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ARGINASE INHIBITION PREVENTS THE DEVELOPMENT OF HYPERTENSION AND IMPROVES INSULIN RESISTANCE IN OBESE RATS

Kelly J. Peyton*, Xiao-ming Liu*, Ahmad R. Shebib*, Fruzsina K. Johnson[†], Robert A. Johnson[†], and William Durante*

*Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO

[†]College of Osteopathic Medicine, William Cary University, Hattiesburg, MS

Abstract

This study investigated the temporal activation of arginase in obese Zucker rats (ZR) and determined if arginase inhibition prevents the development of hypertension and improves insulin resistance in these animals. Arginase activity, plasma arginine and nitric oxide (NO) concentration, blood pressure, and insulin resistance were measured in lean and obese animals. There was a chronological increase in vascular and plasma arginase activity in obese ZR beginning at 8 weeks of age. The increase in arginase activity in obese animals was associated with a decrease in insulin sensitivity and circulating levels of arginine and NO. The rise in arginase activity also preceded the increase in blood pressure in obese ZR detected at 12 weeks of age. Chronic treatment of 8 week old obese animals with an arginase inhibitor or L-arginine for 4 weeks prevented the development of hypertension and improved plasma concentrations of arginine and NO. Arginase inhibition also improved insulin sensitivity in obese ZR while L-arginine supplementation had no effect. In conclusion, arginase inhibition prevents the development of hypertension and improves insulin sensitivity while L-arginine administration only mitigates hypertension in obese animals. Arginase represents a promising therapeutic target in ameliorating obesity-associated vascular and metabolic dysfunction.

Keywords

arginine; arginase; obesity; hypertension

Correspondence: William Durante, Ph.D., Department of Medical Pharmacology and Physiology, School of Medicine, University of Missouri-Columbia, M409 Medical Sciences Building, One Hospital Drive, Columbia, MO 65212, Tel: 573-882-3886, Fax: 573-884-4276, durantew@health.missouri.edu.

Conflict of Interest: The authors declare that they have no conflict of interest

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The article does not contain any studies with human participants performed by any of the authors, thus no informed consent was required in the study.

Introduction

Obesity is a serious public health problem in the United States that affects nearly 40% of the adult population (Flegal et al. 2016). Enhanced caloric consumption combined with diminished physical activity is precipitating an increasing prevalence of obesity in modern societies. This rising obesity epidemic is fueling a battery of human diseases. Obesity is a major risk factor for the development of diabetes, dyslipidemia, cancer, arthritis, and numerous cardiovascular disorders, including hypertension (Malnick and Knobler 2006). Epidemiological data strongly support the relationship between obesity and hypertension in both adults and children. Risk estimates from the Framingham Heart Study indicate that approximately 78% and 65% of the cases of hypertension in men and women, respectively, are directly attributable to excess weight gain (Garrison et al. 1987). Moreover, the combination of obesity and hypertension is a major contributor to cardiovascular morbidity and mortality, underscoring the clinical significance of obesity-related hypertension and the need to prevent its occurrence (Landsberg et al. 2013).

While numerous pathogenic stimuli have been implicated in obesity-associated hypertension, endothelial dysfunction exemplified by reduced bioavailability of nitric oxide (NO) is a critical determinant that links obesity to the rise in blood pressure. Endothelial dysfunction and diminished NO bioavailability precede increases in blood pressure in overweight mice fed a high fat, high sucrose diet (Weisbrod et al. 2013). Furthermore, clinical studies have shown that serum nitrite and nitrate, an index of systemic NO bioavailability, is attenuated in juvenile obese subjects with elevated systolic arterial blood pressure when compared to age- and gender-matched normal weight controls (Gruber et al. 2008). Circulating NO is also reduced in obese hypertensive subjects relative to obese normotensive counterparts (Rajapakse et al. 2014). In addition, whole body NO synthesis is compromised in obese hypertensive patients relative to lean control subjects (Siervo and Bluck 2012). Moreover, a significant negative correlation between plasma nitrite/nitrate and mean arterial pressure has been documented (Rajapakse et al. 2014). Reductions in NO bioavailability can promote the development of hypertension via multiple mechanisms. In particular, loss of NO stimulates arterial stiffness, the activation of the renin-angiotensin-aldosterone system, renal salt retention, and the activation of the sympathetic nervous system which can function in a concerted manner to elevate arterial blood pressure (Rajapakse et al. 2016).

Emerging studies have identified the enzyme arginase, which hydrolyzes arginine to ornithine and urea, as a critical regulator of NO synthesis by competing with endothelial NO synthase (eNOS) for substrate L-arginine (Durante et al. 2007; Durante 2013; Pernow and Jung 2013). Arginase-mediated impairments in NO bioavailability have been demonstrated in patients and animal with diabetes, hypercholesterolemia, atherosclerosis, hypertension, and heart failure (Durante et al. 2007; Durante 2013; Pernow and Jung 2013). In addition, arginase compromises NO production in blood vessels obtained from morbidly obese humans (El Assar et al. 2016). Moreover, we recently reported that vascular arginase activity is upregulated in obese animals and that it impairs NO-mediated arterial vasodilation in obese Zucker rats (ZR), a well-established and highly reproducible genetic model of obesity (Johnson et al. 2015). Due to a non-functional leptin receptor and the resulting hyperphagia,

these animals exhibit the seminal characteristics of human obesity, including hypertension and insulin resistance (Johnson et al. 2015; Johnson et al. 2015). Remarkably, we also found that administration of arginase inhibitors normalizes arterial blood pressure in hypertensive obese Zucker rats, suggesting a prominent role for arginase in obesity-related hypertension (Zucker 1965). Given the potential importance of arginase in modulating blood pressure in obesity, the present study determined the temporal expression of vascular and plasma arginase activity, and the concentration of circulating arginine and NO in obese ZR. In addition, we investigated if arginase inhibition or arginine supplementation could prevent the development of hypertension in obese ZR and improve insulin resistance in these animals.

Methods

Materials

Arginine, heparin, acetylcholine, EDTA, sulfanilamide, H₃PO₄, naphthyl ethylenediamine dihydrochloride, and trichloroacetic acid (TCA) were from Sigma (St. Louis, MO); N^ω-hydroxy-nor-L-arginine (OHNA) was purchased from EMD Biosciences (San Diego, CA); L-[guanido-¹⁴C]Arginine (52 mCi/mmol) was from Amersham (Arlington Heights, IL). All other reagents were from Fisher Scientific (Houston, TX).

Animals

Male obese (fa/fa) and lean (Fa/?) ZR were obtained from Charles River Laboratories (Wilmington, MA) and used for experiments between 4–12 weeks of age. Animals were housed under controlled temperature and humidity, a 12 hour on/off light cycle, with *ad libitum* access to water and standard rodent chow (Harlan Teklad, Madison, WI). In some instances, 8 week old obese Zucker rats received a continuous infusion of the arginase inhibitor OHNA (25mg/kg/day, ip) or isotonic saline via implanted osmotic pumps (ALZA Corporation, Palo Alto, CA) for 4 weeks. Alternatively, another group of animals received L-arginine (20g/L) in the drinking water for 4 weeks.

Blood Chemistry and Tissue Collection

Rats were weighed, anesthetized with isoflurane, and femoral arterial catheters implanted for blood sampling. Fasting blood was collected in tubes containing EDTA and plasma obtained by centrifugation and stored at –80°C. Plasma levels of insulin were determined using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI) and glucose concentrations measured using the glucose oxidase method. Circulating levels of arginine were quantified by ion exchange chromatography, as previously described (Durante et al. 1996). For total NOx (nitrite + nitrate) measurements, plasma was deproteinized with 10% TCA and samples centrifuged at 14,000rpm for 20 minutes to remove precipitated protein. The resulting supernatant was incubated with nitrate reductase and NO oxidation products measured as we previously described (Durante et al. 1993). Following blood collection, animals were heparinized (1000U/kg, iv) and the thoracic aorta removed and stored at –80°C.

Insulin Sensitivity

Insulin sensitivity was determined using the convenient and clinically employed homeostasis model assessment of insulin resistance (HOMA-IR) calculated as [fasting insulin

(microunits/ml) x fasting glucose (mg/dL)]/405, that we have successfully used in another rodent model of metabolic syndrome (Manrique et al. 2011).

Arginase Assay

Vascular arginase activity was determined by monitoring the generation of [^{14}C]urea from L-[*guanido*- ^{14}C]arginine, as we previously described (Johnson et al. 2015). For plasma arginase measurement, plasma samples were depleted of urea using Centricon YM10 filters (Amicon Inc, Beverly, MA) and subsequent urea production from exogenously administered arginine measured by absorption spectroscopy, using a commercially available kit (QuantiChromTM Arginase Assay Kit, BioAssay Systems, Hayward, CA).

Blood Pressure Measurements in Awake Animals

Animals were anesthetized with isoflurane and fitted with femoral arterial catheters (14). Following a three-day recovery interval, inline blood pressure was measured in awake animals using a pressure transducer (TSD 104A, Biopac Systems, Santa Clara, CA) coupled to a polygraph (Biopac Systems, Santa Clara, CA) and a personal computer (Biopac Systems, Santa Clara, CA).

Data Analysis

Results are expressed as the means $\pm$ SEM. Statistical differences between groups were evaluated with a Student's two-tailed t-test or by ANOVA with the Tukey post hoc test when more than two treatment regimens were compared. A value of $P < 0.05$ was considered statistically significant.

Results

There was a progressive increase in body weight in rats between 4 and 12 weeks of age (Figure 1A). However, body weight was significantly greater in obese ZR at 8 and 12 weeks of age relative to lean animals. While whole body insulin sensitivity remained relatively constant between 4 and 12 weeks of age in lean ZR, an age-dependent increase in insulin resistance was noted in obese ZR. Insulin sensitivity between the two strains of mice was equal at 4 weeks of age but a significant reduction in insulin sensitivity was observed in 8 and 12-week old obese ZR compared to lean ZR (Figure 1B). A similar pattern was evident when arginase activity was monitored. Both vascular and plasma arginase activity remained unchanged in lean obese ZR between 4 and 12 weeks of age, however, an age-related increase in vascular and plasma arginase activity was detected in obese ZR beginning at 8 weeks of age (Figures 2A and B). Vascular and plasma arginase activity was significantly greater in 8 and 12 but not 4 week old obese ZR relative to lean animals.

Since plasma arginase activity was elevated in obese ZR, we examined whether circulating levels of its substrate, arginine, were compromised in these animals. Indeed, there was a significant decline in plasma arginine in 8 and 12 week old obese ZR that paralleled the rise in plasma arginase activity in these animals compared to lean controls (Figure 3A). As arginase competes with NO synthase for substrate arginine, we also examined if circulating levels of NO were adversely affected in older obese ZR. In fact, a significantly lower

concentration of plasma NO was observed in both 8 and 12 week old obese ZR relative to lean ZR (Figure 3B). Mean arterial blood pressure was unaffected by aging in lean ZR and was similar to that observed in 4 and 8 week old obese ZR (Figure 4). However, a significant increase in mean arterial pressure was detected in obese ZR at 12 weeks of age.

Given that increases in arginase activity precede the development of hypertension in obese ZR, we determined if arginase was responsible for increasing blood pressure in these animals. Notably, intraperitoneal infusion of the arginase inhibitor OHNA to 8 week old obese ZR for 4 weeks prevented the development of hypertension (Figure 5C). Similarly, administration of L-arginine in the drinking water blocked the rise in blood pressure in obese ZR. The antihypertensive action of OHNA and L-arginine was associated with a significant increase in plasma arginine and NO in obese ZR (Figures 5A and B). Finally, while L-arginine administration had no effect on body weight or insulin resistance in obese ZR, a significant decline in body weight and insulin resistance was noted in animals treated with OHNA (Figure 5D and E).

Discussion

The present study demonstrates that arginase plays an essential role in promoting hypertension and insulin resistance in obesity. We identified an age-dependent increase in arginase activity in blood vessels and plasma of obese ZR that is paralleled by a decline in whole body insulin sensitivity, plasma arginine, and plasma NO concentration. We also discovered that increases in arginase activity precede the development of hypertension in obese ZR. Moreover, we found that chronic administration of an arginase inhibitor or L-arginine to pre-hypertensive obese ZR prevents the increase in blood pressure in these animals and this is associated with improvements in circulating arginine and NO. Furthermore, arginase inhibition, but not L-arginine supplementation, enhances insulin sensitivity in obese ZR. These findings indicate that the induction of arginase activity in obesity contributes to hypertension by curbing arginine availability for NO synthesis and insulin resistance via a NO-independent pathway.

We found that arginase activity increases in blood vessels and plasma of obese ZR beginning at 8 weeks of age. The rise in vascular arginase activity is likely mediated by both arginase I and II since we previously reported that both isoforms are upregulated in the vasculature of obese ZRs (Johnson et al. 2015). The underlying mechanism by which arginase activity is upregulated in the vessel wall of obese animals is not known. However, multiple factors may be involved. In an earlier study (Johnson et al. 2015), we detected increases in circulating tumor necrosis factor- α , oxidized low-density lipoprotein, and insulin in obese ZR. It is possible that all three of these agents may elevate arterial arginase activity in our obese animals since they are known to stimulate arginase expression in vascular cells (Giri et al. 2012; Ryoo et al. 2006; Gao et al. 2007). We also found that plasma arginase activity, which arises from arginase I (Morris 2007), is increased in a similar age-dependent fashion in obese ZR. While the cellular source of arginase 1 responsible for the elevated plasma arginase activity remains uncertain, the extremely high concentration of arginase I in the liver and the presence of age-related hepatic injury in obese ZR make this organ a likely source of the elevated plasma arginase I in obese animals (Sun et al. 2001). However, as

arginase I is augmented in monocytes of overweight adult subjects these cells may also contribute to the rise in plasma arginase activity (Erdely et al. 2010). Consistent with our findings, plasma arginase activity or expression is also upregulated in mice fed a lipid-enriched or a high fat-high sucrose diet and in obese subjects (Erdely et al. 2010; Kim et al. 2012; Jung et al. 2014; Bhatta et al. 2017). Interestingly, arginine transport is also downregulated in obese mice and humans (Rajapakse et al. 2014; Assumpcao et al. 2016), raising the possibility that obesity impairs NO synthesis by targeting both the uptake and catabolism of arginine.

We also found a significant decrease in plasma arginine and NO in 8 and 12 week old obese ZR relative to age-matched lean control animals. A decrease in arginine and NO bioavailability has also been observed in dietary animal models of obesity and in obese subjects (Gruber et al. 2008; Rajapakse et al. 2014; Sailer et al. 2013; She et al. 2007), suggesting a generalized disruption of the arginine-NO signaling pathway in obesity. We previously demonstrated that the decrease in plasma arginine in obese ZR is paralleled by a corresponding increase in circulating ornithine, which is consistent with the increase in plasma arginase activity observed in these animals (Johnson et al. 2015). Furthermore, we showed that the decline in plasma arginine is mediated by increases in plasma arginase activity since it was corrected by pharmacological blockade of arginase (Johnson et al. 2015). Importantly, the reduction in circulating arginine by arginase likely mediates the decrease in circulating NO detected in our obese animals. In support of this notion, decreases in plasma arginine and NO occur in parallel in obese ZR. In addition, oral administration of arginine or arginase inhibition restores the circulating concentration of both arginine and NO in obese ZR. Moreover, a recent population-based study found a positive correlation between arginine intake and serum NO in overweight and obese subjects (Mirmiran et al. 2016), highlighting the intimate association between these two molecules in obesity.

We also show that arginase activity is required for the development of hypertension in obesity. Increases in both vascular and plasma arginase activity are observed in obese ZR prior to the rise in arterial blood pressure. Notably, chronic administration of the arginase inhibitor OHNA to pre-hypertensive obese animals negates the increase in blood pressure in these animals and this is associated with a significant increase in plasma arginine and NO. Similar effects are also noted following the oral administration of L-arginine to pre-hypertensive obese ZR. Collectively, these data indicate that increases in arginase activity in obesity promote the development of hypertension by restricting NO synthesis via the depletion of arginine. In addition, they suggest that serum arginase activity may serve as an early biomarker for the prompt diagnosis and prevention of obesity-related hypertension. The ability of arginase to promote hypertension in obesity is not unique to our genetic model as a recent study found that arginase mediates the increase in systolic blood pressure in a dietary mouse model of obesity (Bhatta et al. 2017). Notably, both studies employed male rodents. Since sex differences in cardiovascular disease have been reported in obesity (Raizi et al. 2007; Nedungadi and Clegg, 2009; Bohm et al. 2013; Manrique et al. 2013), it will be important to extend our current findings with arginase to female rodents. Interestingly, arginase has also been implicated in mediating arterial hypertension in animal models of diabetes, insulin resistance, sickle cell anemia, and essential hypertension, underscoring the

detrimental role of this enzyme in a multitude of hypertensive states (El Bassossy et al. 2012; El Bassissy et al. 2013; Steppan et al. 2016; Bagnost et al. 2008).

Significantly, arginase contributes to the development of insulin resistance in obesity. We found that the chronic delivery of OHNA reduces whole body insulin resistance in obese ZR and this may reflect the small, but significant, decrease in body weight. In addition, the ability of OHNA to correct hepatic lipid abnormalities and adipose tissue inflammation may mediate the improvement in metabolic function in obese animals (Moon et al. 2014; Hu et al. 2015). Our results are in-line with previous work showing that arginase inhibition improves insulin resistance in fructose-fed rats and decreases body weight in high fat-fed mice (El Bassossy et al. 2012; Hu et al. 2015). Surprisingly, L-arginine administration fails to ameliorate insulin resistance or body weight in obese ZR despite studies showing that L-arginine restores insulin and/or glucose sensitivity in other animal models of obesity as well as obese patients (Fu et al. 2005; Jobgen et al. 2009; Monti et al. 2012). Several potential mechanisms may mediate the beneficial metabolic actions of L-arginine including a reduction of white adipose tissue, increased brown adipocyte development, enhanced energy expenditure, and augmented lipolytic enzyme activity and fatty acid oxidation (McNeal et al. 2016). However, the positive effect of L-arginine supplementation on insulin resistance is not universally observed (Kawano et al. 2003; De Castro Barbosa et al. 2006). Failure of L-arginine to improve insulin resistance in our study may be related to the animal model under investigation, the short 4 week duration of L-arginine exposure, and/or the delayed administration of L-arginine until 8 weeks of age. The inability of L-arginine to restore insulin sensitivity suggests that arginase-mediated decreases in insulin resistance are not related to the impaired conversion of arginine to NO in obese ZR. Instead, the arginase-dependent synthesis of arginine metabolites such as polyamines and proline may contribute to insulin resistance in obesity. Alterations in polyamine concentration have been detected in various tissue compartments in obesity (Jamdar et al. 1996; Sjöholm et al. 2001; Ali et al. 2013), and intriguingly, circulating levels of polyamines and proline are elevated in obese subjects warranting further studies in this area (Codoner-Franch et al. 2011; Newgard et al. 2009).

Our study provides further support for the development of arginase inhibitors in clinical medicine. Highly potent and selective arginase inhibitors such as OHNA and boronic acid-based compounds were developed in the late-twentieth century and have been proven extremely useful in dissecting the role of arginase in various pathological states (Abdelkawy et al. 2017; Pudlo et al., 2017). Recently, small-scale clinical studies have illustrated the therapeutic potential of arginase inhibitors in human diseases such as hypertension, diabetes, heart failure, and hypercholesterolemia (Halowatz et al. 2007; Shemyakin et al. 2012; Quitter et al. 2013; Kovamees et al. 2016). The arginase inhibitors are well tolerated and show no reported toxicity with few non-specific actions (Reid et al. 2007; Huynh et al. 2009). In addition, the beneficial effects of the arginase inhibitors on cardiovascular and endocrine system have been recapitulated in arginase-deficient mice, providing confidence that these inhibitors mediate their effect by targeting arginase (Ryoo et al. 2008; Shatanawi et al. 2011; Ming et al. 2012; Bhatta et al. 2017). Current studies are aimed at improving the pharmacokinetic properties and isoform-selectivity of arginase inhibitors.

In conclusion, the present study identified an age-dependent increase in vascular and plasma arginase that precedes the development of hypertension in obese rats. The increase in arginase activity is associated with decreases in insulin sensitivity and circulating levels of arginine and NO. Importantly, chronic administration of an arginase inhibitor or L-arginine to pre-hypertensive obese animals prevents the development of hypertension and this is linked to an improvement in plasma arginine and NO. Moreover, we discovered that chronic treatment with an arginase inhibitor, but not L-arginine, attenuates insulin resistance in obese animals. These results establish arginase as a potential novel biomarker and therapeutic target in treating obesity-related vascular and metabolic disease.

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Fig. 1. Age-dependent changes in body weight (A) and insulin resistance (HOMA-IR) (B) in lean and obese ZR. Results are means $\pm$ SEM (n=6). *Statistically significant increase in obese relative to lean ZR.

Fig. 2. Age-dependent changes in vascular (A) and plasma (B) arginase activity in lean and obese ZR. Results are means $\pm$ SEM (n=6). *Statistically significant increase in obese relative to lean ZR.

Fig. 3. Age-dependent changes in plasma arginine (A) and NO (B) in lean and obese ZR. Results are means $\pm$ SEM (n=6). *Statistically significant change in obese relative to lean ZR.

Fig. 4. Age-dependent changes in mean arterial blood pressure (BP) in lean and obese ZR. Results are means $\pm$ SEM (n=6). *Statistically significant change in obese relative to lean ZR.

Fig. 5.

Effect of L-arginine supplementation or arginase inhibition on plasma arginine concentration (A), plasma NO concentrations (B), and mean arterial blood pressure (BP) (C), body weight (D), and insulin resistance (HOMA-IR) (E) in obese ZR. Obese ZR were treated with vehicle, L-arginine (20g/L) in the drinking water, or intraperitoneal infusion of OHNA (25 $\mu\text{g}/\text{day}$) beginning at 8 weeks of age for 4 weeks. Results are means $\pm$ SEM (n=5–6).

*Statistically significant changes in obese relative to lean ZR.