ORIGINAL ARTICLE

Inhibition of arginase ameliorates experimental ulcerative colitis in mice

Yuji Akazawa[1], Masayuki Kubo[1], Ran Zhang[1], Kaori Matsumoto[1], Fulin Yan[1], Heri Setiawan[1], Hidekazu Takahashi[1], Yoshihisa Fujikura[2], & Keiki Ogino[1]

[1]*Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*

[2]*Department of Molecular Anatomy, Faculty of Medicine, Oita University, Oita, Japan*

Short title: *Effect of nor-NOHA on DSS-induced colitis*

Correspondence: Keiki Ogino, Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558 Japan

Tel: +81-86-235-7179; Fax: +81-86-226-0715; E-mail: kogino@md.okayama-u.ac.jp

Keywords: *nitric oxide; N^{ω}-hydroxy-nor-L-arginine; L-arginine; NOx; dextran sulfate sodium*

Abstract

Nitric oxide (NO) is produced from the conversion of L-arginine by NO synthase (NOS) and regulates a variety of processes in the gastrointestinal tract. Considering the increased activity of arginase in colitis tissue, it is speculated that arginase could inhibit NO synthesis by competing for the same L-arginine substrate, resulting in the exacerbation of colitis. We examined the role of arginase and its relationship to NO metabolism in dextran sulfate sodium (DSS)-induced colitis. Experimental colitis was induced in mice by administration of 2.5% DSS in drinking water for 8 days. Treatment for arginase inhibition was done by once daily intraperitoneal injection of N^{ω}-hydroxy-nor-arginine (nor-NOHA). On day 8, we evaluated clinical parameters (body weight, disease activity index, and colon length), histological features, the activity and expression of arginase, L-arginine content, the expression of NO synthase (NOS), and the concentration of NO end-product (NOx: nitrite + nitrate). Administration of nor-NOHA improved the worsened clinical parameters and histological features in DSS-induced colitis. Treatment with nor-NOHA attenuated the increased activity of arginase, upregulation of arginase I at both mRNA and protein levels, and decreased the content of L-arginine in colonic tissue in the DSS-treated mice. Conversely, despite the decreased expression of NOS2 mRNA, the decreased

concentration of NOx in colonic tissues was restored to almost normal levels. The consumption of L-arginine by arginase could lead to decreased production of NO from NOS, contributing to the pathogenesis of the colonic inflammation; thus, arginase inhibition might be effective for improving colitis.

Introduction

The prevalence of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, has been increasing [1]. These disorders manifest several clinical symptoms, including weight loss, diarrhea, bleeding, and fever, and are characterized by a clinical course with remission and exacerbation [2-3]. Specific histological findings for inflammatory bowel diseases include inflammatory cell infiltration, including eosinophils, neutrophils, monocytes and mast cells into the gut mcucosa, goblet cell depletion, crypt abscesses, and distortion of mucosal glands [4].

Nitric oxide (NO) is known as a free radical that regulates a variety of processes in the gastrointestinal tract, including blood flow, vascular permeability, mucosal defense, leukocyte recruitment, immune regulation, fluid secretion, and intestinal motility [5,6]. NO is largely produced from L-arginine as a substrate by three isoforms of NO synthase (NOS). Two isoforms of NOS, neuronal (nNOS, NOS1) and endothelial (eNOS, NOS3),

are constitutively expressed and are calcium dependent. The third isoform of NOS is inducible NOS (iNOS, NOS2), expressed in macrophages, neurons and endothelial cells and, when induced, can produce a large amount of NO compared to other constitutive-type NOS [7,8]. Interestingly, there is some evidence that supplementation of nitrite [9] or L-arginine [10] ameliorates the colonic injury of experimental colitis, while administration of NOS inhibitor worsens the injury [11]. Given the findings, it is likely that NOS-derived NO plays an important role in healing ulcerative colitis.

In addition to NOS, L-arginine is also used by arginase I or II to maintain the urea cycle. Arginase may inhibit NO synthesis by limiting the supply of intracellular L-arginine to NOS [12-14]. In experimental asthma and human asthmatic patients, it is suggested that upregulated arginase I consumed L-arginine and resulted in the depletion of NO to enlarge bronchial smooth muscle [15-18]. Considering that the activity and expression of arginase was increased in human inflammatory bowel disease [19], in ulcerative colitis it is speculated that overexpression of arginase might modulate NO metabolism by the consumption of L-arginine; however, there is no evidence to clarify the role of arginase and its relationship to NO production in the pathogenesis of ulcerative colitis.

Therefore, the aim of this study was to address the contribution of arginase to

experimental colitis induced by dextran sulfate sodium (DSS). Using an arginase inhibitor, N^{ω}-hydroxy-nor-L-arginine (nor-NOHA), we investigated whether the clinical signs of DSS-induced colitis were improved by the regulation of NO production via modulating L-arginine metabolism.

Methods

Animals

Male 6-week-old C3H mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). All mice were maintained under a 12-h light/12-h dark cycle and had free access to standard laboratory food and tap water. They were acclimatized for at least 1 wk before the experiments. The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shikata Campus of Okayama University and approved by the Okayama University Institutional Animal Care and Use Committee.

Induction of colitis and treatment of nor-NOHA

Experimental ulcerative colitis was induced in mice by administrating 2.5% (w/v) dextran sulfate sodium (molecular weight 5000; Wako Pure Chemicals, Osaka, Japan) in

drinking water for 8 days [9,20]. Treatment for inhibition of arginase was done by once daily intraperitoneal injection of nor-NOHA (8.5 or 17 mg/kg, Bachem, Bubendorf, Switzerland) after DSS administration. The first administration of nor-NOHA was just before the start of DSS administration. Control mice were allowed to drink only water. Body weight of mice was recorded daily and is expressed as a change from baseline.

Evaluation of severity of colon damage

To assess colon damage severity, the disease activity index was determined macroscopically as the sum of the scores from three major clinical signs (body weight loss, diarrhea, and rectal bleeding), as described previously [20]. Body weight, diarrhea score, and bleeding score of each mouse were assessed daily. Change in body weight in the mice was calculated as the difference between the expected and actual weight. The formula for predicted body weight was derived by simple regression using the body weight data for the control group. The following formula was used: $Y = a + kx$, where Y = body weight change (loss or gain), k = daily increase in body weight, x = day, a = starting body weight. Diarrhea was defined as mucus or fecal material adhering to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively. Rectal bleeding was defined as diarrhea containing visible blood and/or mucus or gross rectal

bleeding and was scored as either 1 or 0, respectively.

Harvest of colon tissue and measurement of colon length

All mice were euthanized 8 days after treatment and the colon was harvested. The length of the colon was measured from the ileo-cecal junction to the anal verge.

Preparation of colon specimen

After measuring the colon length, part of the colon tissue samples was used for a histological examination and extraction of total RNA for reverse transcription (RT)-PCR. The remaining colon tissues were homogenized in homogenizing buffer [20mM Tris-HCl, pH7.5, 150 mM NaCl, and 1 mM EDTA containing a protease inhibitor cocktail (Roche, Mannheim, Germany)] with or without 1% Triton X-100, and used for further analysis.

Histological evaluation

The colon tissues were fixed in 10% neutral phosphate-buffered formalin, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. A histological assessment of the colonic mucosa was performed as described previously

[21,22]. The severity of inflammation (0-3), extent of inflammation (0-3), and crypt damage (0-4) were individually scored, and the sum was used as a score of histological injury.

Immunohistochemistry

An immunohistochemical analysis was performed as described previously [15,23] . Briefly, colon tissue sections were incubated in methanol containing 1% H_2O_2 for 30 min. After three washes in Tris-buffered saline, sections were incubated in 5% normal goat serum. The specimens were stained at 4°C overnight with rabbit polyclonal antibodies against arginase (1:40, H-52, Santa Cruz Biotechnology Inc., Santa Cruz, CA), or NOS2 (1:1000, M-19, Santa Cruz Biotechnology Inc.). The specimens were then treated with goat anti-rabbit immunoglobulin conjugated with peroxidase-labeled polymer (DakoCytomation Inc., Carpinteria, CA) for 1 h at room temperature, visualized with 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation Inc.), and counterstained with hematoxylin.

RT-PCR

To examine the expression of mRNA for arginase and NOS isoform, the total RNA of

each sample of colon tissue was extracted with ISOGEN (Nippon Gene, Tokyo, Japan). RT-PCR was performed using TaKaRa RNA PCR kit AMV ver.3.0 (TaKaRa Bio Inc., Otsu, Japan) and a TaKaRa PCR thermal cycler MP (TaKaRa Bio Inc.) with oligo-dT primers according to the manufacturer's instructions. Primer sets and PCR conditions are detailed in Table 1. We preliminarily employed the cycle-dependent linearity of PCR bands. Each PCR product was detected by agarose gel electrophoresis and ethidium bromide staining. The density of each PCR band was quantified by ImageJ software (National Institute of Health, Bethesda, MD) and normalized against GAPDH.

Measurement of arginase activity

Arginase activity was measured as described previously [15,16,23,24]. Briefly, the colon tissue homogenates treated with 1% Triton X-100 were centrifuged at 10,5000 x g for 60 min at 4ºC (Beckman TL-100; Beckman Coulter, Fullerton, CA). Supernatant was preincubated in the presence of $MnCl_2$ at 55°C for 10 min, and then incubated with L-arginine at 37ºC for 60 min. The reaction was stopped by an acid solution (H_2SO_4:H_3PO_4:H_2O = 1:3:7 vol). After the addition of α-isonitrosopropiophenenone, the mixture was heated at 100ºC for 45 min. The amount of reaction product, urea, was measured colorimetrically at 540 nm.

Western blot analysis

To investigate the protein expression of arginase isoform, Western blot analysis was performed as described previously [15,16,23]. An equal amount of protein from colon homogenate with 1% Triton X-100 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA). After blocking with 5% dried skimmed milk in Tris-buffered saline containing 0.5% Tween 20 (TBS-T), membranes were incubated with polyclonal rabbit antibodies for arginase I or arginase II (1:200, Santa Cruz Biotechnology Inc.). Goat horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibody (1:2000; Sigma-Aldrich, St. Louis, MO) was used as a secondary antibody. Antibody-specific bands were detected using an enhanced chemiluminescence Western blot detection system (Perkin-Elmer, Boston, MA). The membranes were stripped and reprobed with anti-β-actin antibody (Sigma-Aldrich). Antibody-specific bands were quantified by Scion Image software (Scion Corp., Frederick, MD), and the level of arginase isoform was normalized to that of β-actin.

Measurement of NOx production

To estimate the generation of NO in colon tissue, the concentration of NOx, nitrite (NO_2^-) and nitrate (NO_3^-), in the colon homogenate was determined using an NO analyzer (Model-280i NOA with a Purge Vessel; Sievers, Boulder, CO), as described previously [15,16,23,25]. Briefly, the homogenate was treated with nitrate reductase (Sigma-Aldrich) to convert NO_3^- to NO_2^- at room temperature for 30 min. The proteins were removed by centrifugation after addition of acetonitrile. NO_2^- in the supernatant was further reduced to NO in a Purge Vessel containing the reducing agent potassium iodide on acetic acid, and NO was subsequently detected by the ozone-chemiluminescence method.

Measurement of L-arginine

The concentration of L-arginine in colon tissue was quantified by high-performance liquid chromatography (HPLC) with fluorescence detection, as described previously [15,16,23,26]. Briefly, colon homogenates were mixed with monomethylarginine as an internal standard. The mixture was applied to Oasis MCX solid phase-extraction cartridges (Waters, Milford, MA). The analyte containing arginine and monomethylarginine was eluted with methanol/water/ammonia solution (50:45:5, vol/vol/vol) and dried in a vacuum centrifuge. The residue was dissolved in water and

mixed with an equal amount of derivatization reagent (1 mg/ml ortho-phthaldialdehyde, 2% methanol, 0.1% 3-mercaptopropionic acid in 200 mM borate buffer, pH 8.5), and equilibrated for 30 min at room temperature. The sample was injected into the HPLC system consisting of a solvent delivery system and fluorometer (HITACHI Ltd, Tokyo, Japan). A Wakosil 5C18 (4.6 × 250 mm, 5 μm; Wako Pure Chemicals) was used as an analytical column. The mobile phase was 9% acetonitrile in acetate buffer, pH 6.3, at a flow rate of 1.5 ml/min, and the fluorescence excitation and emission wavelengths were 340 and 455 nm, respectively.

Statistical analysis

All results are expressed as the mean ± SE and the data were statistically analyzed by one-way ANOVA with a post-hoc test, a multiple comparison test, to determine whether the means differed significantly from each other or the vehicle using the SPSS 11.0 Windows program. The results were considered significantly different at $P < 0.05$.

Results

Effect of nor-NOHA on clinical signs of DSS-induced colitis

We assessed the effects of arginase inhibitor, nor-NOHA, on the clinical parameters of

DSS-induced experimental colitis. Compared with control mice, body weight gradually decreased in DSS-treated mice; however, the loss of weight in DSS-treated mice was dose-dependently attenuated by treatment of nor-NOHA (Figure 1A). We also assessed the disease activity index by scoring the clinical signs, including weight loss, diarrhea and rectal bleeding. DSS treatment resulted in increases in the disease activity index. Interestingly, the increase in the disease activity index was dose-dependently decreased by the administration of nor-NOHA to DSS-treated mice (Figure 1B). Furthermore, treatment with nor-NOHA reduced the shortening of the colon induced by DSS treatment in a dose-dependent manner. All of these findings indicate that the administration of nor-NOHA improves clinical parameters in DSS-induced colitis.

Moreover, given that the treatment with 17 mg/kg of nor-NOHA significantly ameliorated the clinical signs of DSS-induced colitis, which was almost restored to the level of control mice, in further experiments, we decided to treat with17 mg/kg of nor-NOHA.

Effect of nor-NOHA on the histological features of DSS-induced colitis

We evaluated the histopathology of the colonic tissues. A histologial analysis of colonic sections from DSS-treated mice showed marked transmural inflammation, extensive

infiltration of inflammatory cells, and crypt damage. In contrast, reduced inflammation and crypt damage were observed in the colon tissue of DSS plus nor-NOHA-treated mice. Furthermore, histological scores, assessed by the severity and extent of inflammation, and crypt damage were significantly lower in the DSS plus nor-NOHA-treated mice than in the DSS-treated mice (Figure 2). These results suggest that the administration of nor-NOHA attenuated the histological damage in DSS-induced colitis.

Effect of nor-NOHA on activity, mRNA, and protein levels of arginase

We measured the activity of arginase in colonic tissue. A significant increase in arginase activity was observed in DSS-induced colonic tissues compared with the control mice. The increased activity of arginase in DSS-treated mice was significantly reduced by nor-NOHA administration (Figure 3).

Furthermore, we examined the expression of protein and mRNA for arginase isoform in colonic tissues by Western blot and RT-PCR, respectively. Upregulation of protein and mRNA expression of arginase I was observed in the colon of DSS-treated mice. Importantly, the treatment of nor-NOHA significantly attenuated DSS-induced expression of protein and mRNA for arginase I (Figure 4A, B); however, there was no

significant difference in the expression of protein and mRNA of arginase II between DSS and DSS plus nor-NOHA-treated mice.

These results suggest that treatment of nor-NOHA attenuated the increased activity and expression of arginase I, not arginase II, in the colonic tissues of DSS-induced colitis.

Effect of nor-NOHA on L-arginine content

We examined the concentration of L-arginine, a substrate for arginase, in colon tissue by HPLC with fluorescence detection. The concentration of L-arginine in colon tissue was significantly lower in DSS-treated mice than in control mice. Interestingly, additional administration of nor-NOHA to DSS recovered the reduced levels of L-arginine, which were almost restored to normal levels (Figure 5). These data suggest that administration of nor-NOHA restored the decreased concentration of L-arginine in colonic tissues of DSS-induced colitis.

Effect of nor-NOHA on mRNA expression of NOS isoforms and NOx concentrations

Because L-arginine is a substrate for NOS as well as arginase, we investigated the mRNA expression of NOS isoforms in colonic tissues. The mRNA expression of NOS2

was upregulated by DSS treatment, whereas NOS1 and NOS3 mRNA levels were unchanged. Additional administration of nor-NOHA to DSS-treated mice inhibited mRNA expression of NOS2, whereas mRNA levels of NOS1 and NOS3 were not changed (Figure 6).

Moreover, we measured the concentration of NOx, the end product of NO produced by NOS, in colonic tissues. The concentration of NOx was significantly lower in the colonic tissues of DSS-treated mice than in those of control mice. Interestingly, there was a significant increase in NOx in the colonic tissues by additional administration of nor-NOHA to DSS, compared to DSS alone (Figure 7).

These results suggest that administration of nor-NOHA ameliorated the attenuation of NOx in colonic tissues in DSS-induced colitis, which likely did not result from increased expression of NOS.

Immunohistochemistry for arginase I and NOS2

We examined the immunolocalization of arginase I and NOS2 in the colonic tissues of DSS-treated mice. Immunostaining for arginase I and NOS2 was present in the infiltrating inflammatory cells. When compared with serial sections from the same tissues, positive cells for arginase I and NOS2 did not exclusively colocalize (Figure 8).

Discussion and conclusions

Arginase has been suggested to compete with NOS for their common substrate, L-arginine. To elucidate the involvement of arginase in the regulation of NO production in DSS-induced colitis, we examined the enzymatic activity and expression of arginase, L-arginine content, and the concentration of NOx, the end-product of NO produced by NOS, in the colonic tissues by administration of an arginase inhibitor, nor-NOHA, to DSS-induced mice. We demonstrated for the first time that the inhibition of arginase increased NO production and ameliorated clinical signs in colonic tissue in DSS-induced colitis.

In this study, we used a murine model of colitis induced by DSS. DSS is a heparin-like polysaccharide that provokes experimental colon damage similar to the pathophysiological features of colitis, such as extensive ulceration of the epithelial layer, massive bowel wall edema, fibrotic thickening of the mucosa, and a dense cellular infiltrate. DSS has been linked to direct epithelial cytotoxicity and interference with the normal interaction between intestinal lymphocytes and epithelial cells [20,27].

L-arginine is known to be a substrate for arginase as well as NOS. There are two isoforms of arginase: arginase I is abundant in the liver and is important for the urea

cycle, and arginase II is abundant in the kidneys and is located in mitochondria [12]. Arginase is an endogenous antagonist to NOS because it competes for the same L-arginine substrate by metabolizing to L-ornithine and urea, whereas NOS catalyzes the oxidation of L-arginine to citrulline and NO with Nω-hydroxy-L-arginine (NOHA) formed as an intermediate [12-14]. nor-NOHA is a potent, reversible inhibitor of arginase compared to NOHA. In contrast, nor-NOHA is not a substrate for NOS isoforms and does not inhibit the activity of NOS [28,29]. Thus, we used nor-NOHA as an inhibitor of arginase in this study.

We observed significant increased activity and enhanced expression of arginase in the colonic tissue of DSS-treated mice. These results agree with the report that the activity and expression of arginase were increased in human colitis [19]. More importantly, we found that the administration of nor-NOHA resulted in the amelioration of the clinical signs, as shown by the improved weight loss, high disease activity index, and colon shortening. Similarly, treatment with nor-NOHA attenuated the histological features of DSS-induced colitis, including the marked transmural inflammation, extensive infiltration of inflammatory cells, and crypt damage. Taken together, we believe that the induction of arginase could contribute to the pathogenesis of colitis.

Here, we have shown that the decreased concentration of L-arginine and NOx in colonic tissue in DSS-induced colitis was restored by the administration of nor-NOHA. We also found that there was no significant difference in the NOx concentration in colonic tissue between control (without DSS) and control plus nor-NOHA-treated mice (our unpublished data). Considering that arginase is an endogenous competitor of NOS for L-arginine, their common substrate [12-14], we think that inhibition of arginase could lead to increased production of NO by NOS in colitis tissues. Furthermore, our findings that the inhibition of arginase ameliorated DSS-induced colitis indicate the contribution of NO supply to healing colon damage.

Accumulating evidence has demonstrated that the production of L-ornithine and urea by arginase can contribute to tissue remodeling via the subsequent enhanced synthesis of L-proline and polyamine [18,30,31]. However, we did not examine the production of downstream products of arginase in the colonic tissue in the DSS- and DSS plus nor-NOHA-treated mice. Therefore, further studies are needed to uncover whether decreased production of these products by arginase suppression contributes to the protective effect of nor-NOHA on DSS-induced colitis.

We showed that treatment with nor-NOHA ameliorated the clinical signs of DSS-induced colitis in a dose-dependent manner. Furthermore, the decreased

concentration of L-arginine in the colonic tissues of DSS-treated colitis was dose-dependently restored by treatment with nor-NOHA (our unpublished data). Given these findings, it is likely that the protective effect of nor-NOHA on DSS-induced colitis is dose-dependent.

In this study, the administration of nor-NOHA inhibited the upregulation of arginase and NOS2 induced by DSS. These results coincide with our previous study indicating that the increased expression of arginase and NOS2 was reduced by treatment with nor-NOHA in an experimental model of asthma [16]. It has been demonstrated that the transcription factor nuclear factor (NF)-κB is involved in NOS2 induction [32]. Conversely, it has been reported that increased production of NO inhibits NF-κB activity by S-nitrosylation [33]. Considering that treatment with nor-NOHA increased NOx levels in the colonic tissues of the mice with DSS-induced colitis, regulation of the expression of arginase and NOS2 may be attributed to the modification of transcription factors, including NF-κB, by NO. Given that the cells expressing arginase and NOS2 did not exclusively colocalize, it will be interesting for further studies to elucidate the exact mechanisms regulating the expression of these enzymes by the administration of nor-NOHA.

In conclusion, in this study, we propose a new mechanism of DSS-induced colitis that increased the activity and upregulation of arginase I-induced low NO supply in colon microvessels due to the shift of L-arginine from NOS to arginase I. Moreover, our study suggested a potential therapeutic role for arginase inhibitor in the treatment of colitis.

Declaration of interest

The authors declare no conflicts of interest related to this article. This work was supported in part by Grant-in-Aid for Science Research No. 23390163 from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

References

[1] Maher MM. Inflammatory bowel disease: review and future view. Front Biosci (Elite Ed) 2012;4:1638-1647.

[2] Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.

[3] Wilkins T, Jarvis K, Patel J. Diagnosis and management of Crohn's disease. Am Fam Physician 2011;84:1365-1375.

[4] Gersemann M, Wehkamp J, Stange EF. Innate immune dysfunction in inflammatory bowel disease. J Intern Med 2012;271:421-428.

[5] Habib S, Ali A. Biochemistry of nitric oxide. Indian J Clin Biochem 2011;26:3-17.

[6] Radak Z, Naito H, Taylor AW, Goto S. Nitric oxide: is it the cause of muscle soreness? Nitric Oxide 2012;26:89-94.

[7] Yilmaz O, Calka J, Bukowski R, Zalecki M, Wasowicz K, Jaroszewski JJ, et al. Nitric oxide in the bovine oviduct: influence on contractile activity and nitric oxide synthase isoforms localization. Theriogenology 2012;77:1312-1327.

[8] Finkel J, Guptill V, Khaibullina A, Spornick N, Vasconcelos O, Liewehr DJ, et al. The three isoforms of nitric oxide synthase distinctively affect mouse nocifensive behavior. Nitric Oxide 2012;26:81-88.

[9] Ohtake K, Koga M, Uchida H, Sonoda K, Ito J, Uchida M, et al. Oral nitrite ameliorates dextran sulfate sodium-induced acute experimental colitis in mice. Nitric Oxide 2010;23:65-73.

[10] Coburn LA, Gong X, Singh K, Asim M, Scull BP, Allaman MM, et al. L-arginine supplementation improves responses to injury and inflammation in dextran sulfate sodium colitis. PLoS One 2012;7:e33546.

[11] Aoi Y, Terashima S, Ogura M, Nishio H, Kato S, Takeuchi K. Roles of nitric oxide (NO) and NO synthases in healing of dextran sulfate sodium-induced rat colitis. J Physiol Pharmacol 2008;59:315-336.

[12] Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.

[13] Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cell Mol Life Sci 1999;55:1015-1028.

[14] Zimmermann N, Rothenberg ME. The arginine-arginase balance in asthma and lung inflammation. Eur J Pharmacol 2006;533:253-262.

[15] Takemoto K, Ogino K, Shibamori M, Gondo T, Hitomi Y, Takigawa T, et al. Transiently, paralleled upregulation of arginase and nitric oxide synthase and the

effect of both enzymes on the pathology of asthma. Am J Physiol Lung Cell Mol Physiol 2007;293:L1419-1426.

[16] Takahashi N, Ogino K, Takemoto K, Hamanishi S, Wang DH, Takigawa T, et al. Direct inhibition of arginase attenuated airway allergic reactions and inflammation in a Dermatophagoides farinae-induced NC/Nga mouse model. Am J Physiol Lung Cell Mol Physiol 2010;299:L17-24.

[17] North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. Am J Physiol Lung Cell Mol Physiol 2009;296:L911-920.

[18] Benson RC, Hardy KA, Morris CR. Arginase and arginine dysregulation in asthma. J Allergy (Cairo) 2011;2011:736319.

[19] Horowitz S, Binion DG, Nelson VM, Kanaa Y, Javadi P, Lazarova Z, et al. Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. Am J Physiol Gastrointest Liver Physiol 2007;292:G1323-1336.

[20] Forbes E, Murase T, Yang M, Matthaei KI, Lee JJ, Lee NA, et al. Immunopathogenesis of experimental ulcerative colitis is mediated by eosinophil peroxidase. J Immunol 2004;172:5664-5675.

[21] Tamaki H, Nakamura H, Nishio A, Nakase H, Ueno S, Uza N, et al. Human

thioredoxin-1 ameliorates experimental murine colitis in association with suppressed macrophage inhibitory factor production. Gastroenterology 2006;131:1110-1121.

[22] Andujar I, Recio MC, Giner RM, Cienfuegos-Jovellanos E, Laghi S, Muguerza B, et al. Inhibition of ulcerative colitis in mice after oral administration of a polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells. J Agric Food Chem 2011;59:6474-6483.

[23] Imagama T, Ogino K, Takemoto K, Kato Y, Kataoka H, Suzuki H, et al. Regulation of nitric oxide generation by up-regulated arginase I in rat spinal cord injury. J Clin Biochem Nutr 2012;51:68-75.

[24] Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. J Immunol Methods 1994;174:231-235.

[25] Yang BK, Vivas EX, Reiter CD, Gladwin MT. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. Free Radic Res 2003;37:1-10.

[26] Blackwell S, O'Reilly DS, Talwar DK. HPLC analysis of asymmetric dimethylarginine (ADMA) and related arginine metabolites in human plasma using a novel non-endogenous internal standard. Clin Chim Acta 2009;401:14-19.

[27] Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. J Gastroenterol 2002;37:409-417.

[28] Moali C, Boucher JL, Sari MA, Stuehr DJ, Mansuy D. Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their N omega-hydroxy derivatives, and N omega-hydroxynor-L-arginine. Biochemistry 1998;37:10453-10460.

[29] Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D, Boucher JL. Effects of the new arginase inhibitor N(omega)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. Nitric Oxide 1999;3:427-438.

[30] Maarsingh H, Zaagsma J, Meurs H. Arginine homeostasis in allergic asthma. Eur J Pharmacol 2008;585:375-384.

[31] Maarsingh H, Zaagsma J, Meurs H. Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives. Br J Pharmacol 2009;158:652-664.

[32] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705-4708.

[33] Marshall HE, Stamler JS. Inhibition of NF-kappa B by S-nitrosylation. Biochemistry 2001;40:1688-1693.

Figure Legends

Figure 1. Effect of nor-NOHA on body weight loss (A), disease activity index (B), and colon length (C) in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** $P < 0.01$ vs DSS-treated mice.

Figure 2. Effect of nor-NOHA on histological features in DSS-induced colitis. Representative images of hematoxylin and eosin-stained sections and histological scores. Bars indicate 200 μm. Data are expressed as the means ± SE (n = 6 mice/group). *** $P < 0.01$ vs DSS-treated mice.

Figure 3. Effect of nor-NOHA on arginase activity in colonic tissue in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** $P < 0.01$ vs DSS-treated mice.

Figure 4. Effect of nor-NOHA on the expression protein (A) and mRNA (B) of arginase I and II in colonic tissue in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** $P < 0.01$ vs DSS-treated mice.

Figure 5. Effect of nor-NOHA on the concentration of L-arginine in colonic tissue in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** P < 0.01 vs DSS-treated mice.

Figure 6. Effect of nor-NOHA on the mRNA expression of NOS isoforms in colonic tissue in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** P < 0.01 vs DSS-treated mice.

Figure 7. Effect of nor-NOHA on the NOx concentration in colonic tissue in DSS-induced colitis. Data are expressed as the means ± SE (n = 6 mice/group). *** P < 0.01 vs DSS-treated mice.

Figure 8. Immunohistochemistry for arginase I and NOS2. Representative images of hematoxylin and eosin and immunostaining for arginase I and NOS2. Bars indicate 50 μm.

Figure 1

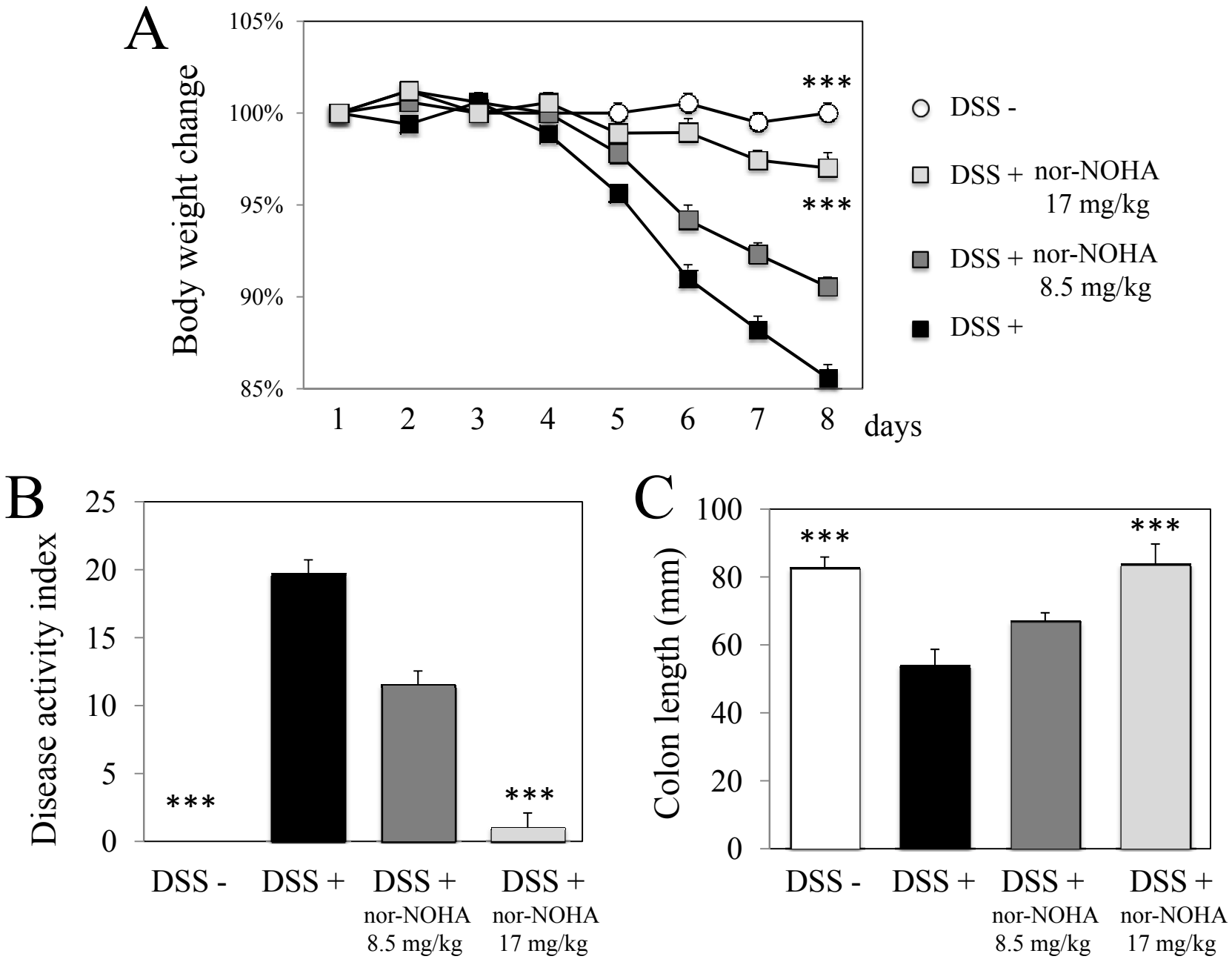

A
105%
100%
95%
90%
85%
Body weight change
1
2
3
4
5
6
7
8
days
***
***
DSS -
DSS + nor-NOHA 17 mg/kg
DSS + nor-NOHA 8.5 mg/kg
DSS +
B
25
20
15
10
5
0
Disease activity index
***
***
DSS -
DSS +
DSS + nor-NOHA 8.5 mg/kg
DSS + nor-NOHA 17 mg/kg
C
100
80
60
40
20
0
Colon length (mm)
***
***
DSS -
DSS +
DSS + nor-NOHA 8.5 mg/kg
DSS + nor-NOHA 17 mg/kg

Figure 2

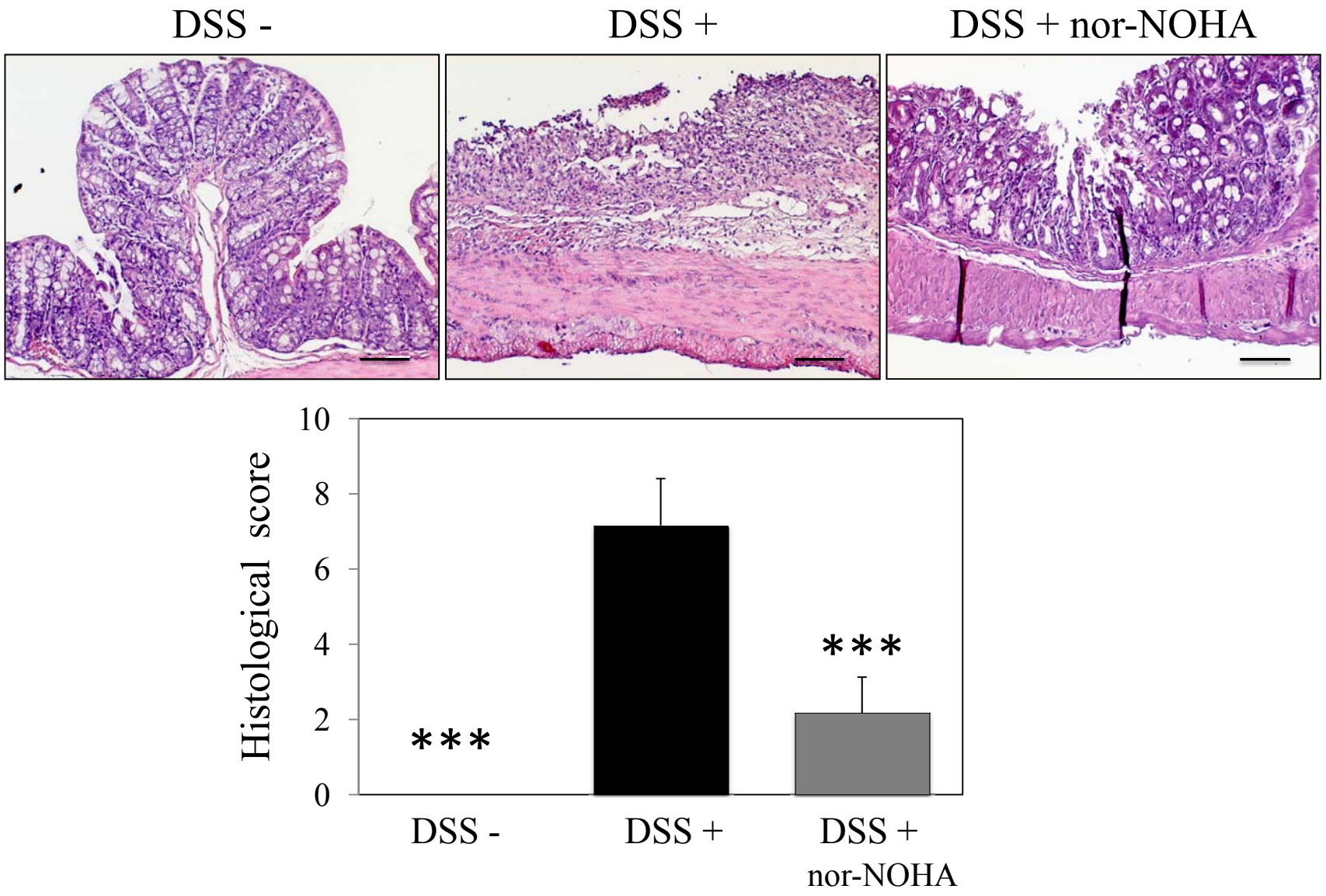

DSS -
DSS +
DSS + nor-NOHA
Histological score
10
8
6
4
2
0
***
***
DSS -
DSS +
DSS +
nor-NOHA

Figure 3

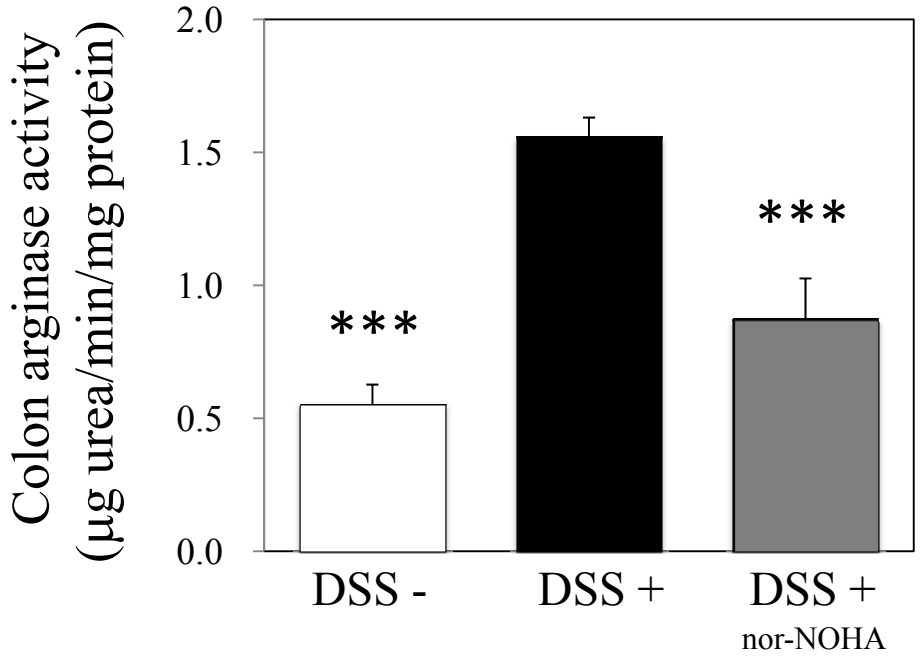

Colon arginase activity
(µg urea/min/mg protein)
2.0
1.5
1.0
0.5
0.0
***
***
DSS -
DSS +
DSS +
nor-NOHA

Figure 4

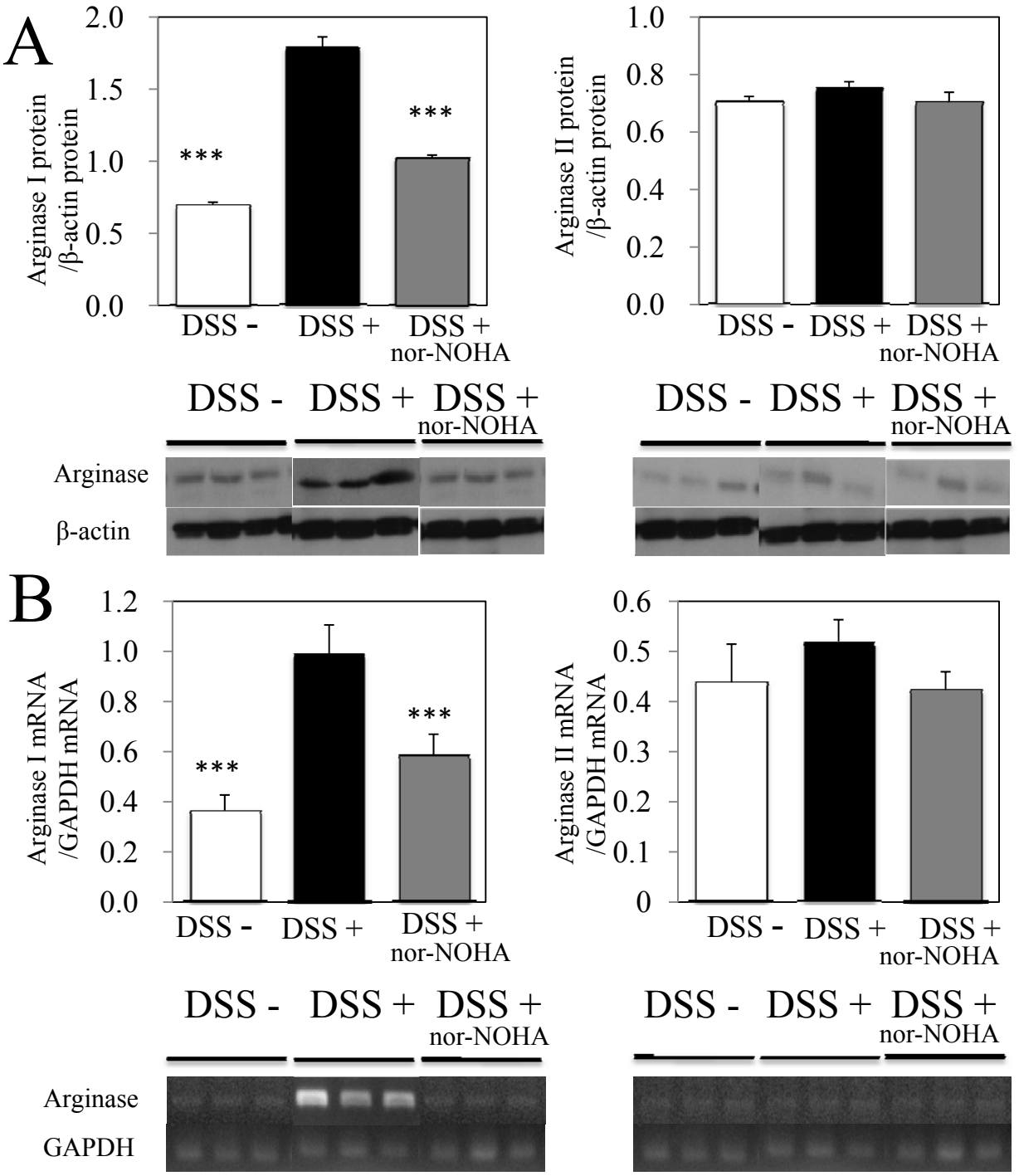

A
Arginase I protein /β-actin protein
2.0
1.5
1.0
0.5
0.0
***
***
DSS -
DSS +
DSS + nor-NOHA
Arginase II protein /β-actin protein
1.0
0.8
0.6
0.4
0.2
0.0
DSS -
DSS +
DSS + nor-NOHA
DSS - DSS + DSS + nor-NOHA
Arginase
β-actin
DSS - DSS + DSS + nor-NOHA
B
Arginase I mRNA /GAPDH mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
DSS -
DSS +
DSS + nor-NOHA
Arginase II mRNA /GAPDH mRNA
0.6
0.5
0.4
0.3
0.2
0.1
0
DSS -
DSS +
DSS + nor-NOHA
DSS - DSS + DSS + nor-NOHA
Arginase
GAPDH
DSS - DSS + DSS + nor-NOHA

Figure 5

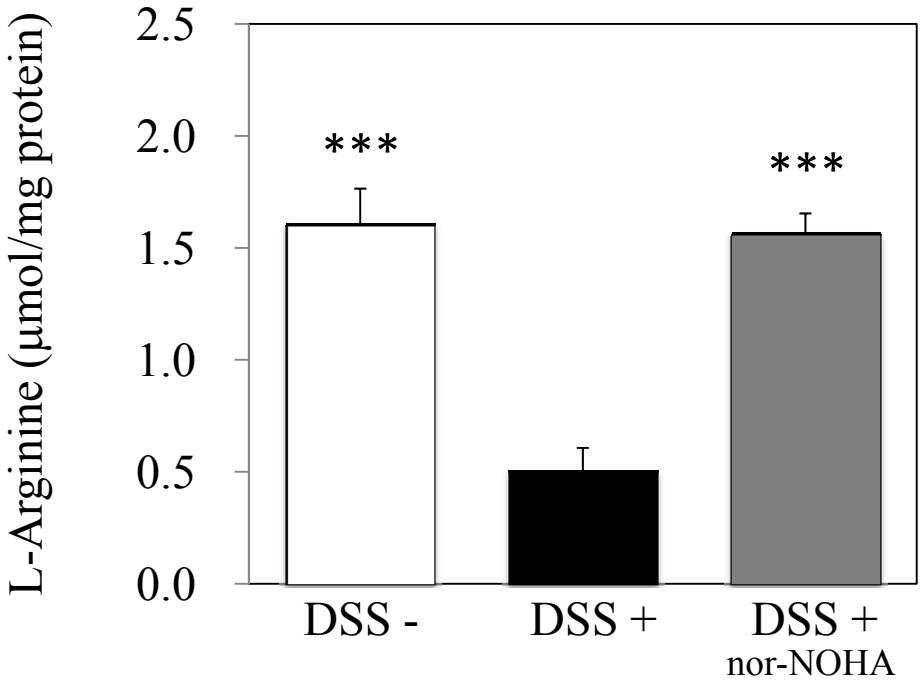
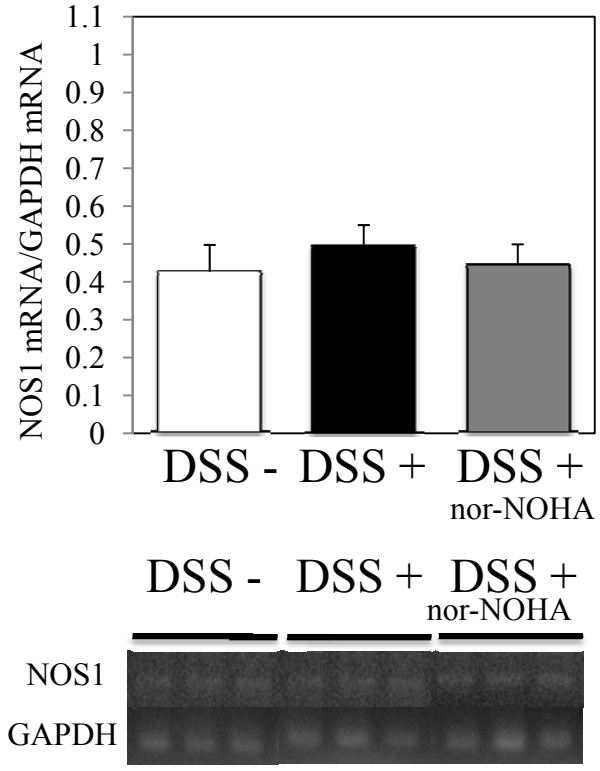
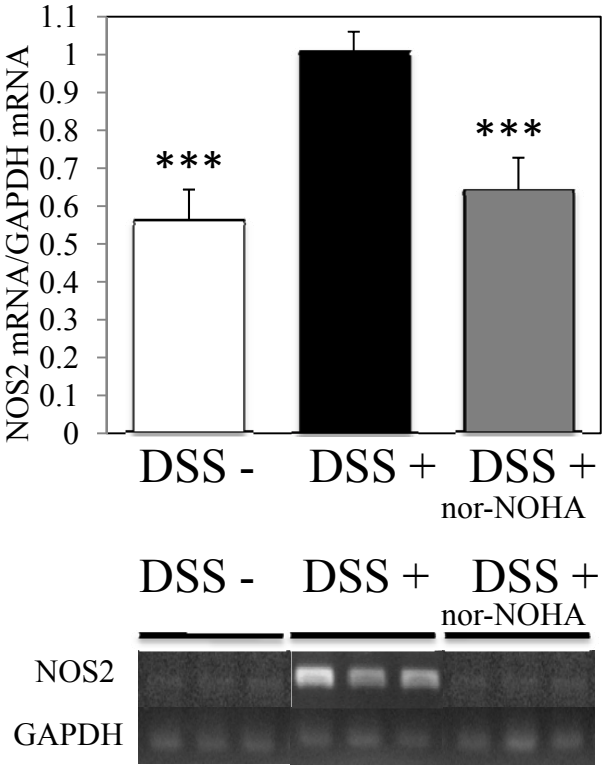

L-Arginine (μmol/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
DSS -
DSS +
DSS +
nor-NOHA

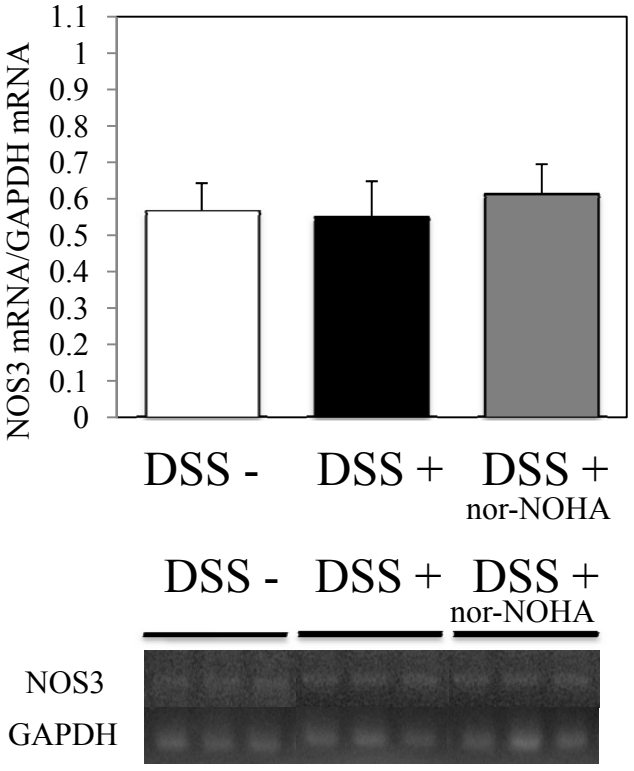
Figure 6


NOS1 mRNA/GAPDH mRNA
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DSS -
DSS +
DSS +
nor-NOHA

DSS -
DSS +
DSS +
nor-NOHA
NOS1
GAPDH


NOS2 mRNA/GAPDH mRNA
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
***
DSS -
DSS +
DSS +
nor-NOHA

DSS -
DSS +
DSS +
nor-NOHA
NOS2
GAPDH


NOS3 mRNA/GAPDH mRNA
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DSS -
DSS +
DSS +
nor-NOHA

DSS -
DSS +
DSS +
nor-NOHA
NOS3
GAPDH

Figure 7

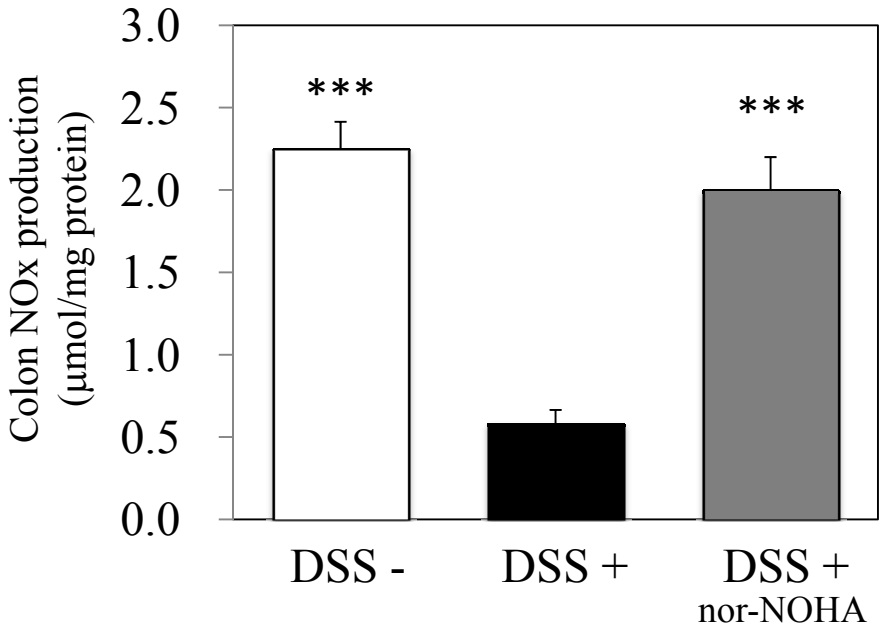

Colon NOx production
(µmol/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
DSS -
DSS +
DSS +
nor-NOHA

Figure 8

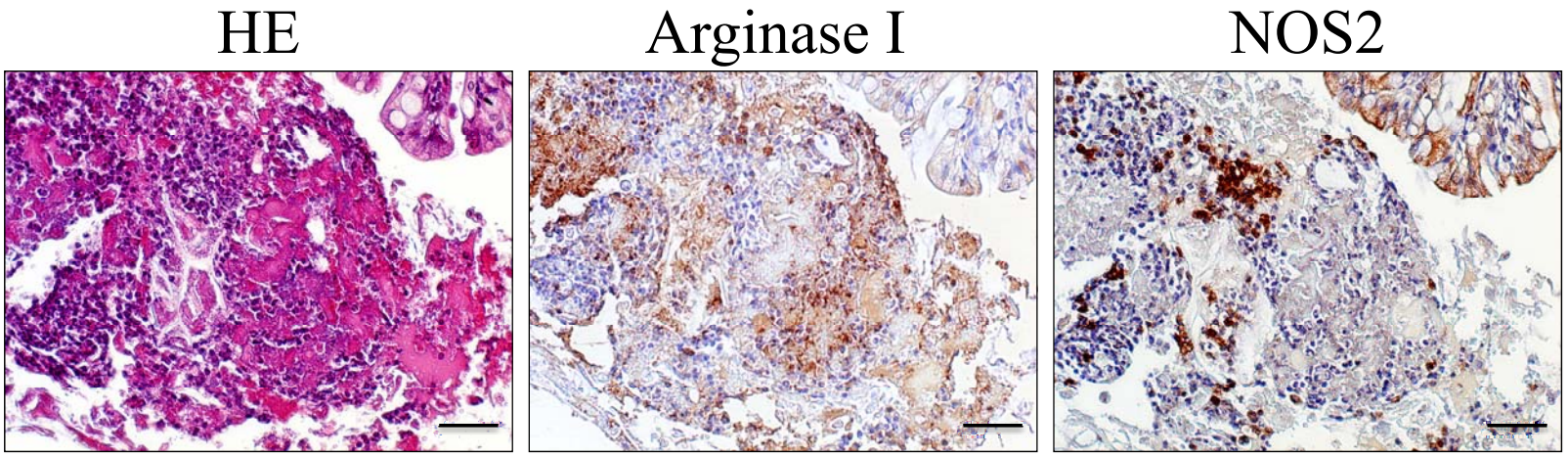


Table1. List of primers and RT-PCR conditions

Target Gene	Sense	Antisense	Denaturation	Amplification			Cycle	Elongation
Arginase I	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	95°C, 5 min	95°C, 20 s	56°C, 20 s	72°C, 30 s	35	72°C, 5 min
Arginase II	TGATTGGCAAAAGGCAGAGG	CTAGGAGTAGGAAGGTGGTC	95°C, 5 min	95°C, 20 s	56°C, 20 s	72°C, 30 s	35	72°C, 5 min
NOS1	CCTTAGAGAGTAAGGAAGGGGGCGGG	GGGCCGATCATTGACGGCGAGAATGATG	94°C, 3 min	94°C, 45 s	60°C, 45 s	72°C, 60 s	35	72°C, 5 min
NOS2	ATGGCTTGCCCCTGGAAGTTTCTC	CCTCTGATGGTGCCATCGGGCATC	94°C, 3 min	94°C, 45 s	60°C, 45 s	72°C, 90 s	31	72°C, 5 min
NOS3	GGGCTCCCTCCTTCCGGCTGCCACC	GGATCCCTGGAAAAGGCGGTGAGG	94°C, 3 min	94°C, 45 s	60°C, 45 s	72°C, 90 s	33	72°C, 5 min
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	94°C, 3 min	94°C, 45 s	60°C, 45 s	72°C, 60 s	30	72°C, 5 min