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Arginase inhibition ameliorates adipose tissue inflammation in mice with diet-induced obesity

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ABSTRACT

This study examined whether oral administration of an arginase inhibitor regulates adipose tissue macrophage infiltration and inflammation in mice with high fat diet (HFD)-induced obesity. Male C57BL/6 mice (n = 30) were randomly assigned to control (CTL, n = 10), HFD only (n = 10), and HFD with arginase inhibitor $N^{(\omega)}$-hydroxy-nor-L-arginine (HFD with nor-NOHA, n = 10) groups. Plasma and mRNA levels of cytokines in epididymal adipose tissues (EAT), macrophage infiltration into EAT, and macrophage phenotype polarization were measured in the animals after 12 weeks. Additionally, the effects of nor-NOHA on adipose tissue macrophage infiltration and mRNA expression of cytokines were measured in co-cultured 3T3-L1 adipocytes and RAW 264.7 macrophages. Macrophage infiltration into the adipocytes was significantly suppressed by nor-NOHA treatment in adipocyte/macrophage co-culture system and mice with HFD-induced obesity. Pro-inflammatory cytokines, including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), were significantly downregulated, and the anti-inflammatory cytokine IL-10 was significantly upregulated in nor-NOHA-treated co-cultured cells. In the mice with HFD-induced obesity, plasma and mRNA levels of MCP-1 significantly reduced after supplementation with nor-NOHA. In addition, oral supplement of nor-NOHA modified M1/M2 phenotype ratio in the EAT. Oral supplementation of an arginase inhibitor, nor-NOHA, altered M1/M2 macrophage phenotype and macrophage infiltration into HFD-induced obese adipose tissue, thereby improved adipose tissue inflammatory response. These results may indicate that arginase inhibition ameliorates obesity-induced adipose tissue inflammation.

1. Introduction

Obesity is a serious global health problem that increases the risk for various metabolic disorders, such as hypertension, hyperlipidemia, type 2 diabetes, and cardiovascular disease [1–4], and is linked to chronic, low-grade systemic inflammatory response [5–7]. This process is associated with increased infiltration of inflammatory cells, macrophages, into the expanded pool of white adipose tissues, which increases production of inflammatory cytokines and adipokines and contributes to the development of insulin resistance (IR) [5–9].

Adipose tissue inflammation is characterized macrophages, which predominantly include a classical pro-inflammatory macrophage (M1) and an anti-inflammatory tissue-residing macrophage (M2) [10]. M1 macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-a), interleukin (IL)-6, and IL-12, and thereby contribute to local and systemic chronic inflammation and IR [10–13]. In obese states, the expanded adipocytes trigger this macrophage

Abbreviations: nor-NOHA, $N^{(\omega)}$-hydroxy-nor-L-arginine; MCP-1, monocyte chemotactic protein 1; ATM, adipose tissue macrophage; IL-10, interleukin-10; EAT, epididymal adipose tissue.

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accumulation [10,11]. M1 macrophages also generate excessive nitric oxide (NO) by activating inducible nitric-oxide synthase (iNOS), which favors the generation of reactive oxygen species (ROS) [10]. Alternatively, M2 macrophages secrete anti-inflammatory cytokine IL-10 and produce arginase-1, which metabolizes L-arginine to urea and L-ornithine. Activation of arginase-1 potentially inhibits NO production by competing with iNOS for the substrate L-arginine [14–16].

Recently, it was reported that increased arginase gene expression and/or arginase activity were related to metabolic disorders such as type 2 diabetes in both animal models and humans [16–18], and the inhibition of arginase has been suggested to be a promising therapeutic target for diabetes and vascular treatments [19–22]. Arginase inhibition may augment NO production by shunting L-arginine from the arginase pathway to the endothelial NOS pathway [19,20], thereby improving endothelial dysfunction [19]. Treatment with an arginase inhibitor, $N^{(\omega)}$-hydroxy-nor-L-arginine (nor-HONA), also restored endothelial function in rat adjuvant-induced arthritis [21]. In addition, we previously reported that arginase inhibition ameliorated obesity-induced hepatic lipid abnormalities and whole body adiposity, possibly because of increased hepatic NO production and subsequent activation of metabolic pathways involved in hepatic lipid metabolism and mitochondrial function [22]. However, little attention has been devoted to the effect of arginase inhibition on adipose tissue inflammation.

As an extended study previously showing ameliorating effect of arginase inhibition on obesity, endothelial dysfunction and metabolic abnormalities [22,23], this study aimed to examine whether oral supplementation of arginase inhibitor nor-NOHA could regulates adipose tissue macrophage infiltration and inflammation in high fat-fed obese mice.

2. Materials and methods

2.1. Animals and experimental protocol

Male C57BL/6 mice (n = 30, 4 weeks old, purchased from DBL, Chungbuk, Korea) were randomly assigned into three groups after a week acclimation period: control (CTL, n = 10), high-fat diet (HFD, 40% fat of total calories, n = 10), and HFD with arginase inhibitor nor-NOHA (HFD with nor-NOHA, n = 10). Mice were maintained under specific pathogen-free (SPF) controlled conditions (18–24 °C, room humidity 50–60 %) and provided a group-specific diet and water for 12 weeks. All mice in the CTL group were fed a normal diet based on the AIN-76 rodent diet. For obesity induction, all mice in the HFD and HFD with nor-NOHA groups were fed the same HFD for 7 weeks. Some fed only a HFD were sham gavaged and others were orally gavaged with 40 mg/kg nor-NOHA (Bachem, Bubendorf, Switzerland) dissolved in 0.9% NaCl solution for 5 weeks. This work is the extended study for our previous report [23] and all experimental procedures were approved by the Institutional Animal Care and Use Committee as governed by the National Institute of Health's "Guide for the Care and Use of Laboratory Animals and by the Committee on Animal Experimentation and Ethics of Korea University" (KUIACUC-2013-96). After the 12 weeks study period, the mice were sacrificed after a 12 h fast. They were anesthetized with 30 mg/kg Zoletil (Virbac, Carros, France) mixed with 10 mg/kg Rompun (Bayer, Seoul, Korea). Epididymal adipose tissue (EAT) was extracted, washed with 1 × phosphate-buffered saline (PBS), weighted, and then placed into 10% formaldehyde solution or rapidly frozen in liquid nitrogen and stored in a deep freezer at −80 °C until analysis.

2.2. Co-culture of 3T3-L1 adipocytes and RAW 264.7 macrophages

Mouse pre-adipocyte 3T3-L1 cells were cultured and differentiated as previously described [24]. Culture media were exchanged after every 2 or 3 days. 3T3-L1 cells were seeded at a density of 1×10^5 cells/well in a 6-well plate until fully differentiated. Mouse macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium that contained 10% heat inactivated fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin. RAW 264.7 cells were stimulated with or without LPS (1 μg/ml) for 12 h, after that, washed and plated onto the fully differentiated adipocytes for 24 h co-culture and starvation [25]. The numbers of macrophages were equal to those of fully differentiated adipocytes in the co-culture. Then, the cells were treated or not treated with nor-NOHA (50 μM) for another 24 h. Subsequently, the cells were harvested for immunofluorescence assay.

2.3. Confocal microscopy analysis

The effects of nor-NOHA and LPS separately or in combination on macrophage infiltration into the adipocytes were detected by immunofluorescence assays using confocal microscopy. Cells were washed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at 4 °C and blocked with a solution of 10% normal goat serum (Sigma, St. Louis, MO, USA) and 0.2% Tween 20 in PBS. Monoclonal antibody F4/80 was diluted 1:200 and incubated overnight at 4 °C. The cells were incubated with Cy5.5-labeled secondary antibodies for 1 h. Nuclei were stained with the fluorescent dye 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St. Louis, MO, USA). Images were taken using an A1Si confocal laser-scanning microscope (Nikon, Tokyo, Japan).

2.4. RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To analyze the mRNA expression of genes in EAT, total RNA was extracted from 500 mg of EAT with QIAzol lysis reagent (RNeasy Lipid Tissue Mini Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Total RNA was also extracted from 3T3-L1 and Raw 264.7 co-culture cells using a MagAttract RNA Cell Mini M48 kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 μg of RNA using oligo-dT and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). One microgram of cDNA was subjected to quantitative real-time PCR amplification using the SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). PCR conditions were as follows: 15 min at 95 °C, followed by 40 cycles at 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 30 s (Step One Plus, Applied Biosystems, Foster City, CA, USA). GAPDH was used as the control in the comparative cycle threshold (Ct) method.

2.5. Plasma cytokine assay

Circulating inflammatory proteins, monocyte chemoattractant protein-1 (MCP-1) and IL-6 were measured using an enzyme linked immunosorbent assay kit (Abcam, Cambridge, MA, USA).

2.6. Immunohistochemistry analysis

To evaluate macrophage infiltration into the adipocytes, EAT samples were fixed with a 10% formaldehyde solution and

embedded in paraffin. Five-micrometer sections were deparaffinized, blocked, sequentially incubated at 4 °C for 24 h with the primary mouse monoclonal antibodies, including anti-F4/80 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD68 antibody, and anti-IL-10 antibody (Abcam, Cambridge, MA, USA), followed by incubating peroxidase conjugated goat anti-mouse secondary antibody for 30 min. These sections were then stained for 3 min with a diaminobenzidine (DAB) kit (Nichirei, Tokyo, Japan), viewed with an optical microscope (Nikon, Tokyo, Japan), and captured at 400× and 1200× magnification. After counting the F4/80-positive cells in each image, the minimum and maximum values were excluded, and the averaged value of six images was calculated. To identify macrophage phenotype, the CD68-positive cells and IL-10-positive cells in five images were counted and averaged, excluding the minimum and maximum values.

2.7. Statistical analysis

Statistical analysis was performed using SPSS 21.0 (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA). The results are represented as mean ± S.E. and the differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) with Duncan's multiple range tests, with $p < 0.05$ as the criterion of significance.

3. Results

3.1. Effects of arginase inhibition on diet-induced obesity

Consistent with our earlier study [22], significant differences in body weight gains between HFD-fed mice and HFD with nor-NOHA-fed mice were observed. HFD with nor-NOHA-fed mice showed a remarkably lower body weight compared to mice fed HFD alone (28.79 ± 2.14 vs 32.55 ± 3.76 g, $p < 0.001$). After 12 weeks, HFD with nor-NOHA-fed mice showed significantly reduced adipose tissue weights compared with the HFD-fed mice (1.16 ± 0.01 vs 1.34 ± 0.02 g, $p < 0.001$).

3.2. Effects of arginase inhibition on macrophage infiltration into adipocytes in the co-cultured cell system and HFD-fed mice

MTT assay shows that nor-NOHA and LPS separately or in combination did not affect cell viability in both 3T3-L1 and RAW 264.7 cells (data not shown). In the co-cultured cell system, immunofluorescence staining assay showed that the expression of macrophage-specific F4/80 increased in LPS-stimulated cells but decreased when treated with nor-NOHA (Fig. 1A). In addition, F4/80 expression was significantly reduced in EAT from mice fed HFD with nor-NOHA compared with those fed only a HFD (Fig. 1B and C).

3.3. Effect of arginase inhibition on cytokine gene expression in 3T3-L1 adipocytes co-cultured with RAW 264.7 cells

Fig. 2 shows the effect of nor-NOHA on mRNA expression of pro-inflammatory cytokines (MCP-1, TNF-α, and IL-6) and an anti-inflammatory cytokine (IL-10) in 3T3-L1 cells co-cultured with RAW 264.7 cells. The mRNA levels of pro-inflammatory cytokines were markedly upregulated in LPS-stimulated cells compared with non-stimulated cells. When LPS-stimulated cells were treated with nor-NOHA, the mRNA expressions were significantly attenuated and close to the levels in the non-stimulated cells (Fig. 2A–C). Alternatively, the mRNA level of anti-inflammatory cytokine, IL-10, was significantly upregulated in the nor-NOHA-treated LPS-stimulated cells compared with the non-stimulated or LPS-only-stimulated cells (Fig. 2D).

3.4. Effect of arginase inhibition on cytokine gene expressions in EAT and plasma cytokines in HFD-fed mice

The mRNA levels of MCP-1 and TNF-α were significantly higher in the mice fed only a HFD compared with the CTL mice. The increased mRNA levels of MCP-1 in the HFD-fed mice were significantly attenuated after the supplementation of nor-NOHA, but those of TNF-α were not attenuated (Fig. 2E and F). Alternatively, IL-6 mRNA levels were not significantly different among the three groups, and the IL-10 mRNA levels were significantly lower in the mice fed a HFD with or without nor-NOHA than the CTL mice (Fig. 2G and H). In addition, plasma levels of MCP-1 were significantly higher in the mice fed only a HFD than the CTL mice and was significantly reduced in those fed HFD with nor-NOHA. However, plasma IL-6 levels were not significantly different among the three groups (Fig. 2I and J).

3.5. Effects of arginase inhibition on the modulation of macrophage phenotype polarization

The number of ETA pro-inflammatory M1 (CD68-positive) macrophages was significantly higher in the HFD-fed mice compared with the CTL mice. The increased M1 numbers were significantly reduced by the supplementation of nor-NOHA, which produced similar results to that of CTL mice (Fig. 3A and B). Alternatively, the number of ETA anti-inflammatory M2 (IL-10-positive) macrophages was significantly reduced in the mice fed only a HFD compared with the CTL mice. The M1 numbers in the HFD-fed mice were slightly reduced by the supplementation of nor-NOHA, but this finding did not reach the statistical significance (Fig. 3C and D). These results demonstrated that oral supplement of nor-NOHA reduced the number of pro-inflammatory M1 macrophages but increased the number of anti-inflammatory M2 macrophages, thereby modifying M1/M2 phenotype ratio in EAT.

4. Discussion

This study showed that oral supplementation of an arginase inhibitor, nor-NOHA, influenced macrophage infiltration into HFD-induced obese adipose tissue and modified M1/M2 macrophage phenotype, thereby improving adipose tissue inflammatory response. These phenomena were also partly confirmed in the 3T3-L1 cells and RAW 264.7 macrophage co-cultured system, indicating that arginase inhibition ameliorates obesity-induced adipose tissue inflammation.

Numerous studies have attempted to determine how to improve obesity and related metabolic disorders, such as hyperglycemia, IR, diabetes, cardiovascular disease, and inflammatory responses [26,27]. Recent studies have suggested benefits of arginase inhibition for treating diabetes, vascular disease, and obesity-related disorders [16,19–23,28–32]. Arginase is expressed in endothelial and vascular smooth muscle cells and is responsible for converting L-arginine to urea and L-ornithine [33]; thus, arginase competes with iNOS for the substrate L-arginine [14–16], thereby down-regulating NO biosynthesis. Therefore, arginase inhibition may increase NO production by shunting L-arginine from the arginase pathway to the endothelial NOS pathway [19,20], which in turn improves endothelial dysfunction [19]. Macrophage infiltration is referred to as a hallmark of inflammation [9], and macrophage infiltration into white adipose tissue (WAT) and secretion of adipokines and cytokines are involved in the initiation of chronic low-grade inflammatory response observed in obesity [5–9]. Thus, suppressing adipose tissue macrophage activation might be a target for the treatment of obesity-induced inflammation. Among the pro-inflammatory cytokines, MCP-1 is mostly produced by infiltrated

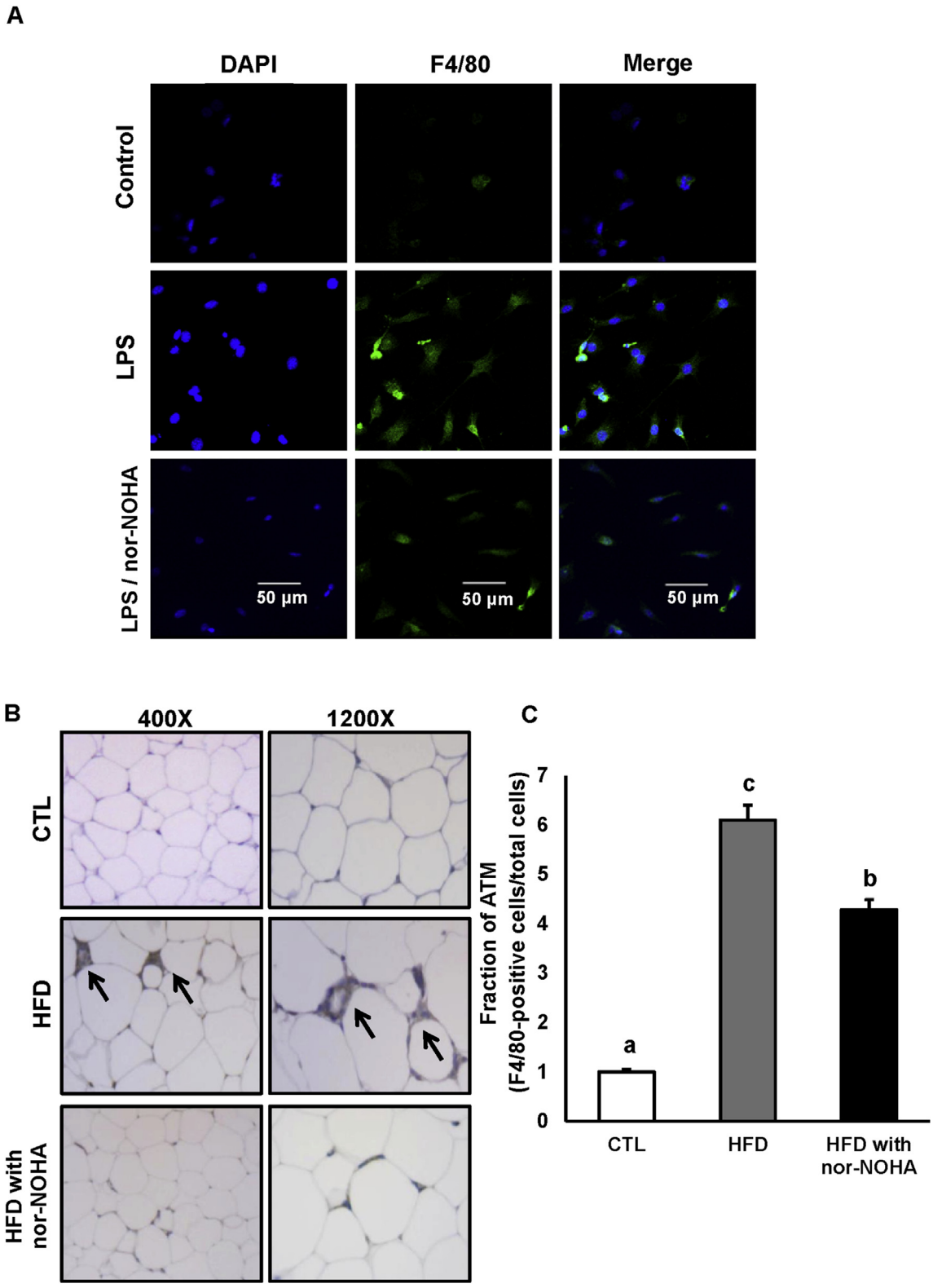

Fig. 1. Effect of arginase inhibitor, nor-NOHA, on macrophage infiltration. (A) Immunofluorescence staining of 3T3-L1 adipocytes and Raw 264.7 macrophages co-culture were performed with an anti-F4/80 antibody, followed by an FITC-conjugated secondary antibody (green). The nuclei were re-dyed with DAPI (blue). (B) Immunohistochemical staining of epididymal adipose tissue with anti-F4/80 antibody. (C) Macrophage content was quantified by analyzing the fraction of F4/80-stained cells relative to the total number of cells. Mice fed with a normal diet or high-fat diets for 12 weeks. The results were expressed by 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 ($p < 0.05$). Bar = 200 um; 20 um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

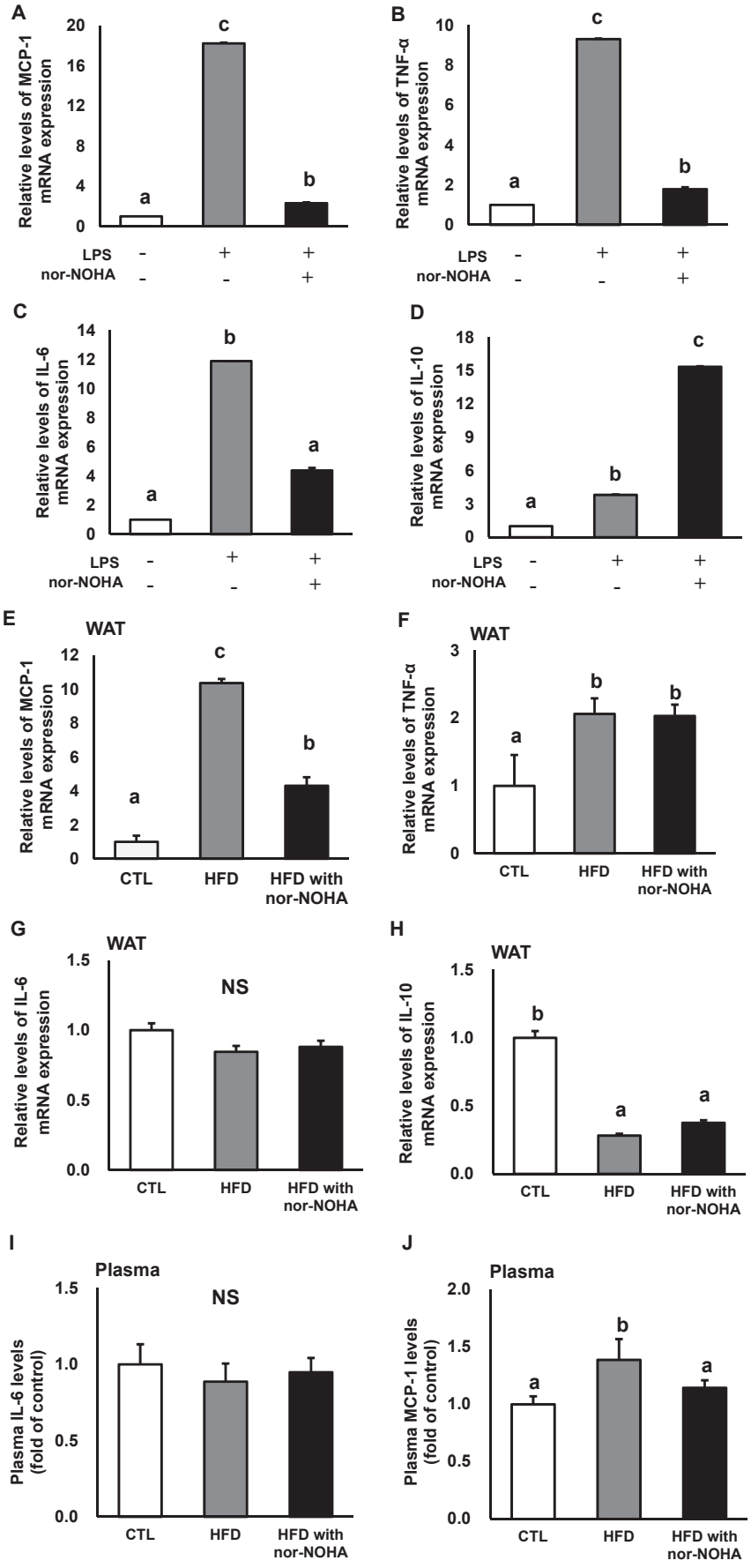

Fig. 2. Effect of arginase inhibitor, nor-NOHA, on gene expression and plasma levels of inflammatory cytokines. The mRNA expression of MCP-1, TNF-α, IL-6, and IL-10 in adipocyte and macrophage co-culture, and epididymal adipose tissue were evaluated by using Real-Time PCR, the plasma levels of inflammatory cytokines were measured by ELISA assay. The values from the independent experiments were quantified, normalized to GAPDH expression level and expressed as fold changes. The results were expressed as means ± S.E. of mice tested by analysis of variance (ANOVA) with Duncan's multiple range tests. Sharing the same alphabet indicates no significant difference between two groups ($p < 0.05$).

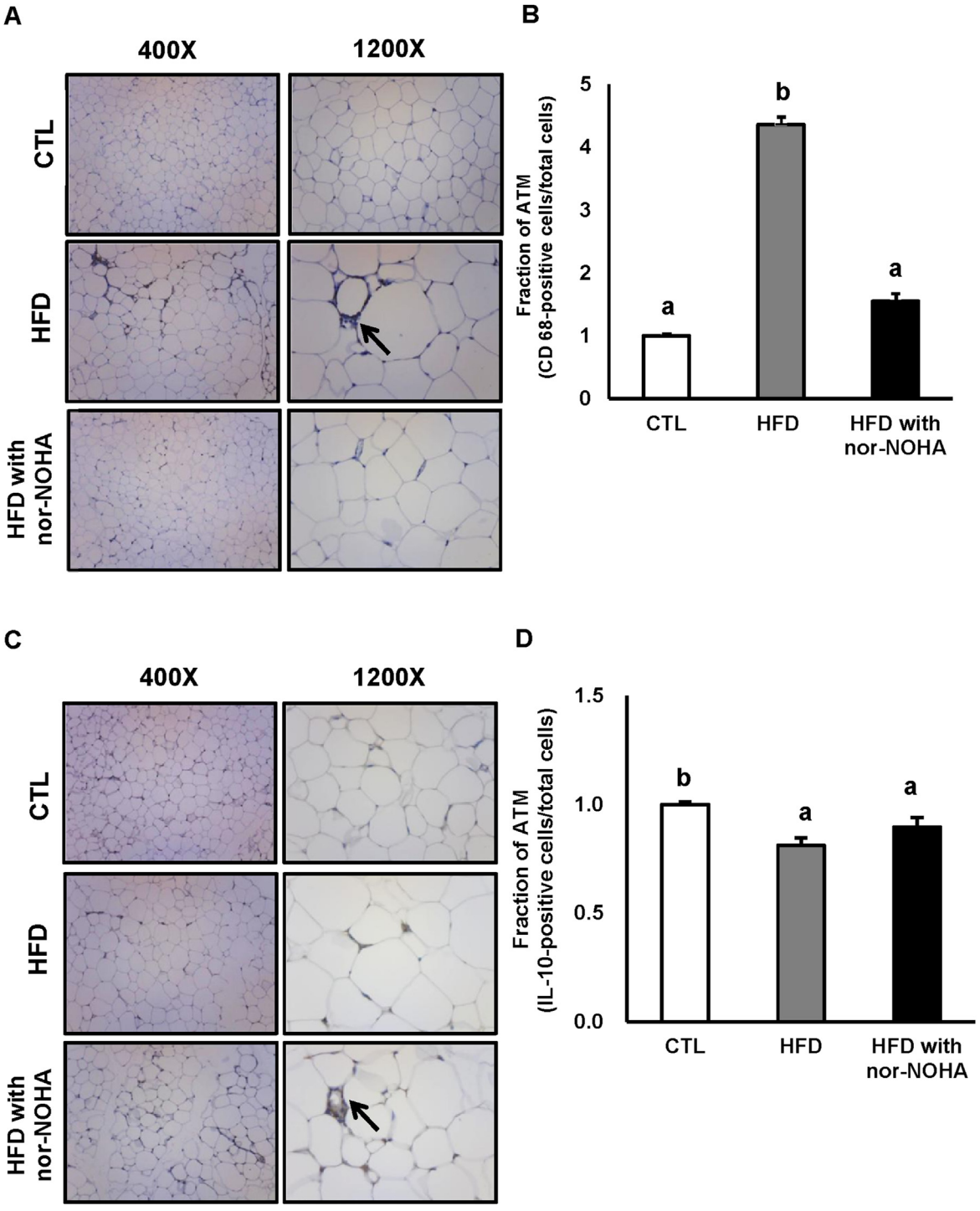

Fig. 3. Effect of arginase inhibitor, nor-NOHA, on macrophage phenotype polarization. Immunohistochemical staining with anti-CD68 (M1 marker) (A), and anti-IL-10 (M2 marker) (C) antibodies in epididymal adipose tissue. Macrophage content was quantified by analyzing the fraction of CD68 (B), IL-10 (D) stained cells relative to the total number of cells in epididymal adipose tissue from mice fed with a normal diet or high-fat diets for 12 weeks. The results were expressed by 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 ($p < 0.05$). Bar = 200 um.

macrophages, and increased MCP-1 expression occurs in both WAT and plasma in obese mice [34,35]. Epidemiological studies have also demonstrated that MCP-1 contributes to regulation of macrophage infiltration, thereby further modulating the process of atherogenesis and IR [35,36].

In the present study, we showed that macrophage infiltration into the adipocytes was significantly reduced by nor-NOHA treatment in both co-cultured cells and HFD-fed mice. In particular, oral supplementation of nor-NOHA reduced macrophage infiltration into EAT and circulating levels of MCP-1. The underlying mechanisms by which arginase inhibition affects macrophage infiltration into adipose tissue are still unknown. Previous studies reported that mice deficient in macrophage-specific chemokines such as MCP-1 and CXCL14 do not exhibit inflammatory responses

even in obese mice [34,37,38]. This might be similar to our results, which showed that plasma and mRNA levels of MCP-1 in HFD-fed mice were significantly reduced by nor-NOHA supplementation, although the increased mRNA levels of TNF-α in the HFD-fed mice were not significantly attenuated after nor-NOHA supplementation. However, we failed to find any significant alteration in plasma and mRNA levels of IL-6 by nor-NOHA supplementation in animal models. In addition, the downregulated mRNA levels of IL-10, the anti-inflammatory marker in HFD-fed mice, were slightly increased by oral supplementation of nor-NOHA, but this did not reach the statistically significance. In fact, the effect of arginase inhibition on pro-inflammatory cytokines (downregulation of MPC-1, TNF-α, and IL-6) and anti-inflammatory cytokine (upregulation of IL-10) were clearly observed in *in vitro* co-cultured cells, but the alterations of these cytokines except MCP-1 in mice models with HFD-induced obesity were not as remarkable as those in an *in vitro* system. The discrepancy in results between the co-cultured cellular models and HFD-induced mice models may be related with the complexity of the experimental model (i.e., *in vitro* or *in vivo*) and characteristics. Therefore, further studies are needed to establish optimal dosages and treatment periods for the efficacy of this arginase inhibitor. Moreover, in obesity-induced inflammation, polarity of macrophage subpopulations occurs, and M1 macrophages are increased, whereas the M2 macrophage fraction is reduced [39]. M1 and M2 macrophage phenotypes can be differentiated by macrophage surface markers such as CD68 and IL-10, respectively [39,40]. In the present study, we tested the hypothesis that the M1 to M2 phenotypic change by arginase inhibition results in inflammation regulation. The results showed that down-regulation of CD68 mRNA, which reflects the presence of the M1 macrophage, and up-regulation of IL-10 mRNA, which reflects the presence of the M2 macrophage, were observed in EAT of mice fed a HFD with nor-NOHA. Thus, these data indicate that oral supplementation of nor-NOHA modulated macrophage phenotypic change from M1 to M2 in EAT. These results demonstrate that oral supplementation of an arginase inhibitor, nor-NOHA, may downregulate adipose tissue pro-inflammatory response by altering macrophage infiltration into HFD-induced obese adipose tissue and the M1/M2 macrophage phenotype.

This study has several limitations. First, the macrophages were not isolated from the adipose tissues. It is known that inflammatory cytokines such as TNF-α, MCP-1, and IL-6 are expressed on adipocytes and macrophages. Therefore, these results should be elucidated using macrophages isolated from adipose tissue. Second, further study on the signaling pathways (i.e., COX2 and NOS) related to adipose tissue inflammation is needed to decipher the underlying mechanisms. Despite these limitations, this study shows that oral supplementation of an arginase inhibitor, nor-NOHA, may alter macrophage infiltration into HFD-induced obese adipose tissue and M1/M2 macrophage phenotype, thereby improving adipose tissue inflammation. In conclusion, these results indicate that arginase inhibition can ameliorate obesity-induced adipose tissue inflammation.

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