine on the expression and phosphorylation of AMPK and ACC as well as oxidation of energy substrates (Chapter V). Specifically, we asked the question of whether arginine regulated glucose and fatty acid metabolism via an NO-dependent mechanism. BNL CL.2 mouse hepatocytes, differentiated C2C12 mouse skeletal muscle cells, and 3T3-L1 mouse adipocytes were treated with various levels of arginine (0-400 μM) or 400 μM of arginine plus 500 μM L-NAME (an NOS inhibitor) in culture medium for 48 h. Increasing extracellular concentrations of arginine increased AMPK phosphorylation as well as both glucose and oleic acid oxidation in all the three cell types. NO synthesis was increased in BNL CL.2 and C2C12 cells cultured in the presence of 400 μM arginine in comparison with its absence. However, NO production did not differ in 3T3-L1 cells cultured in the presence of 0 and 400 μM arginine. Further, an inhibition of NO generation by L-NAME only modestly reduced the arginine-induced increases in AMPK and ACC phosphorylation as well as glucose and oleic acid oxidation in hepatocytes, and had no effect on these parameters in muscle cells or adipocytes. These findings suggest that arginine stimulates AMPK phosphorylation in

a cell-specific manner and that NO is not a major mediator of the arginine action in hepatocytes, skeletal muscle cells, or adipocytes.

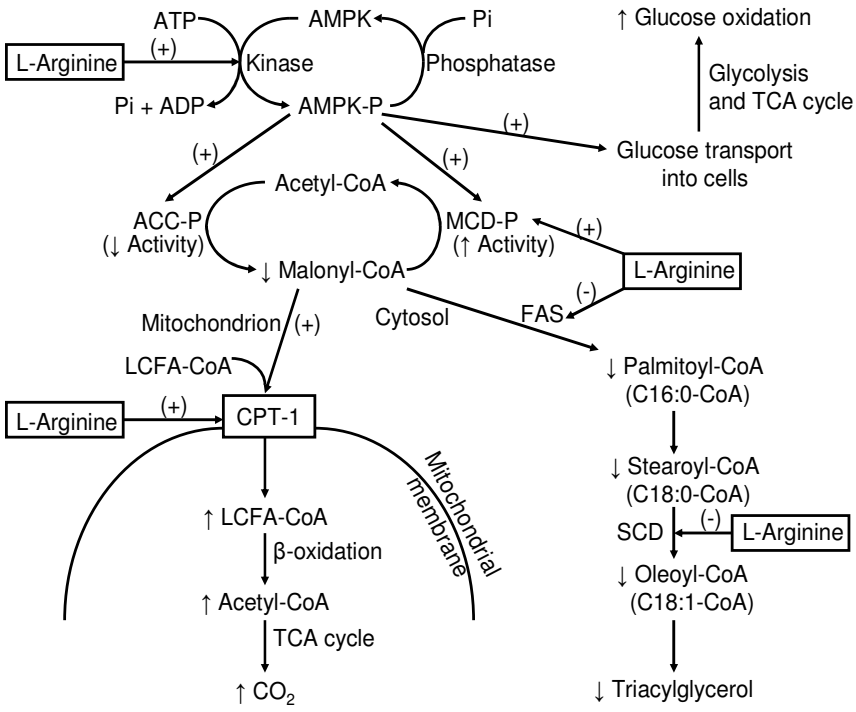


Fig. 6.1. The proposed mechanisms responsible for the beneficial effect of L-arignine on the oxidation of energy substrates and the synthesis of triacylglycerol in animals. The symbol (+) denotes an increase in gene expression and/or enzyme activation. The symbol (-) denotes an inhibition of gene expression and/or enzyme activity.

In conclusion, results of this dissertation research demonstrate that dietary arginine supplementation exerts a beneficial effect on increasing glucose and fatty acid oxidation in skeletal muscle, reducing body fat mass, and improving insulin sensitivity in diet-

induced obese rats. Increases in gene expression of AMPK and its protein phosphorylation in liver may be the key mechanism responsible for the stimulatory action of arginine on substrate oxidation. In addition, dietary arginine supplementation reduces hepatic expression of key lipogenic genes (FAS and SCD-1), thereby attenuating fat accretion in the body. Figure 6.1 outlines the proposed mechanisms responsible for the beneficial effect of L-arignine on the oxidation of energy-substrates and the synthesis of triacylglycerol in animals. Evidence from studies with cell culture models suggests that NO is not a major mediator of the beneficial effect of arginine on the oxidation of energy substrates in insulin-sensitive tissues.

Further research is needed to better understand the mechanism whereby arginine regulates expression of the AMPK gene and its protein phosphorylation in a cell-specific manner. The chemical structure of arginine with a unique guanidino group may play an important role in its action. It is possible that this amino acid may directly regulate expression and phosphorylation of AMPK in various cell types. The use of non-metabolizable analogues of arginine (including D-arginine) may provide a tool to address this question. In addition, arginine supplementation does not affect the cell number of adipocytes in RP adipose tissue, while reducing their cell size due to the loss of intracellular lipids. However, at 12-wk after the initiation of the arginine treatment, no increase in glycerol release from adipose tissue could be observed in diet-induced obese rats. Dietary arginine supplementation may stimulate lipolysis in adipocytes during the initial and mid phases of the treatment, and further studies are warranted to test this hypothesis. Finally, to thoroughly understand substrate metabolism at the whole-

body level, research should be initiated to quantify energy expenditure in lean and obese rats treated with or without arginine.

Obesity is a growing health problem for both humans and companion animals worldwide. In addition, domestic animals have a great capacity for lipogenesis and fat storage, potentially leading to low-quality meat products. Thus, future research on arginine biochemistry and nutrition will provide a much-needed scientific basis for designing a novel, safe and effective means to prevent and treat obesity in mammals and to enhance the efficiency of livestock production.

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