BENEFICIAL EFFECTS OF DIETARY L-ARGININE SUPPLEMENTATION TO DIABETIC RATS

A Thesis

by

RIPLA KOHLI

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

MASTER OF SCIENCE

August 2003

Major Subject: Nutrition

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ABSTRACT

Beneficial Effects of Dietary L-Arginine Supplementation

to Diabetic Rats. (August 2003)

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Diabetic rats exhibit decrease in plasma arginine, NO synthesis and tetrahydrobiopterin in endothelial cells (EC). Treatment with L-arginine may be beneficial for enhancing NO synthesis in diseases associated with endothelial dysfunction. However, little is known about the mechanism responsible for the stimulatory effect of arginine on endothelial NO synthesis. We hypothesized that dietary arginine supplementation increases BH_4 for NO synthesis in EC of diabetic rats, thereby preventing endothelial dysfunction. In experiment I, streptozotocin (STZ) induced-diabetic male Sprague Dawley (SD) rats (a model of type-I diabetes) were individually pair-fed a casein-based diet on the basis of feed intake (per kg body weight) of non-diabetic SD rats. Addition of arginine-HCl or alanine to drinking water for the rats were adjusted daily to ensure isonitrogenous provision per kg body weight. In non-diabetic rats, arginine supplementation increased plasma arginine (144%), plasma insulin (44%), and arginine concentrations (88%), BH_4 concentrations (106%) and NO synthesis (80%) in EC, compared with alanine treatment. In diabetic rats, arginine supplementation

reduced body weight loss (36%), and plasma glucose (54%), and increased plasma arginine (110%), plasma insulin (209%), EC arginine (173%), EC BH_4 (128%) and EC NO synthesis (125%), compared with alanine treatment. In experiment II, male Zucker diabetic fatty (ZDF) rats (a model of type-II diabetes) were individually pair-fed a Purina 5008 diet on the basis of feed intake by alanine-treated diabetic rats (per kg body wt). Addition of arginine-HCl or alanine to drinking water for the rats was adjusted daily to ensure isonitrogenous provision per kg body weight. Arginine supplementation to ZDF rats did not affect plasma levels of glucose and insulin, reduced epididdidmal fat (30%), abdominal fat (43%) and body weight gain (18%), and increased plasma arginine (273%), EC arginine (197%), EC BH_4 (120%) and EC NO synthesis (122%), compared with alanine-treated ZDF rats. These results show that dietary L-arginine supplementation increases BH_4 and NO synthesis in EC of both STZ-diabetic and ZDF rats. Strikingly, arginine treatment prevented hyperglycemia in STZ-diabetic SD rats and reduced obesity in ZDF rats. Collectively, results demonstrate that oral administration of arginine is beneficial for both type-I and type-II diabetic rats.

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CHAPTER I

INTRODUCTION

Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (1). Vascular complications are the major cause of morbidity and mortality in patients with diabetes (2, 3). Diabetic patients are at considerable risk of cardiovascular, cerebrovascular and peripheral vascular disease leading to myocardial infarction, strokes, and amputations (4). It is estimated that 77% of hospitalizations in the United States for chronic complications of diabetes are attributable to cardiovascular disease (CVD) (5).

Multiple factors contribute to the macrovascular abnormalities in diabetes, manifested by accelerated atherosclerosis (6). These factors include oxidation and glycation of proteins and prevalence of traditional risks such as obesity, hypertension, and dyslipidemia, and the state of insulin resistance (5). Furthermore, small-vessel disease, resulting in diabetic retinopathy and nephropathy, also contributes importantly to the morbidity associated with this disease (6).

The endothelium lines the entire vascular system and is comprised of a monolayer of endothelial cells (EC). In an adult human, the endothelium consist of

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This thesis follows the style and format of the Journal of Nutrition.

approximately $1x10^{13}$ cells forming an almost 1 kg "tissue" (7). Endothelium plays a key role in maintaining homeostasis of the vasculature through the synthesis of vasoactive substances that modulate vascular tone, as well as inhibition of platelet aggregation and vascular smooth muscle cell (VSMC) proliferation (8). Loss of the modulatory role of the endothelium could be implicated in the pathogenesis of the diabetic vascular complications.

Endothelial dysfunction in diabetes. There is now substantial evidence indicating that endothelial dysfunction, characterized by diminished endothelium-dependent relaxation, is abnormal in experimental models of diabetes mellitus (9,10,11,12,13). Several other studies have also demonstrated impaired endothelium-dependent vasodilation in human diabetic patients (14,15). Studies designed to investigate the mechanism of this dysfunction have implicated the involvement of various factors. These factors include (1) destruction of endothelium by oxygen-derived free radicals (16); (2) increased release of endothelium-derived constricting factors (17); or (3) decreased release or production of nitric oxide (NO) (13,18). Hyperglycemia contributes directly to above mentioned factors and thus to endothelial dysfunction. There is substantial evidence suggesting that high concentrations of glucose could reduce NO availability due to an increase in superoxide anion production (19). Further, elevated glucose levels may lead to a decrease in cellular concentrations of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) through activation of the polyol pathway (3). Because NADPH is an essential cofactor for NO synthase (NOS) for NO synthesis, its depletion could lead to a reduction in endothelial NO production.

Activation of protein kinase C (PKC) induced by hyperglycemia has also been suggested as a mechanism for endothelial dysfunction and vascular complications in diabetes (3). Additionally, in the presence of high plasma glucose concentrations, circulating or intracellular proteins undergo changes in structure and function, resulting in the formation of advanced glycation end products (AGE) (3). These AGE may affect cellular metabolism and contribute to endothelial dysfunction and diabetic vascular complications.

Although several mechanisms for the defect in diabetic blood vessels have been proposed, much attention has been focused on altered endothelial production of NO. This free radical molecule is the most potent endothelium-derived vasodilator, and is essential for the regulation of vascular tone and integrity. L-Arginine is the substrate for the NOS, which is responsible for NO production in the presence of O_2, tetrahydrobiopterin (BH_4), NADPH, FAD, FMN, Ca^{2+} and calmodulin (20). Endothelial NOS (eNOS) is constitutively expressed in the vasculature. Therefore, endothelial dysfunction could be attributed to a deficiency of arginine or a cofactor of NOS, and/or to the presence of endogenous inhibitors of NOS. This hypothesis leads to the assumption that increased provision of L-arginine could be beneficial for improving endothelial function. Thus, in diseases associated with reduced plasma arginine levels, NO synthesis may be inadequate and endothelium-dependent relaxation may be impaired (21). Several studies have shown that plasma concentrations of arginine are decreased in experimental diabetic animals (10,11,12) and in diabetic humans (21,22). Thus, in recent years, there has been growing interest in the use of L-arginine to prevent

and treat endothelial dysfunction in diabetes. However, little is known about the mechanism responsible for the stimulatory effect of arginine on endothelial NO synthesis in normal or diabetic subjects.

Arginine availability for endothelial cells. L-Arginine is a basic amino acid found in large quantities in fish and other seafood, nuts, meats, and beans. In healthy adults, arginine can be synthesized endogenously from citrulline produced from glutamine and proline in enterocytes of the small intestine; the major tissue responsible for arginine synthesis is the kidney. However, in young animals and in other situations characterized by increased arginine requirements (e.g. infection, trauma and sepsis), the endogenous synthesis of arginine (primarily in the small intestine and kidney) may not be sufficient to meet the demand for this amino acid (23). Approximately 5.4 g of arginine is consumed each day in adults who ingest an average US diet (23).

Endothelial cells form the inner layer of all blood vessels and are in direct contact with circulation. EC derive arginine from plasma, intracellular synthesis and protein degradation. Homeostasis of plasma arginine concentrations is regulated by dietary arginine intake, protein turnover, arginine synthesis and metabolism. This explains why arginine becomes an essential dietary component under certain disease conditions. Due to a relatively high arginase activity in enterocytes, ~40% of dietary arginine is degraded during absorption and the remainder enters the portal vein. However, more than 85% of arginine delivered to liver is not taken up by this organ. Although, the liver is also capable of synthesizing considerable amounts of arginine, this amino acid is rapidly hydrolyzed by arginase via the urea cycle. Thus, the liver does not

contribute to plasma arginine flux (24). The separation between hepatic and systemic arginine pools can be attributed to the fact that the active basic amino acid uptake system, the y+ transporter, has a very low activity in hepatocytes. Normal plasma arginine concentrations in humans and animals range from 95 to 250 μM. Although extracellular arginine is the major source of the arginine for endothelial NO synthesis (20), EC are able to recycle citrulline (a product of NO synthesis) into arginine via the so-called arginine–citrulline cycle (24).

NO-dependent effects of arginine. The vascular effects of arginine are mediated primarily by NO production (20,25). The discovery of endothelial NO synthesis in late 1980's (26) has considerably improved our understanding of vascular biology and pathophysiology. NO mediates the endothelium-dependent vasodilation in response to stimuli such as shear stress, insulin, and actylcholine (27). Recent evidence suggests that NO interacts with other flow-induced vasodilation mediators, including prostacyclin and adenosine, and this interaction results in net hemodynamic changes. NO has also been shown to mediate exercise-induced vasodilation (28). As a potent endogenous vasodilator, NO has numerous beneficial effects that preserve normal vascular function. For example, NO activates guanylate cyclase in vascular smooth muscle, and increases the production of cyclic guanosine monophosphate (cGMP). cGMP, as a second messenger, mediates many of the biological effects of NO, e.g. causing relaxation of smooth muscle and inhibiting platelet aggregation (20,28). In addition, NO inhibits platelet activation and adhesion to the surface of endothelium, the release of vasoconstrictor endothelin-1, smooth muscle cell proliferation, and the synthesis and

expression of cytokines and cell adhesion molecules that attract monocytes and leukocytes to the endothelial surface. NO reduces vascular oxidative stress and inhibits superoxide generation (23,29). In addition, NO stimulates angiogenesis, which plays an important role in wound healing, vascular remodeling, and conditions like myocardial infarction and diabetic retinopathy (23). NO is also a mediator of the immune response, a neurotransmitter, a cytotoxic free radical and a widespread signaling molecule (23,25).

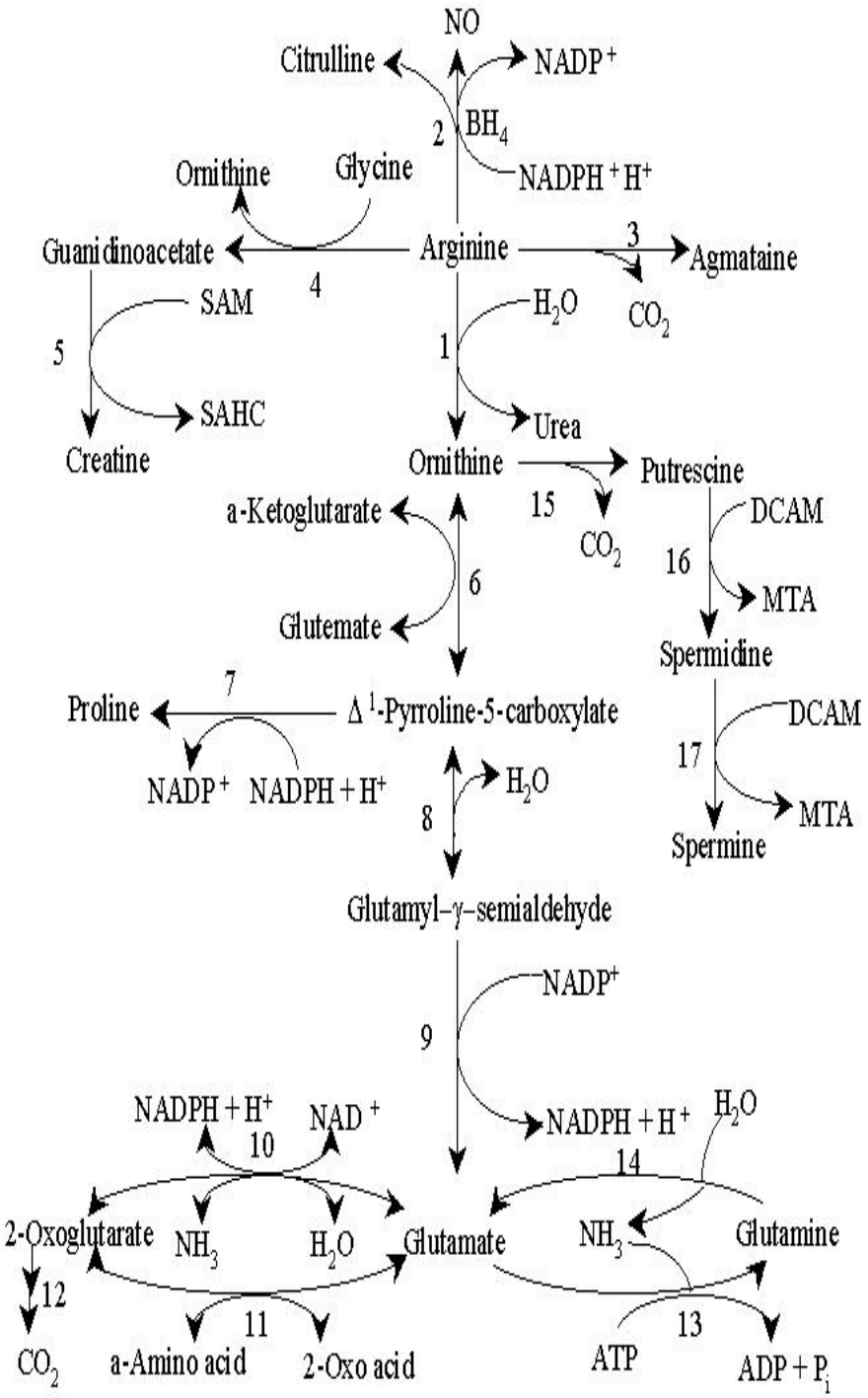
Nisoli et al (30) recently reported that the NO-cGMP-dependent pathway controls mitochondrial biogenesis and body energy balance. For example, NOS null-mutant mice had a reduced metabolic rate and accelerated weight gain (30), insulin resistance (27), hypertension, and hyperlipidemia (31). NO stimulates insulin release from pancreatic ß cells in the presence of glucose (32). These properties suggest that the level of NO production by the endothelium may play a pivotal role in the regulation of vascular disease. Therefore, impaired endothelial NO production may constitute a critical manifestation of proatherogenic events in the vascular wall, including increased vascular tone, platelet aggregation, endothelial barrier dysfunction, vascular inflammation, and smooth muscle cell proliferation. Because one of the factors which regulate NO production, is arginine availability, utilization of L-arginine and its conversion to NO have important implications for the development of endothelial dysfunction.

The arginine paradox. The available evidence indicates that supplemental administration of L-arginine is beneficial to restore endothelium-derived NO production in many cardiovascular disorders. However, some studies have also revealed that

elevating plasma arginine levels may not increase vascular NO synthesis in certain diseases (33). These results suggest the complexity of regulation of endothelial NO synthesis. The Km value is defined as the substrate concentration at which the reaction velocity is half maximal. The Km value of eNOS for L-arginine is 2.9 μM, while the intracellular value of L-arginine in vivo is 1-2 mM (20), which sufficiently saturates NOS. Thus, from an enzyme biochemical viewpoint, it is argued that additional L-arginine could not have any effect on NOS activity, because this enzyme should be saturated with substrate at physiological levels. However, supplementation of L-arginine has consistently been shown to exert a beneficial effect on the endothelium-dependent vasodilation in vivo. The so-called arginine paradox remains to be explained.

NO-independent effects of arginine. In addition to being the precursor of NO and an important component of protein, L-arginine is involved in several biologically important metabolic pathways (**Fig. 1.1)**. Serving as a precursor for the synthesis of urea, creatine, agmatine, proline, polyamines, and glutamate, arginine plays a vital role in the physiology of the organism (23). The high energy phosphate storage compound creatine is essential for sustained skeletal muscle contraction and for energy metabolism in the nervous tissue. Arginine degradation by arginine decarboxylase yields agmatine, which is known to interact with receptors that are normally bound by centrally acting hypertensive agent including clonidine. L-Arginine is an essential component of the urea cycle, the major pathway for elimination of ammonia in mammals (23).

FIGURE 1.1 Pathways of arginine catabolism. Enzymes that catalyse the indicated reactions are: 1, arginase; 2, NOS; 3, arginine decarboxylase; 4, arginine:glycine amidinotransferase; 5, guanidinoacetate N-methyltransferase; 6, Ornithine aminotrasferase; 7, Pyrroline-5-carboxylate reductase; 8, Spontaneous reaction; 9, Pyrroline-5-carboxylate dehydrogenase; 10, glutamate dehydrogenase; 11, alanine aminotransferase, aspartate aminotransferase or branched-chain amino acid aminotransferase; 13, glutamine synthetase; 14, glutaminase; 15, ornithine decarboxylase; 16, spermidine synthase; 17, spermine synthase. Step 8 is a spontaneous, non-enzymic reaction. Abbreviations: DCAM, decarboxylated S-adenosylmethionine; MTA, methylthioadenosine; SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine; BH_4, (6R)-5,6,7,8-tetrahydro-L-biopterin.


NO
Citrulline
NADP+
2
BH4
NADPH + H+
Ornithine
Glycine
Guanidinoacetate
Arginine
3
Agmataine
CO2
4
SAM
5
H2O
1
SAHC
Urea
Creatine
Ornithine
Putrescine
15
CO2
a-Ketoglutarate
DCAM
16
6
MTA
Glutemate
Spermidine
7
Proline
Δ1-Pyrroline-5-carboxylate
DCAM
17
NADP+
NADPH + H+
H2O
8
MTA
Spermine
Glutamyl-γ-semialdehyde
NADP+
9
NADPH + H+
NAD+
10
NADPH + H+
H2O
14
2-Oxoglutarate
NH3
H2O
Glutamate
NH3
Glutamine
12
11
13
CO2
a-Amino acid
2-Oxo acid
ATP
ADP + Pi

Arginine plays an important role in wound healing (34). As a product of arginine catabolism by arginase, ornithine is a precursor for synthesis of polyamines (putrescine, spermidine and spermine) and proline. Polyamines are essential for cell proliferation and differentiation. Proline is critical to collagen synthesis and thus extracellular matrix formation and vessel remodeling (23). Supplementation of arginine to healthy individuals improves matrix synthesis after wounding, whereas an arginine-free diet deteriorates healing in rats (35).

L-Arginine functions as a secretogogue of a number of important hormones. High intravenous doses of L-arginine (30 g) have been used to stimulate growth hormone secretion in humans. In addition, arginine increases the release of insulin, glucagon, prolactin polypeptide, and adrenal catecholamines (23,36), thus regulating the metabolism of protein, amino acids, glucose, and fatty acids. Arginine, independent of NO, is known to decrease lipid peroxidation and thus oxidative stress (37,38). Furthermore, arginine reduces copper-induced lipid peroxidation, scavenges O_2^-, and inhibits O_2^- release by EC (20). Through inhibiting the formation of thromboxane B_2 and the platelet-fibrin complex while enhancing plasmin generation and fibrin degradation, arginine stimulates fibrinogenolysis (23). Arginine may directly decrease leukocyte adhesion to nonendothelial matrix (39), thereby inhibiting the development of atherosclerosis. Further, high concentrations of arginine may decrease blood viscosity at low shear rate independent of NOS activity (40), which contributes to improved blood flow after intravascular administration of L-arginine.

Arginine in diabetes. Diabetes is associated with reduced plasma concentrations of arginine (41,42). Thus, dietary supplementation of L-arginine could be beneficial for the treatment of endothelial dysfunction in diabetic patients. Ozcelikay et al. (43) showed that L-arginine treatment in vivo could prevent diabetes-associated abnormalities in vascular function. These results are consistent with the findings of Pieper et al. (41,42), which reported that L-arginine supplementation normalized the endothelium-dependent relaxation in diabetic aorta by enhancing NO availability and restoring the acetylcholine-stimulated cGMP generation.

In the diabetic state, it is well known that oxidative stress is increased due to excessive production of oxygen free radicals and impaired antioxidant defense mechanisms. Increasing arginine supply to diabetic rats improved vascular reactivity, reduced blood pressure, and normalized lipid peroxidation (43). Further, concentrations of malondialdehyde, a product of lipid peroxidation, may be reduced by arginine in diabetic patients (38) and diabetic rats (44).

It is known that hypertension is an independent risk factor for cardiovascular mortality in patients with diabetes. Giugliano et al (45) found that intravenous L-arginine infusion completely reversed both the increase in blood pressure and the decrease in leg blood flow brought about by short-term elevation of plasma glucose levels in healthy subjects. This effect of arginine is mediated by the generation of NO.

As noted previously, AGE contribute to endothelial dysfunction and diabetic vascular complications by permanent chemical modification of cells and proteins (3). Interestingly, L-arginine supplementation may directly inhibit AGE action on the

vasculature (22). Because AGE is believed to quench NO, arginine administration might be of benefit to improve the endothelium-dependent relaxation by preventing AGE-mediated quenching of the NO-dependent smooth muscle relaxation (22).

Mendez and Balderas (32) recently reported beneficial effects of L-arginine supplementation on reducing serum concentrations of glucose and lipids in diabetic rats. These authors suggested that the beneficial effects of L-arginine treatment are primarily due to the action of polyamines synthesized from the arginine-derived ornithine in extrahepatic tissues. Putrescine and spermidine have antilipolytic action and are necessary for insulin and protein biosynthesis, whereas spermine depletion affects several processes involved in insulin metabolism (32). Importantly, NO stimulates glucose uptake and utilization by skeletal muscle (36). These results provide a biochemical basis for explaining the beneficial effects of L-arginine on hyperglycemia and dyslipidemia in experimental diabetes.

Diabetic healing is impaired, but the mechanisms are not well understood. High blood glucose lowers fibroblast proliferation (46) and affects collagen synthesis. Cross-linking of collagen fibers is reduced in diabetic subjects (47). Concentrations of growth factors, mediators of collagen synthesis, are also low in the wound milieu (48,49) probably secondary to the diminished and delayed inflammatory reaction. In diabetes, NO synthesis is reduced in the wound milieu and hence, arginine supplementation improves wound healing by restoring NO synthesis (50). Conversely, inhibiting wound NO formation lowers collagen formation and wound breaking strength (51), and decreases wound contraction (52) Alternatively, arginine can be metabolized in wounds

via arginase to ornithine. Ornithine is the precursor for polyamines and proline. Polyamines are essential for cell proliferation, whereas proline is necessary for collagen formation. Ornithine supplementation also enhances wound healing, suggesting that at least some of the arginine effect could be mediated through ornithine synthesis (23,34). Interestingly, iNOS knock-out mice supplemented with L-arginine do not demonstrate improved incisional healing, suggesting that the effect of arginine on wound healing involves primarily the arginine-iNOS pathway (50).

Relation between arginine and tetrahydrobiopterin (BH_4). BH_4 has long been recognized as an essential co-factor for all isoforms of the NOS, including eNOS (53). Schmidt et al. (54) were the first to demonstrate that an increase in intracellular BH_4 availability due to an increase in its synthesis stimulated NO production by EC. Since this initial report, there has been growing interest in the role of BH_4 in the therapeutic treatment of endothelial dysfunction (55). Meininger et al. (13) reported that EC from the spontaneously diabetic BB rat had an impaired ability to produce NO due to the deficiency of BH_4 and replenishing BH_4 levels in the EC from diabetic animals restored their ability to make NO and proliferate.

Importantly, administration of either BH_4 or L-arginine improves endothelial function in experimental animals and humans with a variety of major cardiovascular risk factors such as diabetes, insulin resistance, smoking, and hypercholesterolemia (56,57,58). However, a combination of both L-arginine and BH_4 has no additional effect on the endothelium-dependent relaxation, compared with either compound alone (59).

These findings raise an important question of whether BH_4 is deficient in EC of diabetic persons and whether arginine stimulates de novo synthesis of endothelial BH_4.

Summary

Endothelial dysfunction is a major factor contributing to the high rates of morbidity and mortality in diabetic patients. Impaired NO synthesis from arginine in EC plays an important role in the pathogenesis of diabetes-associated cardiovascular complications. Recent studies indicate that a deficiency of BH_4 is the biochemical basis for inadequate generation of NO in EC. Extensive studies demonstrate that arginine administration improves endothelial function in humans with type-I or type-II diabetes mellitus and in animal models of type-I diabetes. However, the underlying mechanisms are not known. Because the Km value of the three isoforms of the NOS for arginine are < 10 μM, it is unlikely that increasing extracellular arginine concentrations would increase substrate concentrations for the enzymes in mammalian cells. Alternatively, arginine may stimulate endothelial BH_4 synthesis and therefore increase endothelial BH_4 availability for NO generation. The available evidence indicates that diabetic subjects have low levels of plasma arginine, and thus dietary arginine supplementation may provide a potentially novel means to increase arginine availability and prevent endothelial dysfunction in diabetic subjects.

Objectives of This Research

In view of the above literature review, we hypothesized that dietary arginine supplementation increases the availability of BH_4 (a critical cofactor for endothelial NOS) for endothelial NO synthesis in diabetic rats, thereby preventing endothelial dysfunction. The major objective of the author's thesis research was to test this hypothesis with the use of streptozotocin (STZ)-induced diabetic rats and Zucker diabetic fatty (ZDF) rats. The specific aims of the investigations were:

1. To determine the effect of dietary arginine supplementation on body weight loss, EC BH_4 availability, EC NO synthesis, and plasma glucose concentrations in STZ diabetic rats; and
2. To determine the effect of dietary arginine supplementation on body weight gain, EC BH_4 availability, EC NO synthesis, and plasma glucose concentrations in ZDF rats.

Significance of This Research

NO is an important product of arginine catabolism by NOS in virtually all animal cells. Although there is a great body of literature regarding the beneficial role of arginine in humans and rats with diabetes and endothelial dysfunction, little is known about the mechanisms responsible for the stimulating effect of arginine on endothelial NO synthesis in normal and diabetic subjects. Elucidating the underlying mechanism(s) will help in the design of new means to treat patients with a wide array of cardiovascular disorders. As arginine may be a potentially novel nutrient to improve endothelial function, it is of crucial importance to demonstrate that dietary arginine supplementation

increases NO synthesis in EC of both normal and diabetic animals. This new knowledge about arginine biochemistry and nutrition in the cardiovascular system will provide a much-needed experimental basis for the clinical application of arginine to prevent endothelial dysfunction in diabetic patients Because obesity and diabetes have become a major public health problem in the U.S. and worldwide, arginine may provide an effective solution for improving life quality and health of obese and diabetic patients while reducing the tremendous health care costs.

CHAPTER II

EFEFCTS OF ORAL ADMINISTRATION OF L-ARGININE ON STREPTOZOTOCIN INDUCED DIABETIC RATS

Synopsis

L-arginine is the substrate for synthesis of nitric oxide (NO), the endothelium-derived relaxing factor essential for regulating vascular tone and hemodynamics. In recent years, there has been growing interest in the use of L-arginine to prevent and treat the impaired endothelium-dependent relaxation associated with major cardiovascular risk factors (e.g. diabetes). However, little is known about the mechanism responsible for the stimulatory effect of arginine on endothelial NO synthesis in diabetic and normal subjects. In the present study, the effect of dietary arginine supplementation on body weight changes, EC BH_4 availability, EC NO synthesis, and plasma glucose concentrations in male Sprague-Dawley (SD) rats was studied. Diabetes in SD rats was induced by intravenous injection of streptozotocin (STZ). STZ-diabetic SD rats were individually pair-fed a casein-based diet on the basis of feed intake (per kg body weight) by non-diabetic rats. Addition of arginine-HCl (1.51%) or alanine (2.55%) to drinking water for the rats were adjusted daily to ensure isonitrogenous provision per kg body weight. In non-diabetic rats, arginine supplementation had no effect on feed intake, body weight or plasma glucose, but increased plasma concentrations of arginine (144%), plasma concentrations of insulin (44%), and arginine concentrations (88%), BH_4 concentrations (106%) and NO synthesis (80%) in EC, compared with alanine treatment. In diabetic rats, arginine supplementation reduced body weight loss (36%), and plasma

glucose levels (54%), and increased plasma arginine levels (110%), plasma insulin levels (209%), and arginine concentrations (173%), BH_4 concentrations (128%), and NO synthesis (125%) in EC, compared with alanine treatment. Results of this study demonstrate that dietary L-arginine supplementation is beneficial for preventing hyperglycemia and enhancing endothelial BH_4 availability for NO synthesis in diabetic rats.

Introduction

A goal of the treatment of diabetes mellitus is to control plasma glucose concentrations. Hyperglycemia, the hallmark of diabetes mellitus, may initiate endothelial dysfunction, which results from decreased production of NO, inactivation of NO by oxygen-derived free radicals, and/or increased production of endothelium-derived contracting factors (opposing the protective activity of NO) (3). L-Arginine is the substrate for the NOS, which is responsible for the endothelial production of NO (20). Thus, utilization of L-arginine and its conversion to NO may contribute to a beneficial role of this amino acid in ameliorating endothelial dysfunction.

L-Arginine supplementation may be beneficial for diabetic subjects. For example, Mendez and Balderas (32) reported that L-arginine supplementation reduced serum glucose and lipid levels in diabetic rats. Interestingly, administration of L-arginine reversed endothelial dysfunction, restored the NO-mediated endothelium-dependent relaxation, and decreased oxidative stress in diabetic rats (41,43). In diabetes, NO

synthesis is reduced in the wound milieu and hence, arginine improves wound healing (an angiogenesis-dependent process), by restoring NO synthesis (51).

Despite the foregoing, little is known about the mechanisms responsible for the stimulating effect of arginine on endothelial NO synthesis in normal and diabetic subjects. Wu and Meininger (60) reported that impaired NO synthesis in EC of diabetic BB rats was not due to alterations in arginine uptake, NOS activity, or intracellular arginine concentrations but might have resulted from a limited availability of cofactors of NOS. Recently, Meininger et al. (13) found that EC from the spontaneously diabetic BB rat have an impaired ability to produce NO due to a deficiency of BH_4 and replenishing BH_4 levels in the EC restores their ability to make NO. On the basis of the recent report (61) that arginine stimulates BH_4 synthesis in cultured EC, we hypothesized that dietary L-arginine supplementation may increase BH_4 availability for NO synthesis in EC of diabetic rats. The major objective of the present study was to test this hypothesis with use of STZ-induced diabetic rats.

Materials and Methods

Chemicals. Hexokinase, glucose-6-phosphate dehydrogenase and nitrate reductase were purchased from Roche (Indianapolis, IN). Joklik's modified minimal essential medium, Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's medium (DMEM), L-glutamine and penicillin/streptomycin/amphotericin B were obtained from GIBCO-BRL (Gaithersburg, MD). Collagenase type-II was purchased from Worthington Biochemical (Freehold, NJ). Heparin sodium was purchased from

Elkins-Sinn (Cherry Hill, NJ), whereas 2,3-daminonaphthalene (DAN), dithioerythritol, and streptozotocin were from Sigma (St. Louis, MO).

Animals. Male Sprague-Dawley (SD) rats (65 days-old) were obtained from Harland (Houston, TX). At 70 days of age, SD rats received i.v. injection of streptozotocin (STZ; 65 mg/kg body wt) to induce diabetes or of vehicle solution (50 mM sodium citrate, pH 4.5). One day after the injection, rats received drinking water containing either 0.6% L-arginine-HCl (equivalent to 0.5% arginine) or 1.01% L-alanine (isonitrogenous control) (n = 8 per treatment, a 2 X 2 factorial design). This dose is chosen because it has shown to prevent endothelial dysfunction in BB rats and STZ-diabetic rats and had no adverse effect on non-diabetic rats. The free arginine base was not used because 0.6% in water yields an alkaline solution (pH 10.2), while 0.6% arginine-HCl in water did not affect pH (5.8). Addition of arginine or alanine to drinking water for rats was adjusted daily to ensure isonitrogenous provision of nitrogen per kg body weight. Diabetic rats were individually pair-fed a casein-based diet on the basis of feed intake by non-diabetic rats (per kg body wt). **Table 2.1** shows the composition of the casein-based diet (62). Tail venous blood samples (0.2 ml) were obtained from SD rats on day 2 post arginine supplementation. Following two-week arginine supplementation to SD rats, blood samples (tail vein and heart) and coronary EC were obtained as described below.

TABLE 2.1

Diet composition for SD rats (casein–based diet)

Ingredient	20% Casein Diet (g/kg diet)
Casein[1]	200
DL-Methionine	3
Corn starch	150
Sucrose	500
Cellulose	50
Corn oil	50
Salt mix[2]	35
Vitamin mix[3]	10
Choline bitartrate	2
TOTAL	1000
Gross Energy (kJ/kg)	16,816

[1]Acid casein (88.1% protein) (New Zealand Milk Products, Inc., Santa Rosa, CA). Amino acid composition of acid casein was as follows (g amino acid/100 g protein): alanine, 2.6; arginine, 3.6; aspartate plus asparagine, 6.5; cysteine plus cystine, 0.4; glutamate plus glutamine, 20.9; glycine, 1.8; histidine, 2.6; isoleucine, 4.8; leucine, 8.8; lysine, 7.4; methionine, 2.6; phenylalanine, 5.0; proline, 11.7; serine, 5.4; threonine, 3.8; tryptophan, 1.2; tyrosine, 5.3; and valine, 5.7.

[2]Containing the following (g/kg salt mix): calcium phosphate dibasic (29.5% Ca and 22.8% P), 500; magnesium oxide (60.3% Mg), 24; manganous carbonate (47.8% Mn), 3.5; potassium citrate.1 H_2O (36.2% K), 220; potassium sulfate (44.9% K and 18.4% S), 52; sodium chloride (39.3% Na and 60.7% Cl), 74; chromium potassium sulfate.12 H_2O (10.4% Cr), 0.55; cupric carbonate (57.5% Cu), 0.3; potassium iodate (59.3% I), 0.01; ferric citrate (21.2% Fe), 6.0; sodium selenite (45.7% Se), 0.01; zinc carbonate (52.1% Zn), 1.6; sucrose, 118.03.

[3]Containing the following (g/kg vitamin mix): retinyl palmitate (500,000 IU/g), 0.8; cholecalciferol (100,000 IU/g), 1.0; all-rac-?tocopheryl acetate (500 IU/g), 10.0; menadione sodium bisulfite (62.5% menadione), 0.08; biotin (1.0%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine.HCl, 0.7; riboflavin, 0.6; thiamin.HCl, 0.6; sucrose, 978.42.

Isolation of coronary EC. Rats were injected with heparin sodium (130 U/100 g body wt) intraperitoneally, 20 min before the rats were euthanized. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35mg/250g rat). The hearts were removed and placed in ice-cold Joklik's modified minimal essential medium containing 60 mM taurine, 20 mM creatine, and 5 mM HEPES. The aortas were cannulated with stainless steel tubing (2-mm inner diameter) and perfused with the above medium from a static 40-mmHg hydrostatic pressure head. The oxygenated (100%) perfusate, which was supplemented with 0.1% dialyzed bovine serum albumin and heparin (1 U/ml), was passed through the hearts once. After a 10-min washout period, new medium with collagenase (0.7 mg/ml) was introduced, and the perfusate was allowed to recirculate until the aortic perfusion pressure decreased to below 40 mmHg (30-40 min). The ventricles were cut from the hearts, minced, and placed in fresh collagenase-containing perfusate. The tissue was shaken at 250 rpm in a water bath for 10 min. $CaCl_2$ (50 μM) was added to the minced tissue, and digestion with collagenase continued for an additional 10 min. The cells were dispersed, filtered through a double layer of cheesecloth, and diluted 1:4 with buffer containing 0.1% dialyzed bovine serum albumin. The resulting suspension was allowed to settle to separate myocytes (which are heavier) from EC. EC were further purified by centrifugation through cushions of 6% and 3% bovine serum albumin in Joklik's modified minimal essential medium. The endothelial identity of the collected cells was confirmed by the uptake of modified low-density lipoprotein.

Measurement of plasma glucose, arginine, and insulin. Plasma glucose concentration was determined by an enzymatic method involving hexokinase and glucose-6-phosphate dehydrogenase (63). Arginine levels in plasma were measured by HPLC (60). Insulin was analyzed using a radioimmunological assay kit from Linco Company (St. Louis, MO).

High-performance liquid chromatography analysis of BH_4 and arginine in freshly isolated EC. Cellular content of BH_4 was determined using a modification of the HPLC method of Fukushima and Nixon (64). EC were lysed in 0.3 ml of 0.1 M phosphoric acid containing 5mM dithioerythritol (an antioxidant) and 35ul of 2M trichloroacetic acid. Cell debris was removed by centrifugation. Extracts were oxidized with acidic or basic iodine. Acidic iodine oxidation quantitatively converts BH_4 and dihydrobiopterin to biopterin; basic iodine oxidation converts dihydrobiopterin to biopterin. Samples were incubated in the dark for 1 hr. Excess iodine was removed by adding ascorbic acid (final concentration 0.1M). The final solution was analyzed on a C18 reversed-phase column using fluorescence detection and authentic biopterin as a standard. The amount of BH_4 in the extracts was determined from the difference in biopterin concentrations generated with acidic and basic iodine oxidation. Arginine levels in EC were measured by HPLC (60). Intracellular concentrations of BH_4 and arginine were calculated on the basis of the average cell volume of rat coronary EC (0.348 $\mu L/10^6$ cells).

NO synthesis in freshly isolated EC. EC were rinsed with the Basal Medium Eagle, and then incubated at 37°C for 6 hours in the Basal Medium Eagle containing 0.2

mM L-arginine, 0.5 mM L-glutamine, 5 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. At the end of the 6-h incubation period, media were analyzed for nitrite and nitrate (two major stable end products of NO oxidation) with HPLC as described by Li et al (65). In all experiments, medium incubated without cells were run as the blank.

Data analysis. Results are expressed as mean ± SEM. Data were statistically analyzed by two-way analysis of variance using SAS (Statistical Analysis System). Differences between means were determined by the Student-Newman-Keul's multiple comparison test. Probability values less than 0.05 were taken to indicate significance.

Results

Body weight. Body weights of rats were recorded daily from the beginning of the experiment. **Table 2.2** summarizes data on initial and final body weights as well as body weight changes. At the conclusion of the study (14 days after the onset of diabetes), in diabetic rats, treatment with L-arginine reduced ($P < 0.01$) the body weight loss by ~36% compared with alanine-treated rats. However, in non-diabetic rats, L-arginine supplementation had no effect ($P > 0.05$) on body weight compared with the corresponding alanine-treated rats. There was a significant decrease ($P<0.01$) in the body weight of the alanine-treated diabetic rats due to uncontrolled diabetes.

TABLE 2.2

Body weights of SD rats (g)

Animals	Treatment	Initial body weight (d 0)	Final body weight (d 14)	Change in body weight gain
Non-diabetic	Alanine	284 ± 9.0	319 ± 12	34 ± 2.7
	Arginine	287 ± 4.9	319 ± 5.7	32 ± 1.5
Diabetic	Alanine	283 ± 4.0	167 ± 3.6 *	-116 ± 3.3 *
	Arginine	282 ± 8.2	208 ± 10 *†	-74 ± 8.1 *†

Data are means ± SEM, n = 8.

* $P < 0.01$ vs the corresponding non-diabetic group.

† $P < 0.01$ vs the corresponding alanine-supplemented group.

TABLE 2.3

Feed, water and calorie intake (g/kg body wt/day) by SD rats

Animals	Treatment	Feed (g/kg body wt/d)	Water (ml/kg body wt/d)	Gross Calorie † (kJ/kg body wt/d)
Non-diabetic	Alanine	56.3 ± 2.2	110 ± 9.1	1008 ± 36
	Arginine	53.2 ± 2.0	101 ± 10.4	933 ± 34
Diabetic	Alanine	53.5 ± 2.3	420 ± 16.3	958 ± 35
	Arginine	54.8 ± 2.4	341 ± 19.3	958 ± 39

Data are means ± SEM, n = 8.

† Including diet and drinking water.

Energy, food and water intake. Food and water consumption were measured every 24 hours. **Table 2.3** summarizes the data on feed intake and water intake of rats. Food intake was similar among the four groups of rats to make their diets isonitrogenuos. Arginine and alanine intake from drinking water **(Table 2.4)** were adjusted daily to ensure isonitrogenous provision per kg body weight. **Table 2.5** summarizes the data on arginine and alanine intake from diet. Energy intake **(Table 2.3)** was calculated from the feed intake and water consumption for all four groups of rats.

TABLE 2.4

Intake of arginine from drinking water (g/kg body wt/d) by SD rats

Animals	Treatment	Arginine from drinking water	Alanine from drinking water
Non-diabetic	Alanine	----	3.44 ± 0.23
	Arginine	1.40 ± 0.12	----
Diabetic	Alanine	----	3.23 ± 0.26
	Arginine	1.33 ± 0.12	----

Data are means ± SEM, n = 8.

TABLE 2.5

Intake of arginine from diet (g/kg body wt/d) by SD rats

Animals	Treatment	Arginine from diet	Alanine from diet
Non-diabetic	Alanine	0.36 ± 0.014	0.25 ± 0.006
	Arginine	0.34 ± 0.013	0.24 ± 0.002
Diabetic	Alanine	0.34 ± 0.015	0.26 ± 0.005
	Arginine	0.35 ± 0.015	0.25 ± 0.004

Data are means ± SEM, n = 8.

Plasma glucose levels. All rats receiving STZ-injection developed diabetes within 24 h of administration on the basis of glucosuria, ketosis, and hyperglycemia and body weight loss, likely due to lot of fluid loss. Plasma glucose levels were higher in both L-arginine-treated and alanine-treated diabetic rats compared with non-diabetic rats at day 2 post onset of diabetes **(Table 2.6)**. At day 14 post onset of diabetes, plasma glucose levels were reduced ($P<0.01$) in arginine-treated rats to values similar to those for non-diabetic rats **(Table 2.6)**.

TABLE 2.6

Plasma concentrations of glucose in SD rats after arginine supplementation

Animals	Treatment	Glucose (mM)	
		day 2	day 14
Non-diabetic	Alanine	8.38 ± 0.8	7.73 ± 0.61
	Arginine	7.91 ± 4.1	7.66 ± 0.68
Diabetic	Alanine	19.8 ± 1.7	17.8 ± 2.10 *
	Arginine	16.9 ± 1.9	8.15 ± 0.99 †

Data are means ± SEM, n = 8.

* $P < 0.01$ vs the corresponding non-diabetic group.

† $P < 0.01$ vs the corresponding alanine-supplemented group.

TABLE 2.7

Plasma concentrations of arginine and insulin in SD rats after 14-day arginine supplementation

Animals	Treatment	Arginine (uM)	Insulin (ng/ml)
Non-diabetic	Alanine	203 ± 7.8	3.42 ± 0.11
	Arginine	496 ± 18 †	4.86 ± 0.31 †
Diabetic	Alanine	137 ± 5.6 *	0.42 ± 0.04 *
	Arginine	289 ± 14 *†	1.30 ± 0.15 *†

Data are means ± SEM, n = 8.

* $P < 0.01$ vs the corresponding non-diabetic group.

† $P < 0.01$ vs the corresponding alanine-supplemented group.

Plasma insulin and arginine levels. Untreated diabetic rats exhibit low levels of plasma insulin and arginine ($P<0.01$), which were restored with arginine supplementation **(Table 2.7)**. In diabetic rats, arginine treatment increased ($P<0.01$) plasma insulin levels by ~209% and plasma arginine levels by ~110% compared with alanine-treated diabetic rats. Additionally, arginine supplementation increased ($P<0.01$) plasma insulin (~44%) and arginine (~ 144%) levels in non-diabetic rats.

Intracellular concentrations of arginine, BH_4, and NO production in rat coronary EC. BH_4 levels, arginine levels and NO production were decreased ($P<0.01$) in EC from diabetic animals, compared with non-diabetic rats **(Table 2.8)**. However, arginine treatment for 14 days increased ($P<0.01$) arginine concentrations, BH_4 concentrations and NO production in coronary EC of both diabetic and non-diabetic rats. In diabetic rats, arginine treatment increased ($P<0.01$) arginine concentrations, BH_4 concentrations and NO production in coronary EC by ~ 173%, 128%, 125%, respectively, compared with the corresponding alanine-treated rats. In non-diabetic rats, arginine supplementation increased ($P<0.01$) arginine concentrations, BH_4 concentrations and NO production in coronary EC by 88%, 106%, and 80%, respectively, compared with the corresponding alanine-treated rats.

TABLE 2.8

Intracellular concentrations of arginine, BH_4 and NO production in SD rat coronary endothelial cells after 14-day arginine supplementation

Animals	Treatment	[Arginine] (mM)	[BH_4] (uM)	NO synthesis (pmol/10^6 cells per h)
Non-diabetic	Alanine	1.29 ± 0.06	2.94 ± 0.20	259 ± 12
	Arginine	2.43 ± 0.10 †	6.03 ± 0.03 †	468 ± 19 †
Diabetic	Alanine	0.49 ± 0.03 *	1.40 ± 0.06 *	118 ± 8.1 *
	Arginine	1.34 ± 0.08 *†	3.22 ± 0.03 *†	266 ± 18 †

Data are means ± SEM, n = 8.

* $P < 0.01$ vs the corresponding non-diabetic group.

† $P < 0.01$ vs the corresponding alanine-supplemented group.

Discussion

Endothelial dysfunction is a major factor contributing to morbidity and mortality in diabetes mellitus. Enhanced production of vasoconstrictors and/or reduced synthesis of the vasodilator, NO may be the most important factors responsible for diabetes-associated cardiovascular complications (13). A deficiency of arginine or BH_4 could impair the endothelium-dependent relaxation. As reported by other investigators (11.12), we observed a marked decrease in plasma concentrations of arginine in diabetic rats. This provides a basis for dietary supplementation of arginine as a potentially novel means to prevent diabetes-associated endothelial dysfunction.

Our results demonstrate that oral administration of L-arginine-HCl via drinking water for 14 days decreased ($P<0.01$) glucose levels in diabetic rats by ~54%. This is a very significant finding, but the mechanism is not known at present. Arginine supplementation may stimulate NO production by skeletal muscle, and enhance NO-mediated blood flow, thereby increasing glucose uptake by skeletal muscle and insulin sensitivity in tissues. Beneficial effects of L-arginine administration on serum glucose levels have also been demonstrated in other studies (32). Indeed, Popov et al (66) reported that oral administration of 622 mg arginine/kg body weight to hamsters reduced the high concentrations of circulating glucose by 63%. L-Arginine is known to stimulate the secretion of insulin from beta cells of the pancreas (23,36). Indeed, dietary supplementation of L-arginine resulted in an increase in the plasma insulin levels in both STZ-induced diabetic (by ~209%) and non-diabetic (by ~44%) SD rats. Note that in the STZ-diabetic rat model, not all the ß-cells are destroyed, and the remaining cells can

secret a physiologically significant quantity of sufficient insulin to keep the animals alive for up to 2 months.

An important finding of this study is that L-arginine supplementation ameliorated weight loss in STZ diabetic rats. SD diabetic rats treated with L-arginine had 36% less weight loss than alanine-treated diabetic rats. The ability of L-arginine to reduce body weight loss in STZ-diabetic rats may result from one or more of the following factors: 1) prevention of hyperglycemia, 2) reduction in skeletal muscle protein degradation, and 3) increase in skeletal muscle protein synthesis. L-Arginine supplementation did not affect the weight gain, plasma glucose levels, food and water intake in normal, non-diabetic rats, indicating a lack of adverse effect of elevated levels of arginine under the experimental conditions of the study.

Previous work (67) in our lab showed that rates of arginine degradation by enterocytes of the small intestine did not differ between normal and STZ-diabetic rats. Thus, similar amounts of dietary arginine would be expected to enter the portal circulation in normal and STZ-diabetic rats. L- Arginine treatment increased arginine levels by almost two folds (~110%) compared with alanine-treated diabetic rats. The reasons for decreased plasma arginine in diabetic state are unclear. However, previous work in our lab suggests that the reduced availability of circulating arginine in diabetic rats may result from impaired renal arginine synthesis owing to reduced availability of aspartate (an amino acid required for the conversion of citrulline into arginine by argininosuccinate synthase). Nevertheless, results of this study indicate that oral administration is an effective means to augment plasma arginine levels in diabetic rats.

Intracellular arginine concentrations are approximately 1-2 mM in freshly isolated endothelial cells. Intracellular values of L-arginine (0.49 mM) in diabetic SD rats were substantially decreased but were still much higher than the Km value of eNOS (2.9 μM) for L-arginine. This observation implies that endothelial NOS was saturated with intracellular L-arginine. However, dietary supplementation of L-arginine did increase NO production in coronary EC of both STZ-diabetic and non-diabetic SD rats **(Table 2.8)**. This data further supports the notion that intracellular or extracellular arginine concentrations are critical for endothelial NO production. Importantly, we found that L-arginine increased endothelial BH_4 availability in both STZ-diabetic and non-diabetic SD rats. This finding is consistent with the report that replenishing BH_4 levels in EC from diabetic animals restores their ability to make NO (13). Through an increase in BH_4 availability, administration of L-arginine increased endothelial NO generation.

In conclusion, dietary arginine supplementation to STZ-diabetic rats increased plasma and EC concentrations of arginine, and enhanced BH_4 availability and NO synthesis in EC. In addition, the arginine treatment prevented hyperglycemia and reduced the loss of body weight in diabetic animals. These data demonstrate that oral administration of arginine is beneficial for an animal model of insulin-dependent diabetes mellitus. The findings also provide a biochemical basis for explaining the beneficial effect of dietary arginine supplementation to diabetic human patients.

CHAPTER III

EFEFCTS OF ORAL ADMINISTRATION OF L-ARGININE ON ZUCKER DIABETIC FATTY RATS

Synopsis

This study was conducted to test the hypothesis that dietary arginine supplementation would increase the availability of tetrahydrobiopterin (BH_4) for NO synthesis in endothelial cells (EC) of type-II diabetic rats, thereby preventing endothelial dysfunction. Male Zucker diabetic fatty (ZDF) rats were individually pair-fed a Purina 5008 diet on the basis of feed intake by alanine-treated diabetic rats (per kg body wt). Addition of arginine-HCL (1.51%) or alanine (2.55%) to drinking water for ZDF rats was adjusted daily to ensure isonitrogenous provision per kg body weight. Plasma glucose levels were determined in tail venous blood samples, obtained at weeks 3, 6 and 10 post arginine supplementation. At the end of a 10-week period of arginine supplementation, blood samples and coronary EC were obtained from rats. Arginine supplementation did not affect ($P>0.05$) plasma levels of glucose and insulin, compared with alanine-treated rats, but reduced ($P<0.01$) epididydmal fat (30%), abdominal fat (43%) and body weight gain and increased ($P<0.01$) plasma concentrations of arginine (273%), and arginine concentrations (197%), BH_4 concentrations (120%) and NO synthesis (122%) in EC, compared with alanine-treated rats. These results indicate that oral administration of arginine enhances BH_4 availability for NO synthesis in EC of ZDF rats and that arginine is a novel anti-obesity nutrient. Collectively, dietary arginine

supplementation may be beneficial for obese subjects with insulin-independent diabetes mellitus.

Introduction

Type-II diabetes mellitus and obesity commonly co-occur. Weight loss in obese-diabetic patients is associated with significant health benefits, including improved glycemic control and reduced blood pressure (68). NO, synthesized from L-arginine by eNOS, has emerged as an important therapeutic molecule for enhancing insulin sensitivity and preventing endothelial dysfunction in diabetic subjects (25). Nisoli et al (30) reported that NO plays a major role in mitochondrial biogenesis in a cGMP-dependent manner. Interestingly, NOS null-mutant mice had a reduced metabolic rate and accelerated weight gain (30), insulin resistance (27), hypertension, and hyperlipidemia (31). Other studies have also shown the evidence of NO role in regulation of lipolysis (69) by facilitating leptin-induced lipolysis (70). These findings suggest a possible role of NO as an anti-obesity agent.

Recent data from our laboratory indicate that BH_4 is deficient in EC of both STZ-diabetic rats and ZDF rats. Results presented in chapter II of this thesis demonstrate that dietary arginine supplementation to STZ-diabetic rats increased BH_4 concentrations and NO production in coronary EC. In view of this finding, we hypothesized that dietary arginine supplementation could also increase the availability of BH_4 for NO synthesis in EC of ZDF rats, thereby preventing endothelial dysfunction.

Materials and Methods

Chemicals. Hexokinase, glucose-6-phosphate dehydrogenase and nitrate reductase were purchased from Roche (Indianapolis, IN). Joklik's modified minimal essential medium, Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's medium (DMEM), L-glutamine and penicillin/streptomycin/amphotericin B were obtained from GIBCO-BRL (Gaithersburg, MD). Collagenase type-II was purchased from Worthington Biochemical (Freehold, NJ). Heparin sodium was purchased from Elkins-Sinn (Cherry Hill, NJ), whereas 2,3-diaminonaphthalene (DAN), dithioerythritol, and streptozotocin were from Sigma (St. Louis, MO).

Animals. Male Zucker diabetic fatty rats (ZDF) (8 week-old) were obtained from Charles River (Wilmington, MA). At 9 weeks of age, ZDF rats received drinking water containing either 1.51% L-arginine-HCl or 2.55% L-alanine (isonitrogenous control) (n = 5 per treatment). This dose is chosen because previous studies have shown that oral administration of arginine (1.2% in drinking water) prevented endothelial dysfunction in BB rats and STZ-diabetic rats and had no adverse effect on non-diabetic rats (41). The free arginine base was not used because 1.2% or 1.51 % in water yields an alkaline solution (pH 10.8), while 1.5% arginine-HCl in water did not affect pH (6.5). Addition of arginine or alanine to drinking water for diabetic rats was adjusted daily to ensure isonitrogenous provision of nitrogen per kg body weight. Diabetic rats were individually pair-fed a Purina 5008 diet on the basis of feed intake by alanine-treated diabetic rats (per kg body wt). The ingredients of the Purina 5008 diet are shown in **Table 3.1** and composition of the diet in **Table 3.2.** Tail venous blood samples (0.2 ml) were obtained

from ZDF rats at weeks 3, 6 and 10 post arginine supplementation. At the end of a 10-week period of arginine supplementation, blood samples and coronary EC were obtained from ZDF rats as described below.

TABLE 3.1

Ingredients of Purina 5008 diet for ZDF rats

Ground corn	Dehulled soybean meal	Ground wheat
Fish meal	Wheat middlings	Animal fat
Cane molasses	Ground oats	Brewers dried yeast
Wheat germ meal	Meat meal	Dried beet pulp
Dehydrated alfalfa	Calcium carbonate	Dried whey
Salt	Cyanocobalamin	DL-methionine
Calcium	Pantothenate	Choline chloride
Folic acid,	Pyridoxine hydrochloride	Riboflavin
Thiamin mononitrate	Nicotinic acid	Vitamin A acetate
Cholecalciferol	DL-alpha tocopheryl acetate	Manganous oxide
Ferrous carbonate	Cobalt carbonate	Calcium iodate
Copper sulphate	Zinc sulphate	Zinc oxide
Vitamin K †		

† Provided as Menadione dimethylpyrimidinol bisulfite.

TABLE 3.2

Nutrient composition of Purina 5008 diet for ZDF rats

Nutrients	
Protein	23.5 %
Arginine	1.44 %
Cystine	0.35 %
Glycine	1.23 %
Histidine	0.58 %
Isoleucine	1.20 %
Leucine	1.87 %
Lysine	1.40 %
Methionine	0.43 %
Phenylalanine	1.08 %
Tyrosine	0.66 %
Threonine	0.90 %
Tryptophan	0.28 %
Valine	1.19 %
Serine	1.20 %
Aspartic Acid	2.60 %
Glutamic Acid	4.77 %
Alanine	1.39 %
Proline	1.63 %
Taurine	0.02 %
Fat (ether extract)	6.5 %
Fat (acid hydrolysis)	7.5 %
Cholesterol, ppm	280
Linoleic acid	1.37 %
Linolenic acid	0.09 %
Arachidonic acid	0.01 %
Omega-3 fatty acids	0.29 %
Total Saturated fatty acids	2.51 %
Total Monounsaturated fatty acids	2.32 %
Fiber	3.8 %
Nitrogen-free extract	49.4 %
Starch	34.9 %
Glucose	0.22 %
Fructose	0.24 %

TABLE 3.2 Continued

Nutrients	
Sucrose	2.57 %
Lactose	0.39 %
Total digestible Nutrients	81.2 %
Minerals	
Ash	6.8 %
Calcium	1.0 %
Phosphorus	0.65 %
Potassium	1.10 %
Magnesium	0.20 %
Sulfur	0.24 %
Sodium	0.28 %
Chlorine	0.48 %
Fluorine, ppm	19
Iron, ppm	230
Zinc, ppm	73
Manganese, ppm	71
Copper, ppm	13
Cobalt, ppm	0.4
Iodine, ppm	0.8
Chromium, ppm	1.4
Selenium, ppm	0.23
Vitamins	
Carotene, ppm	4.0
Vitamin K (as menadione), ppm	3.2
Thiamine Hydrochloride, ppm	16
Riboflavin, ppm	5.0
Niacin, ppm	109
Pantothenic Acid, ppm	15
Choline Chloride, ppm	2000
Folic Acid, ppm	3.0
Pyridoxine, ppm	6.0
Biotin, ppm	0.20
B_{12}, mcg/kg	20
Vitamin A, IU/gm	15
Vitamin D_3 (added), IU/gm	3.3
Vitamin E, IU/kg	55
Ascorbic Acid, mg/gm	---
Gross Energy, kJ/kg	17364

Isolation of coronary EC. Rats were injected with heparin sodium (130 U/100 g body wt) intraperitoneally, 20 min before the rats were euthanized. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35mg/250g rat). The hearts were removed and placed in ice-cold Joklik's modified minimal essential medium containing 60 mM taurine, 20 mM creatine, and 5 mM HEPES. The aortas were cannulated with stainless steel tubing (2-mm inner diameter) and perfused with the above medium from a static 40-mmHg hydrostatic pressure head. The oxygenated (100%) perfusate, which was supplemented with 0.1% dialyzed bovine serum albumin and heparin (1 U/ml), was passed through the hearts once. After a 10-min washout period, new medium with collagenase (0.7 mg/ml) was introduced, and the perfusate was allowed to recirculate until the aortic perfusion pressure decreased to below 40 mmHg (30-40 min). The ventricles were cut from the hearts, minced, and placed in fresh collagenase-containing perfusate. The tissue was shaken at 250 rpm in a water bath for 10 min. $CaCl_2$ (50 μM) was added to the minced tissue, and digestion with collagenase continued for an additional 10 min. The cells were dispersed, filtered through a double layer of cheesecloth, and diluted 1:4 with buffer containing 0.1% dialyzed bovine serum albumin. The resulting suspension was allowed to settle to separate myocytes (which are heavier) from EC. EC were further purified by centrifugation through cushions of 6% and 3% bovine serum albumin in Joklik's modified minimal essential medium. The endothelial identity of the collected cells was confirmed by the uptake of modified low-density lipoprotein.

Measurement of plasma glucose, arginine, and insulin. Plasma glucose concentration was determined by an enzymatic method involving hexokinase and glucose-6-phosphate dehydrogenase (63). Arginine levels in plasma were measured by HPLC (60). Insulin was analyzed using a radioimmunological assay kit from Linco Company (St. Louis, MO).

High-performance liquid chromatography analysis of BH_4 and arginine in freshly isolated EC. Cellular content of BH_4 was determined using a modification of the HPLC method of Fukushima and Nixon (64). EC were lysed in 0.3 ml of 0.1 M phosphoric acid containing 5mM dithioerythritol (an antioxidant) and 35ul of 2M trichloroacetic acid. Cell debris was removed by centrifugation. Extracts were oxidized with acidic or basic iodine. Acidic iodine oxidation quantitatively converts BH_4 and dihydrobiopterin to biopterin; basic iodine oxidation converts dihydrobiopterin to biopterin. Samples were incubated in the dark for 1 hr. Excess iodine was removed by adding ascorbic acid (final concentration 0.1M). The final solution was analyzed on a C18 reversed-phase column using fluorescence detection and authentic biopterin as a standard. The amount of BH_4 in the extracts was determined from the difference in biopterin concentrations generated with acidic and basic iodine oxidation. Arginine levels in EC were measured by HPLC (60). Intracellular concentrations of BH_4 and arginine were calculated on the basis of the average cell volume of rat coronary EC (0.348 $\mu L/10^6$ cells).

NO synthesis in freshly isolated EC. EC were rinsed with the Basal Medium Eagle, and then incubated at 37°C for 6 hours in Basal Medium Eagle containing 0.2

mM L-arginine, 0.5 mM L-glutamine, 5 mM D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. At the end of a 6-h incubation period, media were analyzed for nitrite and nitrate (two major stable end products of NO oxidation) by HPLC as described by Li et al (65). In all experiments, media incubated without cells were run as blanks.

Data analysis. Results are expressed as mean ± SEM. Data were statistically analyzed by unpaired t-test or analysis of variance for repeated measurements using SAS (Statistical Analysis System). Probability values less than 0.05 were taken to indicate significance.

Results

Energy food and water intake. Food and water consumption were measured every 24 hours. **Table 3.3** summarizes the data on feed intake and water intake of ZDF rats. Food intake was matched for L-arginine-treated and alanine-treated rats, to make their diets isonitrogenous. Arginine and alanine intake from drinking water **(Table 3.4)** were also adjusted daily to ensure isonitrogenous provision of nitrogen per kg body weight. **Table 3.5** summarizes the data on arginine and alanine intake from diet. Energy intake **(Table 3.3)** was calculated from feed intake and water consumption for both groups of ZDF rats.

TABLE 3.3

Feed, water and calorie intake by ZDF rats

Treatment	Feed (g/kg body wt/d)	Water (ml/kg body wt/d)	Gross Calorie † (kJ/kg body wt/d)
Alanine	99.0 ± 1.0	423 ± 24	1908 ± 18
Arginine	100.5 ± 1.9	472 ± 25	1894 ± 33

Data are means ± SEM, n = 4 for alanine group and n = 5 for arginine group.

† Including diet and drinking water.

TABLE 3.4

Intake of arginine and alanine from drinking water (g/kg body wt/day) by ZDF rats

Treatment	Arginine from drinking water	Alanine from drinking water
Alanine	----	10.7 ± 0.59
Arginine	5.7 ± 0.28	----

Data are means ± SEM, n = 4 for alanine group and n = 5 for arginine group.

TABLE 3.5

Intake of arginine and alanine from diet (g/kg body wt/day) by ZDF rats

Treatment	Arginine from diet	Alanine from diet
Alanine	1.43 ± 0.015	1.38 ± 0.014
Arginine	1.44 ± 0.026	1.39 ± 0.025

Data are means ± SEM, n = 4 for alanine group and n = 5 for arginine group.

Body and tissue weights. Experiment with ZDF rats was started at 9 weeks of age. Rat weights were recorded daily. **Figure 3.1** shows the change in body weights of ZDF rats receiving dietary supplementation of L-arginine or alanine for 10 weeks. A significant decrease in body weight gain **(Table 3.6)** was observed ($P<0.01$) in arginine-treated ZDF rats from week 4 post dietary supplementation. Data on tissue weights of animals at week 10 after initiating arginine supplementation are summarized in **Table 3.7**. Results indicate a marked decrease in epididymal fat (~30%, $P<0.05$) and abdominal fat (~43%, $P<0.01$) in arginine-supplemented rats, compared with control rats. Except for liver and pancreas, no significant differences were found in the weights of all other non-fat tissues examined ($P>0.05$). The ratio of tissue weight **(Table 3.8)** to whole body weight indicates a marked decrease (43%, $P<0.01$) in abdominal fat and a small decrease ($P<0.05$) in pancreas in arginine-supplemented rats, compared with control rats. There was a significant increase ($P<0.05$) in EDL muscle, soleus muscle, and brain weights in arginine-supplemented rats, compared with control rats. However, for other non-fat tissues examined, the ratio of tissue weight to the whole body weight did not differ ($P>0.05$) between control and arginine treated ZDF rats.

TABLE 3.6

Body weights of ZDF rats (g)

Week	Alanine group	Arginine group
0	338 ± 4.5	326 ± 8.8
1	361 ± 5.3	337 ± 12.1
2	375 ± 5.6	349 ± 12.8
3	386 ± 9.3	344 ± 17.2
4	368 ± 9.9	343 ± 15.3 *
5	397 ± 9.0	349 ± 16.3 *
6	447 ± 13.0	397 ± 15.6 *
7	409 ± 15.6	352 ± 18.0 *
8	404 ± 17.2	333 ± 16.1 *
9	404 ± 19.9	344 ± 13.3 *
10	411 ± 21.8	336 ± 13.0 *

Data are means ± SEM, n = 4 for alanine group and n = 5 for arginine group.

* $P < 0.05$ vs alanine treated ZDF rats group.

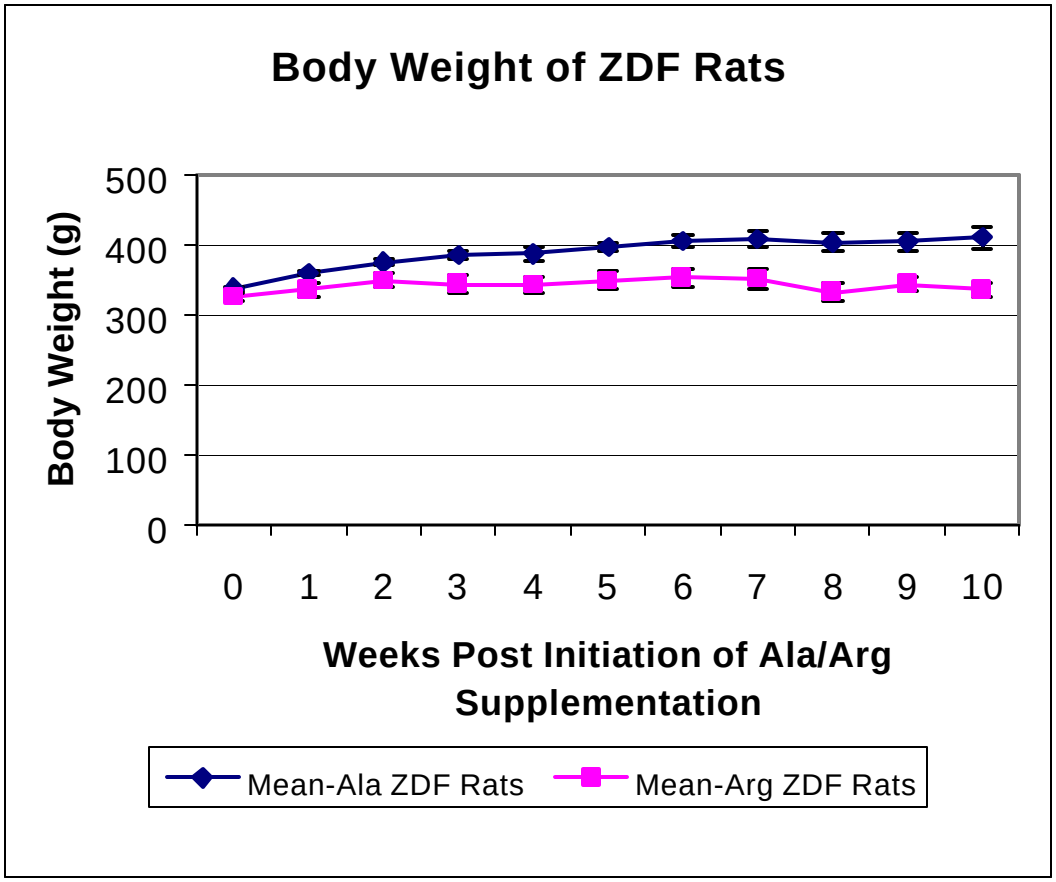


FIGURE 3.1 Changes in body weight of ZDF rats (Data are from **Table 3.5**).

TABLE 3.7

Tissue weight (g) of ZDF rats after 10-week arginine supplementation

Tissue	Treatment	
	Alanine	Arginine
Epdidymal fat	9.38 ± 0.69	6.56 ± 0.57 *
Abdominal fat	15.25 ± 1.34	8.64 ± 0.63 **
Liver	22.85 ± 1.30	17.75 ± 1.06 *
Pancreas	1.69 ± 0.05	1.14 ± 0.06 **
EDL Muscle †	0.12 ± 0.004	0.11 ± 0.005
Soleus Muscle	0.13 ± 0.008	0.12 ± 0.004
Small Intestine	14.28 ± 1.1	12.68 ± 0.35
Kidney	4.21 ± 0.08	3.95 ± 0.18
Brain	1.72 ± 0.05	1.69 ± 0.03
Heart	1.55 ± 0.12	1.47 ± 0.07
Lung	1.05 ± 0.11	1.28 ± 0.05
Spleen	0.62 ± 0.042	0.52 ± 0.032
Testes	2.63 ± 0.13	2.49 ± 0.13

Data are means ± SEM, n = 4 for alanine group and n = 5 for arginine group.

† EDL = Extensor Digitorium Longus Muscle. * * P < 0.01, * P < 0.05 by t-test.

TABLE 3.8

Ratio of tissue weight to whole body weight (g/kg body wt) of ZDF rats after 10-week arginine supplementation

Tissue	Treatment	
	Alanine	Arginine
Epdidymal fat	22.7± 0.97	19.6 ± 1.1
Abdominal fat	36.7 ± 1.5	25.6 ± 1.2 **
Liver	55.4 ± 2.03	53.4 ± 2.7
Pancreas	4.1 ± 0.17	3.4 ± 0.20 *
EDL Muscle †	0.29 ± 0.003	0.33 ± 0.01*
Soleus Muscle	0.31 ± 0.012	0.37 ± 0.004 *
Small Intestine	35.2 ± 4.5	38.4 ± 1.9
Kidney	10.2 ± 0.39	11.9 ± 0.58
Brain	4.2 ± 0.23	5.1 ± 0.08 *
Heart	3.7 ± 0.18	4.5 ± 0.29
Lung	3.5 ± 0.45	4.3 ± 0.10
Spleen	1.5 ± 0.05	1.6 ± 0.13
Testes	6.4 ± 0.52	7.6 ± 0.58

Data are means ± SEM, n=9. † EDL = E**x**tensor Digitorium Longus Muscle.

* * $P < 0.01$, * $P < 0.05$ by t-test.

Plasma glucose levels. Venous plasma was obtained from unanesthetized ZDF rats on weeks 3, 6 and 10 post-arginine supplementation for glucose analysis. Hyperglycemia (19-22 mM glucose) occurred in all ZDF rats. There was no significant difference ($P>0.05$) in plasma glucose levels **(Table 3.9)** between control and L-arginine-treated rats at any sampling day. This was contrary to the results obtained with the STZ-diabetic rat model.

Plasma insulin and arginine levels. At the conclusion of the study (10 week post-arginine supplementation), plasma arginine and insulin concentrations were measured. ZDF rats exhibited low levels of plasma insulin (normal physiological concentrations ~3-4 ng/ml) and plasma arginine (normal ~ 250-300 μM). Arginine supplementation increased ($P<0.01$) plasma arginine concentrations by ~ 274% compared with alanine-treated rats **(Table 3.10)**. However, plasma insulin levels did not differ ($P>0.05$) between alanine and arginine-treated rats.

TABLE 3.9

Plasma concentrations of glucose in 19-week-old ZDF rats after 10-week arginine supplementation

Treatment	Glucose (mM)		
	3 wk	6wk	10 wk
Alanine	19.55 ± 0.66	22.23 ± 0.59	19.52 ± 0.42
Arginine	20.41 ± 0.57	22.24 ± 0.33	18.76 ± 0.59

Data are means SEM, n = 4 for alanine group and n = 5 for arginine group.

TABLE 3.10

Plasma concentrations of arginine and insulin in 19-week-old ZDF rats after 10-week arginine supplementation

Treatment	Arginine (μM)	Insulin (ng/ml)
Alanine	206.9 ± 29.7	1.5 ± 0.3
Arginine	773.56 ± 81.8 *	1.2 ± 0.2

Data are means SEM, n = 4 for alanine group and n = 5 for arginine group.

* $P < 0.01$ by t-test.

Intracellular concentrations of arginine, BH_4, and NO production in rat coronary EC. BH_4 levels, arginine levels and NO production were greater ($P<0.01$) in EC from arginine-treated ZDF rats compared with alanine-treated rats **(Table 3.11)**. The relative increase in arginine concentration, BH_4 concentration, and NO production in EC from ZDF rats, post arginine supplementation, was similar to those for STZ-diabetic SD rats.

TABLE 3.11

Intracellular concentrations of arginine, BH_4 and NO production in coronary endothelial cells of 19-week-old ZDF rats after 10-week arginine supplementation

Rats	[Arginine] (mM)	[BH4] (μM)	NO production (pmol/10^6 cells/h)
Alanine	0.70 ± 0.03	1.12 ± 0.06	71 ± 6
Arginine	2.08 ± 0.10 *	2.47 ± 0.14 *	158 ± 9 *

Data are means SEM, n = 4 for alanine group and n = 5 for arginine group.

* $P < 0.01$ by t-test.

Discussion

This is the first study to determine the effects of dietary L-arginine supplementation on 1) body weight, plasma concentrations of glucose, insulin and arginine in ZDF rats, and 2) concentrations of arginine and BH_4, and NO synthesis in EC of ZDF rats. A novel, important finding of this work is that oral arginine administration to ZDF rats resulted in a significant decrease in epididymal fat (~30%) and abdominal fat (~43%) and in body weight. The reason for this observation is not clear at present. However, arginine or its product (NO) may inhibit lipogenesis and/or increase lipolysis. Importantly, others (30) have reported that a deficiency in the NO-cGMP-dependent pathway reduces mitochondrial biogenesis and promotes weight gain in mice, suggesting a critical role for NO in regulating body energy balance. By increasing NO synthesis, arginine supplementation may promote mitochondrial oxidation of fat in adipose tissue, liver and skeletal muscle, and thereby reducing body fat content. Except for small decreases in liver and pancreas weights, no significant difference was found in the weights of all other non-fat tissues examined. These results imply that the difference in body weights between control and arginine-treated ZDF rats is primarily due to changes in body fat. In addition, the finding suggests that adipose tissue is most sensitive to dietary arginine supplementation and perhaps to NO-mediated mitochondrial biogenesis. Future studies are necessary to determine body composition and energy expenditure in control and arginine-treated ZDF rats.

In contrast to the results found with SD rats, oral supplementation of L-arginine did not affect plasma glucose levels in ZDF rats compared with alanine-treated animals.

This can be due to excess fat in the body, the oxidation of which provides energy and spares glucose. Oxidation of fatty acids, which results in the production of acetyl-CoA, inhibits glycolysis and stimulates gluconeogenesis. Importantly, gluconeogenesis also depends on fatty acid oxidation for ATP provision. Although plasma triglycerides levels were not measured in this study, it is possible, that hydrolysis of high levels of triglycerides may provide glycerol for hepatic gluconeogenesis. In type-II diabetic subjects, insulin-sensitive tissues do not efficiently utilize glucose, which may contribute to the elevated levels of plasma glucose (19-22 mM) in ZDF rats. Interestingly, in contrast to STZ-diabetic rats, arginine did not increase plasma levels of insulin in ZDF rats. This finding may be due to a lack of stimulation of insulin release from pancreatic beta cells of ZDF rats. Plasma arginine levels were increased by arginine supplementation to a greater extent in ZDF rats compared with STZ-diabetic rats. This result may be explained by greater intake of arginine from diet and drinking water in ZDF rats.

As in STZ-diabetic rats, dietary supplementation of L-arginine increased NO production in coronary EC of ZDF rats. Importantly, we found that L-arginine increased endothelial BH_4 availability in ZDF rats. In both of these animal models of diabetes, the cellular mechanism for BH_4 synthesis and the regulation of this pathway appear to be intact, which allows for the response of EC to elevated concentrations of extracellular arginine. Thus, through an increase in BH_4 availability, administration of L-arginine restored the ability of EC to make NO in both type-I and type-II diabetes mellitus. Collectively, the results of this study support the hypothesis that dietary L-arginine

supplementation increases the availability of BH_4 for NO synthesis in EC of diabetic rats, thereby preventing endothelial dysfunction.

In conclusion, oral administration of arginine markedly increased endothelial BH_4 availability and NO synthesis, and reduced abdominal and epididymal fat in ZDF arts. The weight–reducing effect of arginine may be mediated by NO, an activator of mitochondrial biogenesis. Arginine may be a novel anti-obesity nutrient, which has important implications for the prevention and treatment of obese, and diabetic patients.

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSION

Arginine is Beneficial for Diabetic Rats

L-arginine is a versatile amino acid in animal and human cells, serving as a precursor for the synthesis of not only proteins but also NO, creatine, agmatine, proline, polyamines, and other molecules involved in regulating cellular homeostatsis. L-Arginine exerts favorable effects in humans with a number of atherosclerosis risk factors such as hypercholesterolemia, hypertension, smoking, and diabetes. In view of conflicting findings in the literature regarding the effect of arginine on endothelial function in diabetes (71), the present study was conducted to determine the effects of arginine in STZ-diabetic and ZDF rats at cellular, tissue and whole body levels. Results of this thesis work provide evidence for the beneficial effects of dietary intervention with L-arginine in both type-I and type-II diabetic subjects.

An exciting finding of this work is that oral supplementation of L-arginine to STZ-induced diabetic rats prevented hyperglycemia. A daily dose of L-arginine-HCl (equivalent to 0.5 % arginine) in drinking water markedly decreased glucose levels in diabetic rats by ~54%. Hence, L-arginine possesses antihyperglycemic properties, likely due to an NO-mediated increase in blood flow, glucose uptake by skeletal muscle, and tissue insulin sensitivity. This observation is in agreement with recent data of Mendez and Balderas (32), who reported beneficial effects of L-arginine administration on reducing serum glucose levels. Further, Mohan and Das (72) demonstrated that L-arginine and NO prevented beta-cell damage and the severe effects of diabetes. Also,

Popov et al. (66) reported that oral administration of 622 mg arginine/kg body weight to hamsters diminished the high concentrations of circulating glucose by 63%. However, arginine supplementation for 10 weeks is not effective in reducing plasma glucose or increasing plasma insulin levels in ZDF rats. This could be due to excess fat in the body, and hence the body uses lipid oxidation for providing energy and spares glucose. Oxidation of fatty acids, which result in the production of acetyl-CoA, inhibits glycolysis and stimulates gluconeogenesis. At the end of a 10-week period of arginine supplementation, there remains a substantial amount of abdominal and epididymal fat in ZDF rats. We expect that when a period of arginine supplementation is extended beyond 10 weeks to deplete more body fat, arginine treatment may be able to reduce plasma levels of glucose. Further studies are required to test this hypothesis.

The beneficial effect of L-arginine administration has been thought to result from an increase in insulin secretion by pancreatic ß-cells that are not destroyed after STZ injection (73). Consistent with this view, L-arginine increases the secretion of insulin from beta cells of the pancreas (23,36). Additionally, NO synthesis from arginine is known to mediate insulin release from pancreatic cells in the presence of glucose (32). Accordingly, arginine supplementation increased plasma insulin levels in both diabetic (by ~209%) and non-diabetic (by ~44%) STZ-SD animals. Interestingly, L-arginine supplementation had no effect on plasma insulin levels in ZDF rats, probably due to a lack of stimulation of insulin release from pancreatic ß-cells in this type-II diabetic animal model. Another possible reason may be age, as ZDF rats and STZ-diabetic rats

were 19 and 12 weeks old, respectively. In older animals, insulin release from ß-cells may not be sensitive to arginine stimulation.

Another striking finding of this study is that L-arginine treatment reduced the weight loss of STZ-diabetic rats. SD diabetic rats treated with L-arginine had 36% less weight loss than alanine-treated diabetic rats. L-arginine supplementation did not affect the weight gain, food and water intake in normal, non-diabetic rats. Thus, through NO synthesis, arginine may improve the efficiency of utilization of dietary nutrients in type-I diabetic rats. It is possible that an increase in plasma insulin levels in STZ-diabetic rats stimulates protein synthesis and inhibits protein degradation in skeletal muscle and the whole body.

The third novel, important finding of this study is that L-arginine supplementation promoted a significant decrease in body weight gain by ZDF rats, compared with alanine-treated rats. The reason for this finding is not clear at present. However, arginine or its product (NO) may inhibit lipogenesis and/or increase lipolysis **(Fig. 4.1)**. In this regard, it is noteworthy that Nisoli et al (30) reported that the NO-cGMP-dependent pathway controls mitochondrial biogenesis and body energy balance. NOS null-mutant mice had a reduced metabolic rate and accelerated weight gain. In our study, there were marked decreases in epididymal fat (~30%) and abdominal fat (~43%) in arginine-supplemented ZDF rats as compared with the alanine-treated rats. Thus, arginine may be a novel anti-obesity nutrient. Future studies are required to quantify body composition and whole body energy expenditure in control and arginine-treated ZDF rats

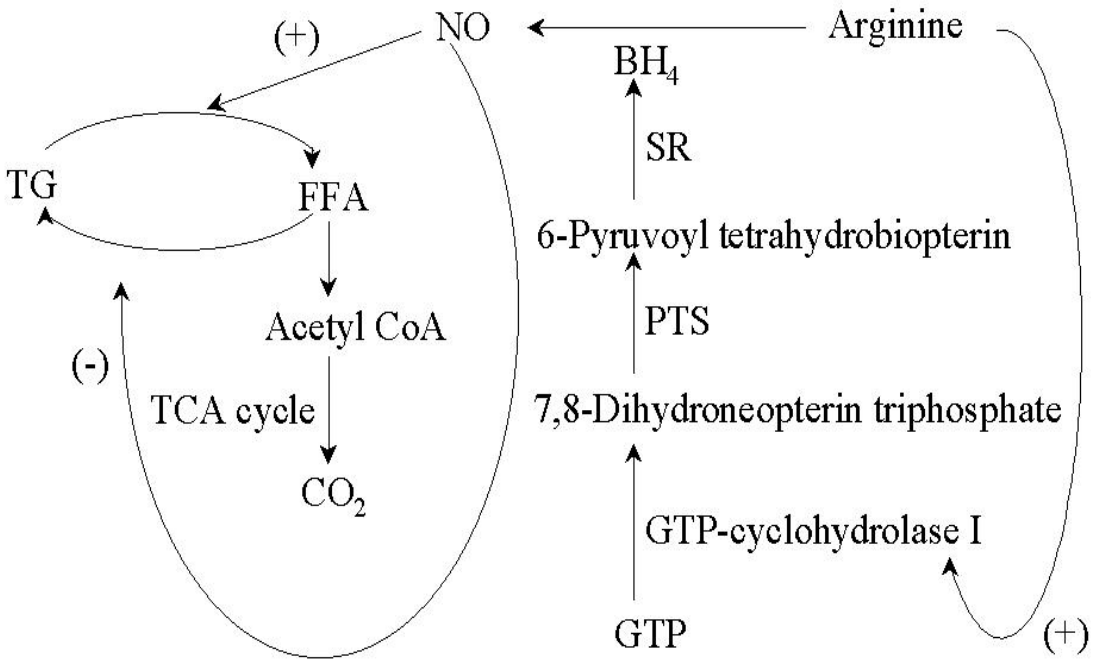


FIGURE 4.1 Role of arginine and NO in lipid metabolism. Abbreviations: TG, triglycerides; FFA, free fatty acids; GTP, guanosine tri phosphate; NO, nitric oxide; BH_4, (6R)- 5,6,7,8-tetrahydro-L- biopterin; SR, Sepiapterin reductase; PTS, 6-Pyruvoyl tetrahydrobiopterin synthase. The sign (+) denotes activation of the reaction and the sign (-) denotes inhibition of the reaction.

Induction of diabetes with STZ injection in Sprague Dawley rats resulted in decreased levels of plasma [arginine] (67). Results of this study also indicated a decrease in plasma arginine levels in control ZDF rats. Impaired arginine synthesis and/or increased arginine degradation may contribute to low levels of circulating arginine in diabetic rats. Whatever the mechanism, arginine supplementation was effective to increase plasma [arginine] in both rat models of diabetes. Similarly, other researchers have reported decreases in plasma concentrations of arginine in experimental diabetic animals (10,11,12) and in diabetic humans (21,22). Thus, reduced plasma levels of arginine in diabetic subjects provide a metabolic basis for dietary arginine supplementation to diabetics.

The "arginine paradox" has been a major conundrum in the NO research field over the past decade. Intracellular values of L-arginine (0.49 mM in SD rats; 0.70 mM in ZDF rats) in control diabetic rats were ~150 to 250 fold higher than the Km value of eNOS (2.9 μM) for L-arginine. Thus, on the basis of kinetics of the purified NOS in the test tube, an increase in intracellular or extracellular concentration of L-arginine would not provide more substrate for NO synthesis because eNOS should be saturated with arginine at levels of 0.49 or 0.70 mM. However, interactions of cellular substrates and cofactors with an enzyme in cells are likely more complex than *in vitro* enzyme reactions would indicate. Findings of this study provide the first line of evidence showing that dietary supplementation of L-arginine increases NO production in coronary EC of both STZ-diabetic SD rats and Zucker rats. This data further supports the notion that extracellular arginine concentrations are critical for endothelial NO production.

A central hypothesis of this thesis work is that dietary L-arginine supplementation increases the availability of BH_4 for NO synthesis in EC of diabetic rats, thereby preventing endothelial dysfunction. Results of the present study confirm and extend the earlier findings from our laboratory that STZ-diabetic rats and ZDF rats exhibit low levels of BH_4 in EC. To our knowledge, this is the first report of the effect of dietary arginine supplementation on increasing endothelial BH_4 availability in both STZ-diabetic SD rats and ZDF rats. Meininger et al. (13) reported that EC from the spontaneously diabetic BB rat have a deficiency of BH_4 and replenishing BH_4 levels in the EC from diabetic animals restores their ability to make NO. As a result of an increase in BH_4 availability, administration of L-arginine restored the ability of EC to make NO in both STZ and ZDF diabetic rat models. Collectively, our results demonstrate that L-arginine improves endothelial function in diabetic subjects through enhancing the availability of BH_4 for NO synthesis by eNOS.

Our findings with diabetic rats may have important implications for reducing body fat in both farm animals and humans. For example, dietary arginine supplementation may provide a means to decrease abdominal, back and intramuscular fat in pigs and poultry, thereby increasing lean tissue content. Likewise, dietary arginine supplementation may be extremely beneficial for obese patients. Obesity is a major public health problem in the U.S. and worldwide. Recent data show that 31 % and 65 % of the U.S. adult population are obese and overweight, respectively (74). Children and adolescents have not been immune to this epidemic, as 15 % of them are obese (74). Worldwide, more than 300 million adults are obese and over one billion are overweight

(74). Obesity is a major risk factor for such life-threatening diseases as type-II diabetes, atherosclerosis, hypertension, and some types of cancer (including colon and breast cancers). Consequently, obesity claims an increasing number of lives and contributes to tremendous costs of medical care. In the U.S. alone, about 300,000 people die of obesity-related diseases every year; incidence of type-II diabetes among children has increased 10-fold, and obesity accounts for 6-8 % of all health care expenditures (74,75). Unfortunately, clinicians have few tools to fight the obesity epidemic, because current antiobesity drugs are not highly effective and are fraught with side effects (76). Identifying arginine supplementation as a new means to reduce body fat will be extremely beneficial.

Summary

This research focuses on the beneficial roles of dietary L-arginine supplementation in diabetic rats. Specifically, we evaluated the effects of dietary L-arginine intervention on 1) body weight, 2) plasma concentrations of glucose, insulin and arginine, 3) concentrations of arginine and BH_4 in EC, and 4) NO synthesis in EC in two rat models of diabetes: STZ-diabetic rats (an animal model for type-I diabetes) and ZDF rats (an animal for type-II diabetes). Our results demonstrate that oral administration of arginine is beneficial for both STZ and ZDF diabetic rats and has no adverse effects in non-diabetic rats. Dietary arginine supplementation increased plasma concentrations of arginine as well as concentrations of arginine and BH_4 in EC, and NO synthesis in EC, in both STZ-diabetic and non-diabetic SD rats, and in ZDF rats.

Additionally, arginine supplementation ameliorates weight loss in STZ diabetic rats, and promotes weight loss in ZDF rats. Thus, we expect that dietary arginine supplementation will be beneficial for diabetic subjects.

The key findings of the present work are as follows:

1. In STZ-diabetic SD rats, L-arginine supplementation for 2 weeks increased plasma concentrations of arginine (110%), plasma concentrations of insulin (209%), and arginine concentrations (173%), BH_4 concentrations (128%), and NO synthesis (125%) in EC, compared with alanine-treated rats.
2. In STZ-diabetic SD rats, L-arginine supplementation reduced body weight loss (36%) and prevented hyperglycemia.
3. In non-diabetic SD rats, L-arginine treatment increased plasma concentrations of arginine (144%), plasma concentrations of insulin (44%), and arginine concentrations (88%), BH_4 concentrations (106%) and EC NO synthesis (80%) in EC, compared with alanine-treated rats.
4. Arginine supplementation for 10 weeks did not affect plasma levels of glucose and insulin in ZDF rats, compared with alanine-treated ZDF rats.
5. In ZDF rats, supplementation with L-arginine for 10 weeks reduced epididymal fat (30%), abdominal fat (43%) and body weight gain (18%).
6. Treatment with L-arginine in ZDF rats increased plasma concentrations of arginine (273%), and arginine concentration (197%), BH_4 concentrations (120%) and NO production (122%) in EC by more than two folds, as compared with alanine-treated ZDF rats.

Results of this thesis work may have important implications for both animal agriculture and human health. Future studies are necessary to determine whether dietary arginine supplementation can reduce body fat in farm animals (e.g. pigs and chicks), companion animals (e.g. dogs and cats), and obese humans.

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