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Arginase inhibition restores endothelial function in diet-induced obesity

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ABSTRACT

Arginase may play a major role in the regulation of vascular function in various cardiovascular disorders by impairing nitric oxide (NO) production. In the current study, we investigated whether supplementation of the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) could restore endothelial function in an animal model of diet-induced obesity. Arginase 1 expression was significantly lower in the aorta of C57BL/6J mice fed a high-fat diet (HFD) supplemented with nor-NOHA (40 mg kg^{-1}/day) than in mice fed HFD without nor-NOHA. Arginase inhibition led to considerable increases in eNOS expression and NO levels and significant decreases in the levels of circulating ICAM-1. These findings were further confirmed by the results of siRNA-mediated knockdown of *Arg* in human umbilical vein endothelial cells. In conclusion, arginase inhibition can help restore dysregulated endothelial function by increasing the eNOS-dependent NO production in the endothelium, indicating that arginase could be a therapeutic target for correcting obesity-induced vascular endothelial dysfunction.

1. Introduction

As a biological messenger, nitric oxide (NO) plays a role in the pathogenesis of many metabolic disorders such as cardiovascular diseases, atherosclerosis, and hypertension [1–4] through regulating various physiological processes, including vasodilation, inflammation, and metabolism [5,6]. In addition, the reduced bioavailability of endothelium-derived NO has been reported to be closely associated with obesity [7]. NO is synthesized by endothelial NO synthase (eNOS) using L-arginine as substrate, and arginase reciprocally regulates NOS and NO production by competing for L-arginine [8]. In various cardiovascular disorders, arginase has been shown to regulate vascular cell functions primarily through impairment of NO production [9–11]. In line, we previously observed significant upregulation of arginase 1 in the peripheral blood mononuclear cells (PBMCs) of overweight/obese individuals [12], which suggested an association between arginase activity in the endothelium, eNOS-dependent NO production, and the endothelial dysfunction evident in obesity.

Although the role of arginase in endothelial dysfunction has been reported in various experimental models such as aging [13], ischemia-reperfusion-induced endothelial dysfunction [14], hypertension [15] and atherosclerosis [16], the effects of arginase blockade on the endothelial function in diet-induced obesity has not been studied. Very recently, we observed that arginase inhibition ameliorates obesity-induced abnormalities in hepatic lipids and whole-body adiposity through the mechanism that activates pathways involved in hepatic triglyceride metabolism and mitochondrial function [17]. Given that obesity has been closely linked to endothelial dysfunction induced by impaired NO release from the endothelium [7], in the present study, we investigated whether oral supplementation of the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) could restore endothelial function in an animal model of diet-induced obesity.

2. Materials and methods

2.1. Cell culture and siRNA-mediated knockdown of arginase

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, MD,

Abbreviations: NO, nitric oxide; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical endothelial cells; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; ICAM-1, intercellular adhesion molecule-1.

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USA). The cells were cultured in Clonetics® EGM® (Endothelial Growth Medium, Lonza, MD, USA) at 37 °C, 5% (v/v) CO_2 and the ones (1×10^5 cells) from passages 6–9 were seeded into each well of 6-well culture plates. After incubation for 24 h, the cells were serum-starved overnight before siRNA treatment. Predesigned and validated Stealth siRNAs were purchased from Invitrogen (Carlsbad, CA, USA). The serum-starved HUVECs were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen, USA) according to the suppliers' standard protocols. Cells showing successful knockdown of the target genes were used in further experiments.

2.2. Animals and study design

Four-week-old male C57BL/6 mice were purchased from DBL (**Chungbuk, Korea**) and, after an adaptation period of 1 week, were randomly assigned to control (ND; $n = 10$), high-fat diet (HFD; $n = 10$), and HFD supplemented with nor-NOHA (HFD with nor-NOHA; $n = 10$) groups and grown for 12 weeks. The control diet was based on the AIN-76 rodent diet. The HFD (40% total energy from fats), which was used for obesity induction, was identical to the control diet, except that it contained 200 g fat/kg (170 g lard, 30 g corn oil) and 1% cholesterol. After 7 weeks on the HFD, for the following 5 weeks, the mice were fed daily with either only sham gavages of HFD with an excipient or oral gavages of HFD supplemented with 40 mg/kg nor-NOHA (Bachem, Bubendorf, Switzerland) dissolved in 0.9% NaCl solution. They were housed in a temperature- (18–24 °C) and humidity-controlled (50–60%) room in a pathogen-free environment. All the experimental procedures were approved by the Committee on Animal Experimentation and Ethics of Korea University (KUIACUC-2013-96).

2.3. Sample collection

At the end of the experimental period, the mice were anesthetized with a mixture of 30 mg/kg zoletil (Virbac, Carros, France) and 10 mg/kg Rompun (Bayerkorea, Seoul, Korea), sacrificed and blood samples were collected from the abdominal inferior vena cava into vacutainer tubes containing EDTA. To obtain plasma, the whole-blood samples were centrifuged at $1390 \times g$ for 15 min at 4 °C. The obtained plasma was aliquoted and stored at −80 °C until further use. Aortic tissue was extracted, washed with $1 \times$ phosphate-buffered saline (PBS), rapidly frozen using liquid nitrogen, and then stored at −80 °C.

2.4. Determination of aortic NO production and plasma concentrations of intercellular adhesion molecule 1 (ICAM-1)

Aortic NO production was evaluated using a commercial colorimetric assay kit (Cayman Chemical, MI, USA); NO levels were estimated by measuring the levels of nitrite, a major stable breakdown product of NO. Plasma ICAM-1 was separated using an enzyme-linked immunosorbent assay kit (Abcam, MA, USA), and its concentration was determined from the absorbance 450 nm; absorbance was read using Victor™ *X*3 multimode plate reader (Perkin-Elmer Life Sciences, MA, USA). Values were corrected for background absorbance.

2.5. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from aortic tissue and HUVECs using the RNeasy Lipid Tissue Mini Kit (Qiagen, USA) and the RibospinTM Kit (GeneAll, Korea), respectively, according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using oligo-dT and SuperscriptTM II reverse transcriptase (Invitrogen, USA). One microgram of the cDNA was amplified by quantitative real-time PCR using the SYBR Green PCR Kit (Qiagen, USA). PCR was conducted in a Step One Plus system (Applied Biosystems, Foster City, CA) and the conditions were as follows: 95 °C for 15 min, 94 °C for 30 s (40 cycles), 58 °C for 20 s, and 72 °C for 30 s. The sequences of the designed primers are shown in Table 1. GAPDH was used as the control in the comparative CT method ($2^{-[\Delta][\Delta]Ct}$).

2.6. Western blot analysis

Total protein was extracted from aortic tissues by homogenization in cold RIPA lysis buffer (Amresco, Solon, USA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). As was stated above, the HUVECs were transfected with siRNA, harvested, and lysed in the same buffer. Protein concentrations were determined by the BCA (Bicinchoninic acid) method (Sigma–Aldrich, St. Louis, MO). Equal amounts of protein lysates were mixed with the loading buffer (5×; 1 M Tris–HCl, 50% glycerol, 10% sodium dodecyl sulfate (SDS), trace amounts of bromophenol blue, and distilled water; pH 6.8) and the lysis buffer, denatured at 95 °C for 5 min, and then loaded on a 10% SDS–polyacrylamide gel for electrophoresis. The separated proteins were then transferred to a PVDF membrane through electrophoresis at 0.3 mA/cm^2 for 90 min at room temperature (RT); the transfer buffer (pH 8.3) was composed of 2.5 mM Tris, and 19.2 mM glycine. Residual binding sites on the membrane were blocked by incubation (60 min at RT) with TBS (pH 7.6) containing 0.1% Tween 20, 5% nonfat dry milk. The blots were washed in TBS containing 0.1% Tween 20 and incubated with the appropriate antibody (antibodies against arginase 1 and 2, eNOS, and β-actin [control]; Santa Cruz Biotechnology, CA, USA) overnight at 4 °C. After washing, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse IgG antibody, and the bands were visualized using enhanced chemiluminescence buffer (Young in Frontier Co. Ltd, Seoul, Korea), western blot detection kit. Proteins were quantified by densitometry using Alphaview® software (Alpha Innotech, USA).

2.7. Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA). Results were represented as mean ± SE, and the differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) with Duncan's multiple range analysis. A p value < 0.05 was considered significant.

3. Results

3.1. Effect of arginase inhibition on expression of arginase 1, eNOS and nitrite release in the aorta of HFD-fed mice

Fig. 1 showed the effects of arginase inhibition on the expressions of arginase 1 and eNOS in the aorta of HFD fed mice. In the aorta of HFD-fed mice, arginase 1 expression was found to be significantly elevated, and arginase inhibition lowered these elevated levels (Fig, 1A). The expression of eNOS was lower in the aorta of mice from the HFD group and significantly higher in the HFD with nor-NOHA group than in those of the control group. (Fig. 1B). NO production was evaluated by measuring nitrite levels, a major stable breakdown product of NO. The aorta of the HFD-fed mice showed low levels of nitrite; these levels increased following arginase inhibition (Fig. 1C).

Table 1
Primers used in the experiment.

Gene	Forward primer	Reverse primer
eNOS	5′-CAGTGTCCAACATGCTGCTGGAAATTG-3′	5′-TAAAGGTCTTCTTGGTGATGCC-3′
Arginase 1	5′-GGCTGGTCTGCTTGAGAAAC-3′	5′-ATTGCCAAACTGTGGTCTCC-3′
Arginase 2	5′-GGAACTGGCTGAGGTGGTTA-3′	5′-CTGGCTGTCCATGGAGATTT-3′
GAPDH	5′-TCCACCACCCTGTTGCTGTA-3′	5′-ACCACAGTCCATGCCATCAC-3′

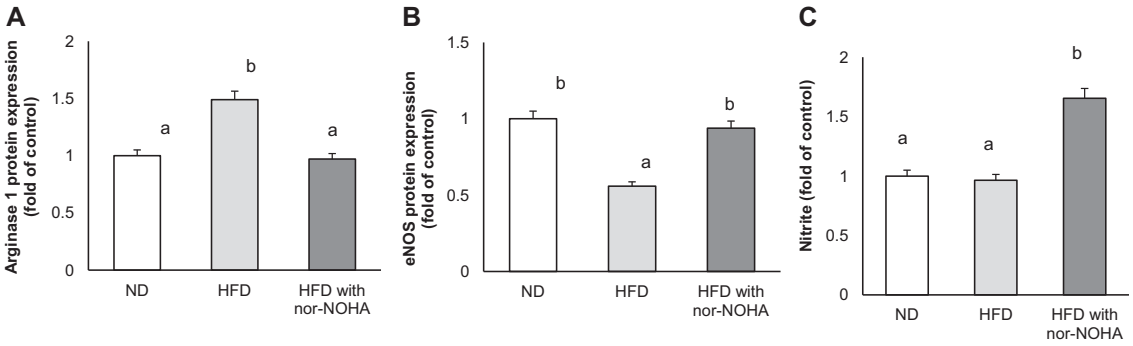

Fig. 1. Effect of arginase inhibitor, nor-NOHA, on the protein levels of arginase 1 (A) and eNOS (B) using representative western blot and on level of nitrite (C) in aorta of mice fed with a normal diet or HFD for 12 weeks. ND: control group; HFD: high-fat diet group; HFD with nor-NOHA: high-fat diet group treated with arginase inhibitor (nor-NOHA, 40 mg/kg/d). β-Actin was used as loading control. The representative image was shown. The results were expressed as means ± S.E. of mice tested by analysis of variance (ANOVA) with Duncan's multiple range test. Sharing the same alphabet indicates no significant difference between two groups ($p < 0.05$).

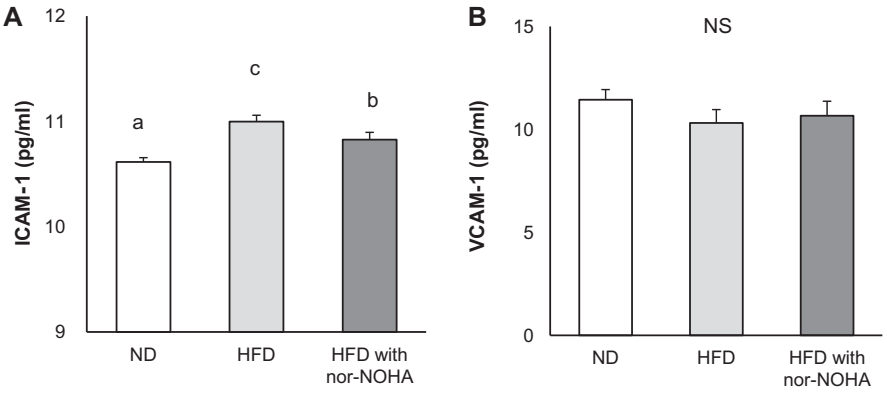

Fig. 2. Effect of arginase inhibitor, nor-NOHA, on plasma ICAM-1 (A) and VCAM-1 (B) in mice fed with a normal diet or high-fat diets for 12 weeks. The results were expressed as means ± S.E. of mice tested by analysis of variance (ANOVA) with Duncan's multiple range test. Sharing the same alphabet indicates no significant difference between two groups ($p < 0.05$).

3.2. Effect of arginase inhibition on the levels of circulating ICAM-1

The HFD-fed mice showed higher plasma levels of ICAM-1 than did mice from the control group (Fig. 2). In mice from the HFD with nor-NOHA group, arginase inhibition resulted in significantly lower plasma levels of ICAM-1.

3.3. Effect of knockdown of Arg1 and Arg2 on the expression eNOS in HUVECs

Fig. 3 shows the confirmation of siRNA knockdown of *Arg* 1 and *Arg* 2 in HUVEC. The protein and mRNA expression of arginase 1 was not detectable in HUVECs (data not shown). On the other hand, eNOS production was upregulated in *Arg2* knockout HUVECs.

4. Discussion

The vascular endothelium plays a key role in the maintenance of normal vascular function by modulating vascular tone, inflammation, and hemostasis [18,19]. Endothelial dysfunction, an important early marker of cardiovascular disease, can also predict the progression of atherosclerosis [20]. Decreased bioavailability of the vasoprotective endothelial NO is known to indicate a dysfunctional endothelium or endothelial dysfunction under pathological conditions and in the presence of risk factors [21–23]. Obesity is a global health problem [24,25] that is closely linked to endothelial dysfunction, and obesity-induced endothelial dysfunction is associated with decreased NO production due to impaired eNOS activity and expression [26]. Arginase has emerged as an important regulator of NO bioavailability; it regulates eNOS production by

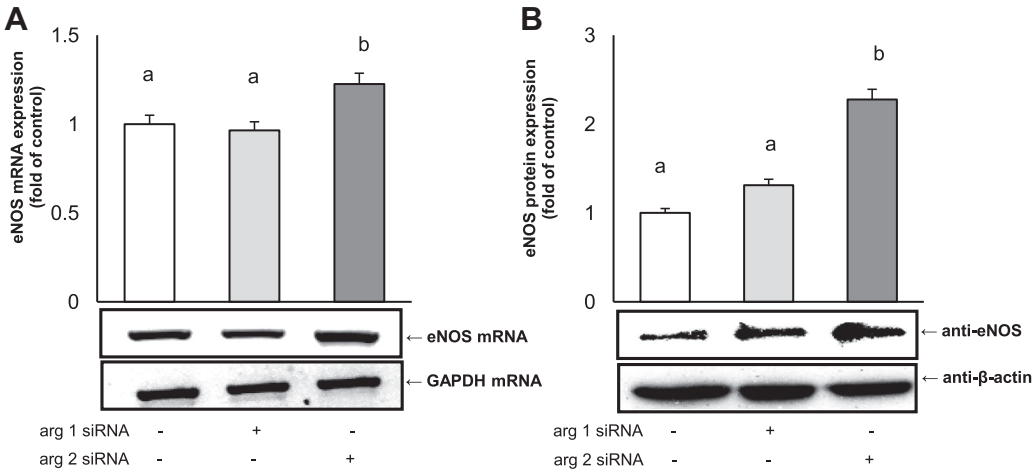

Fig. 3. Effects of siRNA-mediated knockdown of arginase on expression of eNOS in HUVEC cells. The mRNA expression of eNOS (A) using Real-Time PCR and the protein expression of eNOS (B) using immunoblotting in transfected HUVEC cells. The values from the independent experiments were quantified, normalized to GAPDH expression level and expressed as fold changes. β-actin was used as loading control. The representative image was shown. The results were expressed as means ± S.E. of mice tested by analysis of variance (ANOVA) with Duncan's multiple range test. Sharing the same alphabet indicates no significant difference between two groups ($p < 0.05$).

competing for L-arginine, the common substrate for the two enzymes. Increased arginase expression and activity have been observed in diabetes (in animals and humans) [27,28] and obesity (humans) [12]. These findings suggest that arginase expression and/or activity may be responsible for many of the pathological changes associated with the vascular dysfunction possibly via interference with NO bioavailability by limiting L-arginine sources. Arginase 1, encoded by *Arg1*, is a cytosolic enzyme that is abundantly expressed in the liver. *Arg1* expression can be induced by a variety of stimuli such as cAMP, IL-4, and TGF-β [29]. In contrast, arginase 2 is a mitochondrial protein that has a wide tissue distribution, with the expression being the highest in the kidney and prostate and the lowest in the liver.

The aim of this study was to assess the effect of arginase blockade on endothelial function in diet-induced obesity; the influence was estimated by analyzing eNOS expression, NO release in the aorta, and the levels of circulating markers for endothelial function. Expression of arginase 1 was significantly elevated in the aorta of HFD-fed mice, and arginase inhibition with nor-NOHA significantly lowered this elevated expression. This finding is in agreement with the finding that *Arg1* is the predominant isoform expressed in endothelial aortic rings [30] and that it regulates substrate availability for eNOS. In addition, arginase inhibition with nor-NOHA increased the eNOS expression in the aorta and significantly reduced the plasma levels of ICAM-1, a biomarker for endothelial function, in this mouse model of diet-induced obesity. Inhibition of arginase activity by nor-NOHA also restored the NO levels in the aorta of HFD-fed mice. The finding that arginase knockdown led to upregulation of eNOS expression was confirmed by the results of the *in vitro* analysis in HUVECs. These results are consistent with previous findings that arginase inhibition could restore endothelial function to some extent in the vasculature of experimental models of atherosclerosis, myocardial ischemia, hypertension, and aging [31–34]. However, our findings in this mouse model of obesity clearly support the important role of *Arg1* in vascular endothelial dysfunction and regulation of NO bioavailability, a role that has not been studied to date. In the present study, the association between arginase inhibition and systemic endothelial function was analyzed by estimating the plasma levels of ICAM-1, because endothelial NO is an important anti-inflammatory molecule that suppresses the expression of adhesion molecules such as VCAM-1 and ICAM-1 [35]. In our previous study, a positive association was observed between arginase mRNA level in PBMCs and the levels of soluble VCAM-1 and ICAM-1 [12,36]. Our results showed that the high plasma levels of ICAM-1 in the HFD-fed mice were reduced by nor-NOHA supplementation. ICAM is a cytokine expressed in the vascular endothelium and elevation of its levels is indicative of endothelial dysfunction [37]. Furthermore, circulating soluble cell adhesion molecules have been known to be elevated in patients with chronic diseases and metabolic disorders, including obesity and atherosclerosis [37]. The negative association between obesity and the levels of ICAM-1 could explain the mechanism by which obesity is a risk factor for atherosclerotic diseases.

In our HFD-induced obesity model, we found that the presence of an arginase inhibitor lowered the elevated ICAM-1 levels that are seen in dysfunctional endothelium. These observations were further supported by the results of siRNA-mediated knockdown of arginase. Arginase 1 was not expressed in detectable levels in the siRNA-transfected HUVECs. This is consistent with the previous finding that the predominant arginase isoform in HUVECs was arginase 2 [38]. However, eNOS expression was upregulated when HUVECs were transfected with arginase 2 siRNA. This result was similar to that of another study in which a flavanol-rich cocoa, which is known to increase circulating NO levels, was found to reduce vascular arginase 2 activity in HUVECs [39]. In conclusion, the upregulation of arginase activity may play a key role in the endothelial dysfunction observed in diet-induced obesity. Arginase inhibition restored dysregulated endothelial function both *in vitro* and *in vivo*. Thus, reduced arginase activity and/or expression and increased eNOS-dependent NO production in the endothelium might contribute to this restorative effect. Arginase can therefore be considered a therapeutic target for the correction of vascular endothelial dysfunction induced by obesity.

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