Orotic Acid Induces Hypertension Associated with Impaired Endothelial Nitric Oxide Synthesis


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ABSTRACT

Orotic acid (OA) is an intermediate of pyrimidine nucleotide biosynthesis. Hereditary deficiencies in some enzymes associated with pyrimidine synthesis or the urea cycle induce OA accumulation, resulting in orotic aciduria. A link between patients with orotic aciduria and hypertension has been reported; however, the molecular mechanisms remain elusive. In this study, to elucidate the role of OA in vascular insulin resistance, we investigated whether OA induced endothelial dysfunction and hypertension. OA inhibited insulin- or metformin-stimulated nitric oxide (NO) production and endothelial NO synthase (eNOS) phosphorylation in human umbilical vein endothelial cells. A decreased insulin response by OA was mediated by impairment of the insulin-stimulated phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB/Akt) signaling pathway in cells overexpressing the p110-PI3K catalytic subunit. Impaired effects of metformin on eNOS phosphorylation and NO production were reversed in cells transfected with constitutively active AMP-activated protein kinase. Moreover, experimental induction of orotic aciduria in rats caused insulin resistance, measured as a 125% increase in the homeostasis model assessment, and hypertension, measured as a 25% increase in systolic blood pressure. OA increased the plasma concentration of endothelin-1 by 201% and significantly inhibited insulin- or metformin-induced vasodilation. A compromised insulin or metformin response on the Akt/eNOS and AMP-activated protein kinase/eNOS pathway was observed in aortic rings of OA-fed rats. Taken together, we showed that OA induces endothelial dysfunction by contributing to vascular and systemic insulin resistance that affects insulin- or metformin-induced NO production, leading to the development of hypertension.

Key words: orotic acid; insulin resistance; endothelial dysfunction; eNOS; hypertension.

Orotic acid (OA) is an anabolic intermediate of pyrimidine nucleotide biosynthesis. All nucleated cells contain certain levels of OA, and dietary intake and de novo synthesis constitute the major sources of OA in the body. Although OA is efficiently excreted via the kidneys, a number of pathologic situations cause cellular OA accumulation, ultimately leading to orotic aciduria. Inborn errors in pyrimidine biosynthesis, as seen by mutation of the multifunctional protein uridine 5'-monophosphate (UMP) synthase, prevent conversion of OA to UMP, leading to the accumulation of OA in plasma (Smith et al., 1961). Hereditary enzyme deficiencies involved in the urea cycle, such as ornithine transcarbamoylase, cause car bamoyl...
phosphate to accumulate in the mitochondria, resulting in uncontrolled synthesis of pyrimidine nucleotides, as well as OA (Wendler et al., 1983). Impaired endogenous arginine and/or ornithine synthesis as a result of deficiencies in argininosuccinate synthase or arginosuccinate lyase in the face of hyperammonemia also induce orotic aciduria (Clery et al., 2005; Salerno and Crifo, 2002). Orotic aciduria is also seen in hepatic fibrosis, in which impaired hepatic protein synthesis increases NH₃ concentration, and urea cycle disturbances inhibit arginine synthesis (Ju et al., 2013).

Studies have indicated an increased prevalence of essential hypertension in patients with arginosuccinic aciduria induced by argininosuccinate lyase deficiency, the second most common urea cycle disorder (Brunetti-Pierri et al., 2009). Moreover, a single-nucleotide polymorphism in carbamoyl phosphate synthase I, a rate-determining enzyme of the urea cycle, may be associated with altered vascular resistance and persistent pulmonary hypertension in infants (Pearson et al., 2001). Decreased arginine synthesis due to defects in the urea cycle and thus limited nitric oxide (NO) availability accounted for an increased risk of hypertension (Pearson et al., 2001). However, the direct contribution of OA to the development of endothelial dysfunction and hypertension has not been evaluated.

One of the most important vascular actions of insulin is production of the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1) in endothelial cells (Kang, 2014). Phosphorylation of insulin receptor substrate (IRS)-1 by insulin activates phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB/Akt) signaling to induce NO production via endothelial NO synthase (eNOS) phosphorylation (Montagnani et al., 2002). Activation of AMP-activated protein kinase (AMPK) increased eNOS phosphorylation in endothelial cells, indicating that AMPK also plays an important role in vascular homeostasis (Hien et al., 2011; Lan et al., 2008; Xie et al., 2008). Insulin induces the expression of ET-1 via the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) pathway (Eringga et al., 2004). Therefore, the net hemodynamic effect of insulin on blood pressure is minimal in insulin-sensitive, healthy humans (Muscelli et al., 1998). In an insulin-resistant state, however, the balance between vasodilator and vasoconstrictor actions of insulin is altered in favor of ET-1-dependent functions that lead to endothelial dysfunction and hypertension (Cusi et al., 2000; Montagnani et al., 2002).

OA administration has been found to induce fatty liver in rats, as well as in humans (Clery et al., 2005; Creasey et al., 1961). We previously reported that OA-induced fatty liver is mediated predominantly by the AMPK-mammalian target of rapamycin-sterol response element binding protein-1 pathway (Jung et al., 2011). Higher serum uric acid levels are a strong and independent predictor of incident metabolic syndrome in men and women (Sui et al., 2008). We recently found that uric acid induced endothelial dysfunction and hypertension by suppressing insulin-stimulated eNOS phosphorylation and NO production (Choi et al., 2014). Uric acid and OA share many similarities in terms of their chemistry and metabolism. In the present study, we investigated the effects of OA on hypertension-associated vascular insulin resistance and endothelial dysfunction. We found that OA suppressed insulin- or AMPK-stimulated eNOS phosphorylation and NO production in endothelial cells. Moreover, OA-induced hypertension and insulin resistance was associated with impaired insulin action on vasodilation in rats.

MATERIALS AND METHODS

Cell culture and chemicals. Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe et al. (1973) and cultured in M199 medium containing 10 units/ml heparin, 20% fetal bovine serum, and 20 ng/ml fibroblast growth factor. ECV304 cells (ATCC, Manassas, Virginia) were maintained in Dulbecco's Modified Eagle's Medium (GIBCO BRL, Grand Island, New York) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO₂ atmosphere at 37°C. All chemicals were obtained from Sigma-Aldrich (St Louis, Missouri).

Measurement of NO production. NO production was determined by using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, Michigan). Cells were incubated in 96-well black-wall, clear-bottom plates. After treatment, cells were loaded with DAF-2 DA (1 μM) for 30 min at 37°C, rinsed 3 times with Dulbecco's Modified Eagle's Medium. The cells were fixed in 4% paraformaldehyde for 5 min. The fluorescence was measured with a microplate fluorescence reader (Molecular Devices, Sunny-Vale, California) at excitation 480 nm and emission 510 nm.

Western blot analysis. Cells and sorts were harvested and washed with ice-cold phosphate-buffered saline. They were lysed on ice for 60 min in lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EGTA, 50 mM glycerophosphate, 20 mM NaF, 1% Triton X-100, and 10% glycerol) and centrifuged at 12 000 rpm for 10 min. Supernatants were collected, electrophoresed on 10% SDS-polyacrylamide gels, and transferred onto polyvinylene difluoride membrane (Millipore, Bedford, Massachusetts). Western blots were probed with the following antibodies: p-eNOS (Ser1177; No. 9271), Akt (No. 9272), p-AMPK (Thr172, No. 2531), and GAPDH (No. 2118) from Cell Signaling Technology (Beverly, Massachusetts). Detection was performed by enhanced chemiluminescence Western blotting detection reagents (Amersham, Piscataway, New Jersey).

Adenoviral infection and plasmid transfection. Myc-tagged AdCA-AMPK was provided by Dr Joohun Ha from Kyung Hee University. Cells were infected with adenovirus for 6 h and incubated for 48 h after medium change. p110 plasmid, the expression constructs of phosphatidylinositol 3-kinase (PI3K), was a kind gift from Dr Toker A (The Boston Biomedical Research Institute, Boston, Massachusetts). Western blots were probed with the following antibodies: p-eNOS (Ser1177; No. 9570), eNOS (No. 9586), p-Akt (Ser273; No. 9271), Akt (No. 9272), p-AMPK (Thr172, No. 2531), p-Akt (Ser273; No. 9271), Akt (No. 9272), p-AMPK (Thr172, No. 2531), and GAPDH (No. 2118) from Cell Signaling Technology (Beverly, Massachusetts). Detection was performed by enhanced chemiluminescence Western blotting detection reagents (Amersham, Piscataway, New Jersey).

Animal treatments. Specific pathogen-free male Sprague Dawley rats, 10 weeks old, were obtained from SCL Inc (Shizuoka, Japan) and allowed ad libitum access to standard chow and tap water. They were kept in temperature-controlled, filter-sterilized animal quarters under a 12-h light:12-h dark cycle. After 1 week of adaption period, animals were divided into 2 groups. Animals were given normal purified rodent diet (AIN-93G) or OA diet prepared by supplementation of 1% OA to the normal diet for 21 days. They were euthanized after being fasted for 12 h to measure the level of fasting plasma glucose and insulin from serum. The use of animals was in compliance with the guidelines established by the animal care committee of our institute.
Measurements of blood pressure and serum biochemistry. Blood pressure was assessed as the mean value of 3 consecutive measurements obtained in the morning using the CODA mouse rat tail-cuff system (Kent Scientific, Torrington, Connecticut). Serum biochemical parameters were monitored by standard clinical chemistry assays on an Automated Chemistry Analyzer (Prestige 24i; Tokyo Boeki Medical System, Tokyo, Japan). Rat insulin and ET-1 were measured by ELISA (Millipore; R&D Systems, Minneapolis, Minnesota). Insulin resistance was determined from fasting insulin and glucose levels and the previously validated homeostasis model assessment of insulin resistance (HOMA-IR) according to Matthews et al. (1985).

Determination of urinary OA. Determination and quantification of urinary OA were performed according to a previous validated method for quantitative analysis of urinary organic acid with minor modification of instrumental condition (Duez et al., 1996). The gas chromatography coupled with mass spectrometry (GC-MS) was used for the separation of OA from complex urinary matrix and appropriate internal standard was used to quantify OA. The detailed analytical condition was described in Supplementary Method 1.

Determination of serum arginine, citrulline, and ornithine levels. To quantify arginine, citrulline, and ornithine concentration in rat serum sample, LC-MS analysis was performed using o-phthalaldehyde derivatization method (Jones and Gilligan, 1983). Matrix effect of serum sample was assessed by the slope comparison method (Garcia-Villar et al., 2009) for accurate quantification of target compounds. The detailed method was explained in Supplementary Method 2.

Organ chamber study. Thoracic aortas were carefully removed from rats and placed in a modified Krebs-Ringer bicarbonate solution containing 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25.0 mM NaHCO₃, 0.016 mM Ca⁡²⁺ EDTA, and 11.1 mM glucose. The aortas were cleaned of loose connective tissue and then cut into 2 mm wide rings. The aortic rings were suspended horizontally between 2 stainless steel stirrups in the organ chambers filled with Krebs-Ringer solution (37°C, pH 7.4) and bubbled with 95% O₂ and 5% CO₂. The change in tension was measured isometrically with Grass FT03 force transducers (Grass Instrument Co, Quincy, Massachusetts) and data were analyzed with a PowerLab 8/30 Data Acquisition System and LabChart pro software (AD Instruments, Colorado Springs, Colorado). The rings were stretched progressively to the optimal tension (2 g); 1 μM acetylcholine-mediated relaxation was tested in the precontracted rings by 1 μM phenylephrine to check endothelium-dependent relaxation responsiveness. After 30 min incubation, 15 or 30 μM insulin or 1 or 3 mM metformin was added and the relaxation response was monitored in the precontracted aortic rings after addition of phenylephrine.

Statistical analysis. All data were expressed as mean ± SD. Statistical analysis was performed using Student’s t test, 1-way analysis of variance (ANOVA) and 2-way ANOVA where appropriate. Differences between groups were considered to be statistically significant at P < 0.05. Multiple comparisons were evaluated by 1-way ANOVA followed by Tukey’s multiple comparison or 2-way ANOVA followed by Bonferroni’s post hoc test.

RESULTS

OA Inhibits Insulin-Stimulated eNOS Phosphorylation and NO Production in Endothelial Cells via the PI3K/Akt Pathway

We first examined whether OA is capable of affecting eNOS expression or phosphorylation. Neither the expression nor the phosphorylation of eNOS was affected by 5–100 μM OA treatment in HUVEC (Fig. 1A). Insulin activates eNOS by phosphorylation of the protein at Ser1177 and increases NO production in endothelial cells via the PI3K/Akt pathway (Montagnani et al., 2001). To investigate whether OA affects insulin-induced phosphorylation of eNOS, HUVECs were incubated with OA for 1 h prior to insulin stimulation. Insulin-induced stimulation of eNOS phosphorylation at Ser1177 was markedly impaired by OA at concentrations as low as 5 μM. Phosphorylation of the upstream kinase Akt was also suppressed by the same
concentrations of OA (Fig. 1B). Decreased eNOS phosphorylation by OA led to diminished NO production in HUVECs, determined by the fluorescence intensity of DAF-2 DA staining (Fig. 1C). The effects of OA on insulin-induced Akt and eNOS phosphorylation and NO production were reproduced similarly in ECV304 cells (Supplementary Fig. 1). Although the use of ECV304 cells for the study of endothelial function is controversial, these cells present many features characteristic of endothelial cells and are frequently used for the study of vascular biology (Xiong et al., 2011; You et al., 2010). To demonstrate that the suppressive effect of OA on insulin-stimulated eNOS phosphorylation was mediated by the canonical PI3K/Akt pathway, we transfected ECV304 cells and HUVECs with p110, the catalytic unit of PI3K. As shown in Figures 2A and 2B, overexpression of p110 completely restored eNOS and Akt phosphorylation, as well as NO production. These data indicate that OA inhibits insulin-induced NO production via PI3K/Akt-dependent phosphorylation of eNOS in endothelial cells.

OA Inhibits Metformin-Induced eNOS Phosphorylation and NO Production in Endothelial Cells

AMPK, the key regulator of energy and metabolic homeostasis, plays a role in regulating vascular tone mediated by NO (Fisslthaler and Fleming, 2009; Nagata and Hirata, 2010). Activation of AMPK by adiponectin results in enhanced NO production in endothelial cells (Chen et al., 2003). Based on our previous report demonstrating OA inhibition of AMPK phosphorylation in human and rat hepatocytes (Jung et al., 2011), we hypothesized that OA inhibits AMPK phosphorylation and consequently NO production in endothelial cells. However, in HUVECs, OA did not inhibit AMPK expression or phosphorylation (Fig. 3A). To investigate whether OA affected AMPK-induced phosphorylation of eNOS, HUVECs were incubated with OA for 1 h prior to metformin treatment for 30 min. Upon metformin stimulation, phosphorylation of AMPK at Thr172 and eNOS at Ser1177, as well as NO production, was increased significantly and was suppressed by preincubation with OA (Figs. 3B and 3C). The effects of OA on metformin-induced AMPK and eNOS phosphorylation and NO production were also reproduced in ECV304 cells (Supplementary Fig. 2). Introduction of a constitutively active mutant of AMPK (Ad-CA-AMPK) using an adenovirus vector increased the basal level of eNOS phosphorylation and NO production. In these cells, the effects of metformin were not suppressed by preincubation with OA (Figs. 4A and 4B). Taken together, these data indicate that both insulin- and AMPK-induced eNOS phosphorylation are impaired by OA in endothelial cells.

Rats with Orotic Aciduria Develop Systemic Insulin Resistance and Hypertension

Based on observations that OA-impaired insulin responses affect Akt and eNOS phosphorylation, as well as NO production, in HUVECs, we investigated whether experimental orotic
Aciduria induced systemic insulin resistance and hypertension. Experimental orotic aciduria was induced by feeding rats a diet containing 1% OA for 3 weeks. Systolic blood pressure was measured every week, and the OA concentration in urine was determined at the end of the experiment. Compared with controls, urinary excretion of OA was increased in OA-fed rats by 4-fold, from 89.5 ± 33.0 to 348.4 ± 180.2 μmol/g creatinine (Fig. 5A).

Due to a large difference in the basal urinary excretion level of OA between humans and rats, it is difficult to compare our animal model with hereditary human orotic aciduria (Seiler et al., 1994). However, our model showed very mild effects considering the 3- to 46-fold increase in OA excretion in ornithine transcarbamylase (OTC) mutant mice or OTC-deficient individuals (Ogino et al., 2007; Qureshi et al., 1979; Tim-Aroon et al., 2014). Systolic blood pressure was increased significantly by 20.1 and 31.8 mmHg at 2 and 3 weeks, respectively (Fig. 5B). Table 1 summarizes the results of the serum biochemical analysis. Consistent with the previous report, orotic aciduria increased serum alanine aminotransferase levels. Fasting glucose and insulin were increased in rats with orotic aciduria by 61.6% (P < 0.05) and 46.1%, respectively. The HOMA-IR, which is calculated from basal plasma glucose and insulin concentrations, is a valuable alternative to the clamp test in the evaluation of in vivo insulin sensitivity (Bonora et al., 2000). In rats with orotic aciduria, the HOMA-IR was increased by 2.2-fold (P < 0.05) compared with the control group, indicating the development of insulin resistance. Elevated plasma ET-1 levels have been reported in patients with insulin resistance and hypertension (Kohno et al., 1990; Takahashi et al., 1990). Experimental orotic aciduria increased the serum ET-1 level by 2-fold (Table 1). These results indicate that orotic aciduria induces insulin resistance and hypertension in rats.

Rats with Orotic Aciduria Develop Vascular Insulin- and Metformin Resistance with Respect to NO Synthesis and Vasodilation

Next, we investigated whether the Akt/eNOS and/or AMPK/eNOS signaling pathway was impaired in the vasculature of orotic aciduria rats. Our organ chamber studies using endothelium-intact aortic rings demonstrated that insulin-induced vasodilation was inhibited significantly in rats with orotic aciduria,
while acetylcholine-induced vasodilation was not changed (Figs. 6A and 6B). Mesenteric artery is frequently used for the study of vascular response to insulin. However, conduit artery also responds to insulin very well and is frequently used for the study of insulin-mediated vasodilation (Elgebaly et al., 2008; Lee et al., 2009). Insulin-stimulated phosphorylation of Akt and eNOS was also suppressed substantially in these rats (Fig. 6C). Similar results were obtained when the aortic rings were stimulated with metformin. Metformin-induced vasodilation as well as AMPK and eNOS phosphorylation were inhibited in rats with orotic aciduria (Figs. 7A and 7B).

Availability of the urea cycle intermediates and NO precursors play role in the regulation of vascular resistance (Scaglia et al., 2004). The mean serum concentrations of arginine and citrulline were significantly lower in rats with orotic aciduria than in the controls (Table 2). The citrulline/arginine ratio, a quantitative indicator of NOS activity was increased in rats with orotic aciduria. The increased citrulline/arginine ratio was not due to increased arginine activity as we found that the ornithine/citrulline ratio was rather decreased in rats with orotic aciduria (Table 2). Together, these data suggest that OA impairs the PI3K/Akt/eNOS and AMPK/eNOS signaling pathways and causes vascular insulin and metformin resistance, leading to impaired vasodilation and hypertension. The decreased serum arginine concentration might also contribute to the development of hypertension.

**DISCUSSION**

In this study, we showed that OA, an anabolic intermediate of pyrimidine nucleotide biosynthesis, induced systemic and vascular insulin resistance and hypertension in rats. OA inhibited insulin- and metformin-stimulated eNOS phosphorylation and NO production in HUVECs. A decreased insulin response by OA was mediated by impaired insulin-stimulated PI3K/Akt signaling pathways. Rats with orotic aciduria exhibited hyperglycemia and hyperinsulinemia together with an increased HOMA-IR, indicating systemic insulin resistance. The rats exhibited increased systolic blood pressure with significantly impaired insulin and metformin actions on eNOS phosphorylation and vasodilation. Thus, this study provides evidence that OA induces endothelial dysfunction and hypertension as mediated by insulin resistance and insulin-induced NO production.

OA is consumed in the diet and synthesized de novo. OA is a minor dietary constituent with the richest sources found in cow’s milk and dairy products (Motyl et al., 1991). Although OA is excreted efficiently via the kidney, a number of situations cause OA accumulation in the liver leading to orotic aciduria. These include inborn errors in the pyrimidine biosynthetic pathway, drugs affecting pyrimidine synthesis, hereditary enzyme deficiencies involved in the urea cycle and impaired endogenous arginine synthesis in the face of insufficient dietary arginine or of increased arginine catabolism (Brosnan and Brosnan, 2007). While the normal plasma concentration of OA ranges from 0 to 2.5 μM (Mills et al., 1979), it is increased to 86.5 μM in patients deficient in arginosuccinic acid synthase, an essential enzyme in the urea cycle (Sass and Skladal, 1999). In the present study, we showed that OA inhibited insulin- or metformin-induced eNOS phosphorylation and NO production at concentrations as low as 5 μM or 50 μM in HUVECs, respectively. Thus, the results obtained from in vitro studies are comparable to the conditions found in orotic aciduria patients. Moreover, the urinary OA concentration in our orotic aciduria model was well within the range found in many pathophysiological situations. Our results clearly suggest orotic aciduria is a risk factor for insulin resistance and hypertension whether it is caused by a hereditary disorder in the urea cycle or by accidental overdose of OA.

![FIG. 5. Rats with orotic aciduria develop systemic insulin resistance and hypertension. A, Rats were fed with 1% OA-mixed powder diet for 3 weeks. Determination and quantification of urinary OA were performed using GC-MS as described in the Materials and Methods section. B, Systolic blood pressure was measured every week using a tail-cuff system. Data represents the mean ± SD (n = 8). Asterisk (*) represents significance (P < 0.05) compared with the control using Student’s t-test.](image)

| TABLE 1. Serum Biochemical Parameters in Rats with Orotic Aciduria |
|---|---|---|
| **Characteristics** | **Control** | **Orotic Acid** |
| ALT (U/l) | 26.6 ± 5.3 | 45.0 ± 10.4* |
| AST (U/l) | 118.4 ± 32.6 | 122.0 ± 24.9 |
| GGT (U/l) | 6.9 ± 1.1 | 7.6 ± 1.1 |
| ALP (U/l) | 668.9 ± 185.4 | 668.9 ± 102.7 |
| CPK (U/l) | 1851.1 ± 3238.9 | 907.9 ± 173.7 |
| LDH (mg/dl) | 3360.6 ± 599.2 | 3233.7 ± 783.8 |
| Glucose (mg/dl) | 164.0 ± 57.4 | 265.1 ± 108.0* |
| Insulin (μU/ml) | 21.7 ± 4.2 | 31.7 ± 13.5 |
| HOMA-IR | 9.7 ± 4.1 | 21.8 ± 11.1 |
| ET-1 (pg/ml) | 1.0 ± 0.2 | 2.0 ± 0.8 |
| TG (mg/dl) | 65.1 ± 25.0 | 60.6 ± 43.5 |
| Total cholesterol (mg/dl) | 59.4 ± 0.7 | 62.4 ± 12.4 |
| HDL (mg/dl) | 37.6 ± 7.8 | 44.7 ± 11.7 |
| Total bilirubin (mg/dl) | 0.6 ± 0.1 | 0.5 ± 0.1 |
| BUN (mg/dl) | 11.0 ± 2.5 | 11.6 ± 2.4 |
| Creatinine (mg/dl) | 0.5 ± 0.2 | 0.3 ± 0.1 |

Notes: ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; ET-1, endothelin-1; TG, triglyceride; HDL, high-density lipoprotein; BUN, blood urea nitrogen. Values are mean ± SD (n = 7–8). *P < 0.05 compared with control group using Student’s t-test.
Wakabayashi et al. (1994) reported that a deficiency in endogenous arginine synthesis provoked hypertension by exhausting arginine as a substrate for NO synthesis. They determined that rats with a resected small intestine fed an arginine-free diet show reduced arginine concentrations and elevated blood pressure. Because arginine is not only a substrate for NO synthase but also a critical intermediate in the urea cycle, arginine deficiency-induced orotic aciduria. Although urinary OA concentration was elevated strikingly in arginine-deficient rats, the authors concluded that hypertension was induced by arginine deficiency during endothelial NO synthesis, and that the elevated level of OA was regarded only as an index of arginine deficiency (Milner and Visek, 1973). Links between urea cycle disorders and hypertension have been reported in several cases, including arginosuccinate lyase deficiency and single-nucleotide polymorphism in carbamoyl phosphate synthase I (Brunetti-Pierri et al., 2009; Pearson et al., 2001). However, they also concluded that deficiencies in endogenously synthesized arginine and, consequently, NO were responsible for the increase in blood pressure. In contrast, in this study, we clearly demonstrated that OA was not only a marker of arginine deficiency but also an intermediate in pyrimidine synthesis, playing a central role in inducing vascular and systemic insulin resistance, endothelial dysfunction, and hypertension. At present, we do not know why serum arginine level was decreased and whether the decreased arginine level contributes to the increased blood pressure in the rats with orotic aciduria. We can speculate, however, that impaired insulin response to NO synthesis may increase NOS activity to compensate for the low NO bioavailability. Further studies are needed to clear the cause and effect relationship.

Insulin has important biological effects in metabolic tissues, as well as in the vasculature. In endothelial cells, insulin maintains vascular homeostasis by releasing various mediators including the vasodilator NO and the vasoconstrictor ET-1. These two mediators are well balanced, maintaining normal blood pressure in the healthy state. During insulin resistance, however, the action of insulin is impaired selectively during eNOS-mediated vasodilation in the face of unaltered or even increased vasoconstrictive effects on ET-1 production (Jiang et al., 1999; Montagnani et al., 2002). Our in vivo data show that in the aortic ring of rats with orotic aciduria, insulin-induced eNOS phosphorylation and vasodilation were reduced significantly, while the plasma ET-1 level was increased 2-fold. ET-1 expression and secretion in response to insulin stimulation is mediated by ERK/MAPK signaling pathway in vascular endothelium (Gogg et al., 2009; Zeng et al., 2000). However, the ERK/MAPK signal was not changed in response to insulin in aorta of OA-fed rats (data not shown). ET-1 production in human vascular endothelial cells is also regulated by increased ET-1 promoter activity such as AP-1 and GATA-2 (Hsu et al., 2004; Kawana et al., 1995). Activation of peroxisome proliferator-activated receptor (PPAR) negatively regulates ET-1 expression by interfering with AP-1 activation (Delerive et al., 1999). Although we do not know the

**FIG. 6.** Insulin-induced vasodilation is decreased in OA-treated rat aortic rings. A and B, Endothelium-intact aortic rings were isolated from rats and anchored to the organ chamber. Acetylcholine- and insulin-induced vascular relaxation was monitored in the precontracted aortic rings using phenylephrine. Data are expressed as percentages of relaxation relative to acetylcholine-mediated relaxation and represent the mean ± SD (n = 4). Two-way ANOVA with Bonferroni’s post hoc test revealed a significant effect on insulin (P < 0.05) and OA (P < 0.05) and interaction between the 2 (P < 0.05). Asterisk (*) represents significance (P < 0.05) compared with the aortic rings from the control group with insulin. C, Rat aortic rings were incubated with insulin for 30 min and aortic ring homogenates were incubated with antibodies. Each bar represents the mean ± SD (n = 4). Two-way ANOVA with Bonferroni’s post hoc test revealed a significant effect on insulin (P < 0.05) and OA (P < 0.05) and interaction between the 2 (P < 0.05). Asterisk (*) represents significance (P < 0.05) compared with the aortic rings from the control group without insulin; hash (#) represents significance (P < 0.05) when compared with the aortic rings from the control group with insulin.
reason why OA increases the serum ET-1 level, we cannot
eclude the possibility that OA-induced suppression of PPARα
expression in rat liver (Ferreira et al., 2008) might contribute
to the ET-1 change. Insulin resistance in the rats was accompa-
nied by endothelial dysfunction in mesenteric vessels with
impaired PI3-kinase-dependent NO production and enhanced
MAPK-dependent ET-1 secretion (Potenza et al., 2005).
Hyperinsulinemia, together with hypertriglyceridemia, are
potent inducers of ET-1 release, which is one of the pathogenic
causes of hypertension and cardiovascular diseases in patients
with metabolic syndrome (Piatti et al., 1996). Therefore, an
increase in ET-1 production and vascular insulin resistance in
rats with orotic aciduria might be the consequence of hyperin-
sulinemia and systemic insulin resistance. However, it is also
possible that impaired endothelial function due to vascular
insulin resistance can induce systemic insulin resistance by
reducing capillary recruitment and blood flow in skeletal

TABLE 2. Serum Levels of Arginine, Citrulline and Ornithine in Rats with Orotic Aciduria

<table>
<thead>
<tr>
<th>Group</th>
<th>Arginine (μM)</th>
<th>Citrulline (μM)</th>
<th>Ornithine (μM)</th>
<th>Citrulline/Arginine</th>
<th>Ornithine/Citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.0 ± 1.1</td>
<td>10.8 ± 0.6</td>
<td>28.9 ± 12.3</td>
<td>0.64 ± 0.06</td>
<td>2.65 ± 1.09</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>11.6 ± 1.4*</td>
<td>10.0 ± 0.5*</td>
<td>21.1 ± 6.1</td>
<td>0.87 ± 0.10*</td>
<td>2.11 ± 0.60</td>
</tr>
</tbody>
</table>

Notes: Values are mean ± SD (n = 7-8). *P < 0.05 compared with control group using Student’s t test.
between them (Youn et al., 2009). We showed that OA attenuated metformin-induced AMPK phosphorylation and insulin-induced Akt phosphorylation collectively leading to the reduced NO production. We are not sure whether OA inhibition of insulin- and AMPK-induced eNOS phosphorylation cross-talk each other. Further studies are needed for elucidating the molecular network of interaction in OA-induced hypertension associated with impaired endothelial NO synthesis.

Rats with orotic aciduria also developed hyperglycemia, which is well known to attenuate blood flow and insulin-induced vasodilation (Saini et al., 2004). A pathogenic role of hyperglycemia in vascular cells remains unclear, but there are suggestive evidences that high glucose develops endothelial dysfunction. It may relate to decreased production of NO (Salt et al., 2003), reduction of NO availability by oxygen-derived free radicals (Brodsky et al., 2001), and enhanced production of vasoconstrictor (Tesfamariam et al., 1990). Therefore, increased glucose level contributes to endothelial dysfunction as well as the development of insulin resistance. Considering the role of hyperglycemia in endothelial dysfunction, it is presently unclear how high glucose level might act in hypertension in rats with orotic aciduria and further study is needed.

Some compelling evidence suggests that vascular insulin resistance precedes various vascular dysfunctions, including hypertension. Muscle or coronary arterioles of Zucker obese rats showed impaired insulin-induced vasodilation in the absence of or prior to impaired dilation by acetylcholine (Eringa et al., 2007; Oltman et al., 2006). The same is true in the case of hypertension and aging. Vascular insulin resistance in spontaneously hypertensive rats is a risk factor preceding phenotypic manifestations of hypertension (Li et al., 2010). Despite normal fasting plasma levels of insulin and glucose, as well as normal metabolic insulin sensitivity, aged rats exhibited impaired aortic relaxation in response to insulin, but not to acetylcholine, indicating selective vascular insulin resistance (Schulman et al., 2007). The role of vascular insulin resistance in the development of endothelial dysfunction has been clearly demonstrated in transgenic mice with endothelial-targeted overexpression of a dominant-negative mutant human insulin receptor and in humans carrying an Arg⁹⁷² to Gln⁹⁷² polymorphism (Perticone et al., 2004; Duncan et al., 2008). Thus, vascular insulin resistance induced by OA is an early vascular defect that can develop into widespread vascular disease.

Clinical and epidemiological studies have shown a close linkage between hypertension and insulin resistance, which together increase the risk of cardiovascular disease and the incidence of type 2 diabetes (Alberti et al., 2005; Schulman and Zhou, 2009). Although OA is not a component of the urea cycle, per se, blood and urinary OA concentration is increased in some urea cycle disorders. The present study indicated for the first time that OA at concentrations observed in humans with genetic disorders associated with the urea cycle caused endothelial dysfunction via vascular insulin resistance. We surmise that the development of hypertension in rats with orotic aciduria was caused by vascular and systemic insulin resistance. We suggest that subjects with hereditary, accidental, or drug-induced orotic aciduria are at risk of endothelial dysfunction, hypertension, insulin resistance, and metabolic syndrome. The OA levels of the subjects should be monitored regularly and proper nutritional care recommended to reduce the OA intake via food, drugs, or dietary supplements. Finally, impairment of the insulin response during vasodilation in subjects with orotic aciduria may add to the increased susceptibility to vascular diseases induced by various cardiovascular risk factors.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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