ENDOTHELIAL ARGININOSUCCINATE SYNTHETASE 1 REGULATES NITRIC OXIDE PRODUCTION AND MONOCYTE ADHESION UNDER STATIC AND LAMINAR SHEAR STRESS CONDITIONS

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Laminar shear stress (LSS) is known to increase endothelial nitric oxide (NO) production, which is essential for vascular health, through expression and activation of nitric oxide synthase 3 (NOS3). Recent studies demonstrated that LSS also increases the expression of argininosuccinate synthetase 1 (ASS1) that regulates the provision of L-arginine, the substrate of NOS3. It was thus hypothesized that ASS1 might contribute to vascular health by enhancing NO production in response to LSS. This hypothesis was pursued in the present study by modulating NOS3 and ASS1 levels in cultured endothelial cells. Exogenous expression of either NOS3 or ASS1 in human umbilical vein endothelial cells (HUVECs) increased NO production and decreased monocyte adhesion stimulated by tumor necrosis factor-α (TNF-α). The latter effect of over-expressed ASS1 was reduced when HUVECs were co-treated with small interfering RNAs (siRNAs) for ASS1 or NOS3. SiRNAs of NOS3 and ASS1 attenuated the increase of NO production in human aortic endothelial cells (HAECs) stimulated by LSS (12 dyn cm⁻²) for 24 h. LSS inhibited monocyte adhesion to HAECs stimulated by TNF-α, but this effect of LSS was abrogated by siRNAs of NOS3 and ASS1 that recovered the expression of vascular cell adhesion molecule-1. The current study suggests that the expression of ASS1 harmonized with that of NOS3 may be important for the optimized endothelial NO production and the prevention of the inflammatory monocyte adhesion to endothelial cells.

Atherosclerosis is a chronic disease characterized by the accumulation of lipids and fibrous elements in the intimal lining of the large and medium-sized arteries. Its development takes place step-by-step involving endothelial cell injury, migration of inflammatory cells, deposition of lipids and proliferation of smooth muscle cells, growth of this mass (atheroma) into the vessel lumen, and rupture of the plaque with subsequent thrombosis (1). When endothelial cells are activated by an inflammatory stimulus, they express intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and other selective adhesion molecules that mediate the heterotypic binding between leukocytes and endothelial cells, the primary event of atherosclerotic lesion formation (2). Endothelial cell senescence also increases monocyte recruitment, independently of acute inflammatory stimuli, by increasing the expression of adhesion molecules such as CD44 (3). Therefore, the ‘health’ of endothelial cells is essential for the prevention of the initial events of atherosclerosis.

Laminar shear stress (LSS) due to pulsatile blood flow is known to provide beneficial effects on vascular health (4-6). Indeed, the straight regions of the arteries that experience pulsatile LSS are usually protected from the formation of atherosclerotic lesions (7-8). The anti-atherogenic effects of pulsatile LSS and steady LSS (the latter does not occur in arteries in vivo, but is being studied in vitro) have been attributed to their ability to increase endothelial nitric oxide (NO) production (9). For example, LSS has been
shown to inhibit monocyte adhesion to endothelial cells in a NO-dependent manner (10).

The activation of endothelial nitric oxide synthase (NOS3) by Ca\textsuperscript{2+} or phosphorylation on multiple sites including Ser\textsuperscript{1177} has been identified as the primary mechanism responsible for the increased NO production under LSS conditions (11). Moreover, the protein level of NOS3 was shown to be increased by chronic LSS in cultured endothelial cells (12) and by exercise training in mice (13). NOS3 requires tetrahydrobiopterin as an essential cofactor and recent studies demonstrated that GTP cyclohydrolase-1, a rate limiting enzyme for \textit{de novo} synthesis of tetrahydrobiopterin, was activated through the phosphorylation on serine 81 in response to LSS (14). Another study pointed out that the GTP cyclohydrolase-1 expression level was also increased by chronic LSS in cultured endothelial cells (15).

Argininosuccinate synthetase 1 (ASS1) is the key enzyme responsible for the provision of L-arginine, the substrate of NOS3 (16) and this enzyme might play a role in the endothelial NO production in response to LSS. In support of this notion, cDNA microarray analyses identified ASS1 as one of the genes induced by LSS (17). Functional association of ASS1 with altered NO production has recently been verified in young and senescent endothelial cells under static and LSS conditions (18). Therefore, it was hypothesized that ASS1 might contribute to vascular health by playing a role in NO production in response to LSS. This hypothesis was pursued in the present study by examining whether endothelial ASS1 is required for the LSS effects of increasing NO production and decreasing monocyte adhesion.

**Experimental procedures**

\textit{Cultivation of endothelial cells-} Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Cambrex (Rockland, ME, USA) were cultured in EBM-2 medium containing endothelial growth supplements (Clonetics Cambrex), 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 U\cdot ml\textsuperscript{-1} penicillin, 100 \mu g\cdot ml\textsuperscript{-1} streptomycin, 0.25 \mu g\cdot ml\textsuperscript{-1} amphotericin B) on 0.2% gelatin-coated 6-well tissue culture plates (Nunc, Roskilde, Denmark) at 37 °C and 5% CO\textsubscript{2}. Human aortic endothelial cells (HAECs) were purchased from Cascade biologics (Portland, OR, USA) and cultured in Medium 200 with low serum growth supplements (Cascade biologics, Portland, OR, USA) and antibiotics, on 0.2% gelatin-coated 100 mm-culture dishes (BD Biosciences, San Jose, CA, USA).

\textit{Cultivation and fluorescence labeling of THP-1 cells-} THP-1 cells (Human acute monocytic leukemia cell line) from the Korea Cell Line Bank (Seoul, Korea) were cultured in RPMI 1640 medium (Gibco BRL) supplemented with fetal bovine serum (10%), antibiotics and \beta\textsuperscript{-}mercaptoethanol (0.05 mM). Cells were cultured in T-25 flasks (Nunc, Roskilde, Denmark) in an upright position. For fluorescence labeling, monocytes were washed with phosphate-buffered saline (PBS) twice and suspended at 5 x 10\textsuperscript{6} cells\cdot ml\textsuperscript{-1} in PBS containing 5 \mu g\cdot ml\textsuperscript{-1} 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Carlsbad, CA, USA), followed by incubation at 37 °C for 45 min. The labeled cells were then harvested, washed with PBS twice, and suspended in RPMI medium to be added to endothelial cell culture.

\textit{Transfection of HUVECs with plasmid constructs-} The full coding sequence of human ASS1 was polymerase chain reaction-amplified from a clone (IMAGE ID 30340813, the American Type Culture Collection, Manassas, VA, USA) and inserted into the pcDNA3.1(+) (Invitrogen, Grand Island, CA, USA) vector to generate the ASS1 plasmid construct (pcDNA-ASS1), as described in the previous study (18). The pcDNA-NOS3 construct encoding bovine NOS3 has been described previously (19). Transient transfection of HUVECs with plasmids was performed using NeoFectin\textsuperscript{TM} (Mid-Atlantic BioLabs Inc., West Bethesda, MD, USA). Briefly, cells at ~90%...
confluency on a 6-well plate were treated with a mixture of 1 µg DNA and 3 µl NeoFectin™ in 1 ml Opti-MEM (Gibco BRL) for 4 h. For co-transfection studies, cells were treated with a mixture of 1 µg DNA, 25 picomoles of small interfering RNA (siRNA), and 3 µl NeoFectin™.

Transfection of HAECs with siRNAs- Human ASS1 siRNA (#1299001, HSS100763) with the nucleotide sequences corresponding to the coding region of a human ASS1 gene transcript (NCBI GenBank accession number, NM_000050), human NOS3 siRNA (1299001, HSS107237) with nucleotide sequences corresponding to the coding region of a human NOS3 gene transcript (NM_000603.3), and a negative control oligoribonucleotide duplex (#12935200) with scrambled sequences were purchased from Invitrogen. The nucleotide sequences of ASS1 siRNA and NOS3 siRNA were as follows: ASS1 siRNA, 5'-UCA UUG GAA UGA AGU CCC GAG GUA U-3' (sense) and 5'-AUA CCU CGG GAC UUC AUU CCA AUG A-3' (antisense); NOS3 siRNA, 5'-GAA GAG GAA GGA GUC CAG UAA CAC A-3' (sense) and 5'-UGU GUU ACU GGA CUC CUU CCU CUU C-3' (antisense).

Transfection of HAECs with siRNAs was performed using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instruction. In brief, cells at ~50% confluency on an 100 mm-culture dish were treated with a mixture of 25 nM siRNA and 1.25 µl · ml⁻¹ Lipofectamine RNAiMAX in 5 ml Opti-MEM for 3 h.

LSS treatment of HAECs- After changing the growth medium with a fresh one, HAECs on an 100 mm-culture dish were exposed to steady LSS at 12 dyn·cm⁻² or kept under static conditions for 24 h. LSS was provided by rotating a Teflon cone (0.5° cone angle) mounted onto a culture dish, as described previously (20-21).

Western blotting- Western blotting of the cell lysates was conducted as previously described (22). Cells were lysed in a buffer (10 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% TritonX-100, and 1% deoxycholate, pH 7.2) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were centrifuged at 13,000 rpm for 15 min to obtain clear supernatants. Proteins were denatured in the Laemmli sample buffer for 5 min at 95 °C, electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Little Chalfont, UK). The membrane was incubated overnight with a primary antibody (1:1000 dilution) at 4 °C, and then with a secondary antibody conjugated to horseradish peroxidase (HRP) (1:3000 dilution) for 1 h at room temperature. The immunoreactive bands were detected using an ECL kit (Amersham Pharmacia, Little Chalfont, UK) and analyzed using the NIH Image program. Mouse monoclonal antibodies for ASS1 and NOS3 were purchased from BD Transduction Laboratories (San Diego, CA, USA). Rabbit polyclonal antibodies for Ser¹¹⁷²-phosphorylated NOS3 and inducible nitric oxide synthase (NOS2) were from Cell signaling (Danvers, MA, USA). Rabbit polyclonal antibody for cationic amino acid transporter 1 (CAT1) was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal β-actin antibody was purchased from Sigma-Aldrich. Goat anti-mouse and anti rabbit antibodies conjugated to HRP were purchased from Cell signaling and Santa Cruz Biotech (Santa Cruz, CA, USA), respectively.

Reverse transcriptase-polymerase chain reaction (RT-PCR)- Total cellular RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA, USA). RT-PCR was conducted using the GeneAmp® PCR system 9700 (Applied Biosystems, Foster city, CA, USA) in a reaction mixture (20 µl) containing Prime RT-PCR Premix (GENETBIO, Nonsan, Korea), 250 ng RNA, and 10 pmole of gene-specific primer sets (Bioneer, Daejeon, Korea). The sequences of PCR primers were: ASS1 (GeneBank accession number, NM_000050.4) 5'-TCC TGG AGA ACC CCA AGA AC-3' (sense) and 5'-CTC AGC AAA TTT CAA GCC CA-3' (antisense); NOS3 (NM_000603.3) 5'-TGC TGG CAT ACA GGA CTC AG-3' (sense) and 5'-TAG
GTC TTG GGG TTG TCA GG-3’ (antisense); CAT1 (NM_003045.4) 5’-AAC TTA ATC TCC TAC ATC ATC G-3’ (sense) and 5’-GCA TGA ATC CAC CAA CAC C-3’ (antisense); GAPDH (NM_002046.3) 5’-GCC AAA AGG GTC ATC ATC TC-3’ (sense) and 5’-GCA TGA ATC CAC CAA CAC C-3’ (antisense); The PCR products were electrophoresed in 1.0% agarose gel a DNA Ladder Marker (ELPIS-BIOTECH, Daejeon, Korea). The gel was ethidium bromide-stained and the band intensities were determined using a Gel Doc system (BioRad, Hercules, CA, USA).

**Assay of NO production**- NO production from endothelial cells was determined by measuring NO metabolites, nitrite plus nitrate, accumulated in a culture medium as described previously (23). After cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), with or without LSS at 12 dyn·cm⁻² for 24 h, and the conditioned medium were harvested for analysis. An aliquot (100 µl) of the conditioned medium was pretreated with a solution (100 µl) of 0.2 units·ml⁻¹ nitrate reductase, 0.4 units·ml⁻¹ glucose 6-phosphate dehydrogenase, 0.25 mM glucose 6-phosphate, and 0.2 µM NADPH in 14 mM Na-PO₄ buffer (pH 7.4), to reduce nitrate to nitrite. After a 45 min-incubation at room temperature, samples (50~100 µl) were added into 20 mM potassium iodide in 0.1 N sulfuric acid (10 ml) to convert nitrite to NO while monitoring the current change due to NO generation with an amperometric NO sensor (AmiNO700, Innovative Instruments, Inc., Tampa, FL, USA). A calibration curve was generated using sodium nitrite.

**Cell adhesion assay**- After LSS treatment or incubation at a static condition, endothelial cells were either stimulated with 4 ng·ml⁻¹ tumor necrosis factor-α (TNF-α) for 4 h or treated with a vehicle in a growth medium. Then the growth medium was replaced by RPMI medium, and the fluorescence-labeled THP-1 cells suspended in RPMI medium were added at a 1:1 ratio. After co-incubation of endothelial cells and THP-1 cells for 1 h, non-adherent THP-1 cells were washed out with PBS twice. Fresh RPMI medium was supplied to the remaining cells. Florescence-labeled THP-1 cells adhering to the endothelial cell surface were observed with a Nikon eclipse TE2000-U microscope. For quantification, adherent cells were lysed in 200 µl of 0.1 M Tris containing 0.1% Triton X-100 and centrifuged at 13,000 rpm for 15 min to obtain the supernatant. The fluorescence intensity of supernatants was determined at an excitation wavelength of 485 nm and emission wavelength of 535 nm, using an LS 55 Fluorescence Spectrometer (Perkin Elmer instruments, Waltham, MA, USA), and normalized for the number of endothelial cells.

**Enzyme-linked immunosorbent assay (ELISA) of VCAM-1**- After HAECs were exposed to LSS and/or TNF-α, cell surface expression of VCAM-1 was determined by ELISA (24). Cells were fixed with 4% paraformaldehyde in PBS for 10 min, and blocked with 0.1% BSA in 0.3% Triton X-100/PBS for 1 h at room temperature. Cells were incubated with goat polyclonal VCAM-1 antibody (Santa Cruz Biotech ) diluted 1:300 overnight at 4 °C followed by 1 h-incubation with donkey anti-goat antibody conjugated to HRP (Santa Cruz Biotech) diluted 1:1000. Then, cells were washed with PBS twice and treated with a substrate for HRP, 3,3',5,5'-tetramethyl benzidine (TMB) from Sigma-Aldrich. The reaction was stopped with 2 N HCl, forming a yellow reaction product. The colorimetric conversion at 450 nm of TMB was determined with a BioRad Model 680 microplate reader.

**Statistical analysis**- Data are presented as the Means ± SEM of experiments performed in triplicate. Significant differences among the groups were determined using the one-way ANOVA. The Duncan's multiple-range test was conducted if differences were identified between the groups at a significance level of p < 0.05.

**RESULTS**

Over-expression of ASS1 increases NO production and inhibits monocyte adhesion by a NOS3-dependent mechanism. Endothelial NO production should be
governed by the enzyme activity of NOS3 and the supply of its substrate, L-arginine. To confirm the critical role of ASS1 in the intracellular L-arginine supply to NOS3 enzyme, it was first determined if the increased expression of ASS1 can increase NO production. As shown in Fig. 1A, transfection of HUVECs with a plasmid construct encoding ASS1 (pcDNA-ASS1) increased protein levels of ASS1. The NO production was much higher in cells transfected with pcDNA-ASS1 as compared with those transfected with an empty vector (pcDNA). Transfection of cells with pcDNA-ASS1 did not alter NOS3 protein level nor the phosphorylation of Ser\textsuperscript{1177} of NOS3 (Fig. 1A), which is known as one of the major mechanisms leading to the activation of the enzyme (11).

If NO is inhibitory against monocyte adhesion, as has been reported previously (10), ASS1, which increased NO production, should be able to inhibit monocyte adhesion. Supporting this notion, TNF-\(\alpha\)-stimulated monocyte adhesion was significantly reduced in cells over-expressing ASS1 (Fig. 1B). As expected, transfection of cells with a plasmid construct encoding NOS3 (pcDNA-NOS3) resulted in an increase of NOS3 protein level, an increase of NO production and a decrease of monocyte adhesion, providing a positive control (Fig. 1A and B). Both pcDNA-ASS1 and pcDNA-NOS3 tended to decrease the basal monocyte adhesion in the absence of TNF-\(\alpha\) although the changes were not statistically significant (Fig. 1B).

Because the over-expression of ASS1 resulted in an increase of NO production and a decrease of monocyte adhesion, it was further tested whether these two consequences are related to each other. If ASS1 inhibited monocyte adhesion through enhancing NO production, this effect of ASS1 should be dependent on the expression of NOS3. To verify this notion, cells were co-transfected with the pcDNA-ASS1or pcDNA vector in combination with a negative control oligoribonucleotide duplex (NC) or ASS1 siRNA or NOS3 siRNA (Fig. 2).

HUVECs are one of the cells that are difficult to transfect with long nucleic acids and thus co-transfection conditions were optimized for plasmid constructs not for siRNAs. As shown in Fig. 2, endogenous NOS3 was decreased by NOS3 siRNA but not fully knocked down, probably because the transfection conditions might not be optimal for siRNAs. However, the expression of ASS1, mainly of exogenous form, in the transfected cells was effectively suppressed by co-transfected ASS1 siRNA. In this Western blotting condition, the change of ASS1 expression levels in the control cells transfected with the pcDNA vector could not be definitively monitored.

ASS1 siRNA increased both basal and TNF-\(\alpha\)-stimulated monocyte adhesion significantly in cells transfected with the pcDNA-ASS1or pcDNA vector, indicating the changes of the ASS1 protein level could influence on monocyte adhesion (Fig. 2). The effects of NOS3 siRNA were also of interest because it increased monocyte adhesion in both cells; the increase of monocyte adhesion by NOS3 siRNA was reduced by ASS1 over-expression, and the decrease of monocyte adhesion by over-expressed ASS1 was reduced by NOS3 siRNA (Fig. 2). These results indicated that NOS3 might be required for the inhibitory action of ASS1 on monocyte adhesion, and vice versa.

Not only NOS3 but also ASS1 is required for the regulatory action of LSS on NO production and monocyte adhesion. The importance of ASS1 in endothelial NO production was further examined under LSS conditions. HAECs were chosen for this experimental purpose because they are normally exposed to a high level of LSS \textit{in vivo}. As shown in Fig. 3A, chronic LSS (12 dyn cm\textsuperscript{-2} for 24 h) increased the mRNA levels of ASS1 and NOS3 in HAECs but these changes were significantly inhibited in cells pretreated with their siRNAs. The protein levels of ASS1 and NOS3 also significantly increased by LSS (Fig. 3B) and decreased by siRNAs of ASS1 and NOS3, respectively. LSS increased the phosphorylation of Ser\textsuperscript{1177} of NOS3 as expected and it was not affected by ASS1 siRNA (Fig. 3B). Of course, the
phosphorylated form of NOS3 was not detected in NOS3-depleted cells.

The mRNA and protein levels of CAT1 were also examined because this protein is known to be involved in the transport of L-arginine (25). The results showed that its mRNA and protein levels were unaltered by the exposure to LSS or the treatments with siRNA of NOS3 or ASS1 (Figs. 3A, B).

LSS also increased NO production by twofold in the NC-treated cells (Fig. 3C). However, pretreatment of cells with either ASS1 siRNA or NOS3 siRNA reduced the NO production levels by half under both static and LSS conditions (Fig. 3C). These results indicated that ASS1 actively participates in the regulation of endothelial NO production in response to LSS, by providing L-arginine to NOS3. Potentially ASS1 could supply L-arginine to other NOS enzymes including NOS2. However, NOS2 protein could not be detected in HAECs under static and LSS conditions (Fig. 3B). In contrast, NOS2 protein was clearly detected in THP-1 cells stimulated with TNF-α (Fig. 3B). Thus it was indicated that ASS1 may not be functionally associated with NOS2 in the endothelial cells.

It was then examined whether ASS1 contributes to the inhibition of monocyte adhesion by LSS. HAECs were pretreated with ASS1 or NOS3 siRNA (25 nM), exposed to LSS (12 dyn cm⁻²), and stimulated with TNF-α (4 ng·ml⁻¹) prior to co-incubation with the fluorescence labeled monocytes. The experiments included several lines of control cells treated with NC instead of a siRNA, those without LSS exposure, and those without TNF-α. As shown in Fig. 4A and B, exposure of HAECs to LSS decreased monocyte adhesion in the absence and presence of TNF-α. In addition, these changes were abrogated by ASS1 siRNA as well as NOS3 siRNA. These results indicated that the expression of NOS3 and ASS1 is a necessary condition for inhibition of monocyte adhesion.

Effects of NOS3 and ASS1 siRNAs on the cell surface expression of VCAM-1 expressions were also examined because this cell adhesion molecule is known to be most consistently down-regulated by LSS and NO (10, 24). The results showed that LSS decreased the cell surface expression of VCAM-1 in endothelial cells in the absence and presence of TNF-α (Fig. 4C). Of special interest, VCAM-1 expression in LSS-exposed cells was maintained high if the cells were pretreated with siRNA of ASS1 or NOS3 (Fig. 4C).

DISCUSSION

The inhibitory action of LSS on monocyte binding, an initial event involved in the development of atherosclerosis, has been attributed to NO which inhibits the gene expression of certain adhesion molecules (10). Chronic LSS is known to increase endothelial NO production through the up-regulation of NOS3 protein expression and activity (11). By providing evidence that the ASS1 expression is required for the increase of NO production and the inhibition of monocyte adhesion by LSS (Figs. 3 and 4), the current study established a sequential event occurring in response to LSS: LSS → increase of ASS1 → increase of NO production by NOS3 → suppression of cell surface expression of VCAM-1 → inhibition of monocyte adhesion. The current study also demonstrated that over-expression of ASS1 alone led to an increase of NO production without altering NOS3 protein expression or phosphorylation (Fig. 1A) and a decrease of monocyte adhesion (Fig. 1B). The latter effect of ASS1 was quite dependent on the presence of NOS3 (Fig 2). And NOS2 was not induced by LSS in HAECs (Fig. 3) Therefore, ASS1 is considered to be functionally associated with NOS3, providing an effective way to improve vascular health.

If L-arginine is not sufficiently provided, NOS3 reaction might be uncoupled to produce reactive oxygen species (ROS) instead of NO. This notion has been experimentally demonstrated in a previous study using HEK 293 cells stably transfected with rat neuronal nitric oxide synthase (NOS1) and stimulated with a calcium ionophore (26). In the present study, the possibility of NOS3 uncoupling was
examined by monitoring intracellular ROS production with an oxidant-sensitive probe, dihydrorhodamine (DHR) 123 (15, 27). The results indicated that ROS formation in cultured HAECs was not significantly altered by LSS or ASS1 siRNA treatments (Supplementary Fig. S1). Therefore it was considered that limited L-arginine supply might not cause significant NOS3 coupling in normal cells unless they were stimulated to a supraphysiological level.

Although normal levels of L-arginine in endothelial cells (> 800 μM) (28) is far higher than the reported Michaelis constant ($K_M$) of purified NOS3 for L-arginine (~ 2.9 μM) (29), the endothelial NOS3 activity still appears to be dependent on the extracellular availability of L-arginine in vivo (30). This phenomenon, termed the ‘arginine paradox’ (31), has led to a hypothesis that L-arginine is compartmentalized in cytosol and its local concentration in the vicinity of NOS3 may be much lower than the mean value of the entire cell (25). According to this hypothesis, the ‘arginine paradox’ has been explained in part by the regulated uptake of extracellular L-arginine into a compartment that contains NOS3 and that is segregated from the bulk cytosolic L-arginine (32). The ‘arginine paradox’ has also been explained by other mechanisms including the antagonistic competition between supplemented L-arginine and endogenous asymmetric dimethylarginine, an inhibitor of NOS (33).

In accordance with the ‘compartmentalization’ hypothesis, NOS3, Cat1 and L-arginine regeneration enzymes are all co-localized at caveolae in endothelial cells (25, 34-35). Caveolae are small invaginations of the plasma membrane found in many cell types (36). ASS1 catalyzes ATP-dependent condensation reaction between L-citrulline and L-aspartic acid producing L-argininosuccinate that is in turn used by argininosuccinate lyase to produce L-arginine; the L-arginine produced in this way can be directed to NO synthesis by NOS3 (16). Supporting the presence of this citrulline-NO cycle, L-citrulline could increase cellular NO production as effectively as L-arginine (37). Thus both L-arginine and L-citrulline are expected to provide beneficial effects on vascular health (38).

ASS1 is a rate limiting enzyme of the citrulline-NO cycle (16) and its expression may be required to maintain endothelial NO production that is essential for vascular health (39). Together with previous studies (17-18), the present study clearly demonstrated that LSS increases both NOS3 and ASS1 expressions in endothelial cells while Cat1 expression level was unchanged (Fig. 3). Certain strategies to enhance LSS may be potentially useful for the maintenance of vascular health. In this regard, frequent exercise would be one of the best choices because it can provide repeated episodes of elevated shear stress (40).

Additionally, pharmacological approaches that can mimic LSS effects would be helpful to maintain vascular health. Previous studies have shown that glutamine and glucosamine increased ASS1 gene expression by activating Sp1 transcription factor that can bind to the GC boxes in the ASS1 promoter region (41) and that glucosamine inhibited atherosclerosis in apoE null mice by increasing heparin sulfate proteoglycans and decreasing monocyte adhesion (42). Thus, it would be interesting to examine whether the anti-atherogenic effects of glucosamine also involve induction of ASS1 expression and enhanced NO production. Further studies are also warranted to examine whether Sp1 transcription factor is commonly involved in the regulation of the ASS1 gene expression in response to LSS and other stimulants known to induce ASS1 gene expression (43).

Previous studies have also revealed that Kruppel-like factor 2 (KLF2) coordinates transcriptional program associated with the vasoprotective effects of LSS (44). KLF2 is known to be transcriptionally activated in response to LSS by myocyte enhancer binding factor 2 (MEF2) which is in turn regulated by mitogen activated protein kinase (MAPK) signaling cascade where MAPK kinase 5 (MEK5) and extracellular-signaling kinase 5 (ERK5) are involved (45). KLF2 transcription factor plays a key role in the
induction of NOS3 and trombomodulin in response to LSS, and thus contribute to the establishment of anti-inflammatory, vasodilatory and anti-thrombotic endothelial phenotypes (46). A recent study also demonstrated that ASS1 is a common transcriptional target of KLF2 and KLF4 (47). Therefore it may be assumed that ASS1 expression can be induced by LSS through a signal transduction pathway, LSS → MEK5 → ERK5 → MEF2 → KLF2/4 → ASS1.

The observed effects of LSS and NO on the expression of cell adhesion molecules vary between studies (10, 24, 48). This is not surprising because cell adhesion may require diverse adhesion molecules whose expression and interaction are coordinated in a time and space-dependent manner. Among the cell-adhesion molecules highly expressed in endothelial cells stimulated by inflammatory cytokines, VCAM-1 has been observed to be commonly down-regulated by LSS and NO at the transcriptional level (10, 24). The results of current study further showed that cytokine-stimulated VCAM-1 expression was suppressed by LSS in control cells but not in ASS1 or NOS3-depleted cells (Fig. 4C), indicating ASS1-assisted NOS3-catalyzed NO production could suppress VCAM-1 expression involved in the inflammatory leukocyte recruitments. Although VCAM-1 expression levels and monocyte adhesion activities of endothelial cells appeared to correlate each other, it should be mentioned that VCAM-1 is just an example of cell adhesion molecules regulated by a mechanism involving ASS1 and the potential association of other cell adhesion molecules may not be excluded.

In conclusion, the current study demonstrated that endothelial ASS1 mediates the inhibitory action of LSS on monocyte adhesion, by enhancing NO production. The regulated expression of ASS1 together with NOS3 should help the optimized endothelial NO production in response to LSS and the prevention of inflammatory processes leading to atherosclerosis.

REFERENCES


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**FOOTNOTERS**

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The abbreviations used are: ASS1, argininosuccinate synthetase 1; CAT1, cationic amino acid transporter 1; DHR, dihydrorhodamine; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; HRP, horse radish peroxidase; ICAM-1, intercellular adhesion molecule-1; KLF2, Kruppel-like factor 2; LSS, laminar shear stress; MAPK, mitogen activated protein kinase; MEK5, MAPK kinase 5; ERK5, extracellular-signaling kinase 5; MEF2, myocyte enhancer binding factor 2; NC, negative control oligoribonucleotide duplex; NO, nitric oxide; NOS1, neuronal nitric oxide synthase; NOS2, inducible nitric oxide synthase; NOS3, endothelial nitric oxide synthase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; TMB, 3,3′,5,5′-tetramethyl benzidine; VCAM-1, vascular cell adhesion molecule-1
FIGURE LEGENDS

Fig. 1. Exogenous expression of ASS1 or NOS3 in HUVECs enhances NO production and inhibits monocyte adhesion stimulated by TNF-α. HUVECs were transfected with a plasmid construct encoding ASS1 (pcDNA-ASS1) or NOS3 (pcDNA-NOS3) or an empty vector (pcDNA). In panel A, the transfected cells were incubated for 24 h in a growth medium and then in DMEM for 24 h to obtain the conditioned medium. NO production was assessed by measuring nitrite plus nitrate accumulated in the conditioned medium. Cell lysates were subjected to Western blotting to monitor ASS1, NOS3, Ser\textsuperscript{1177}-phosphorylated NOS3 and β-actin protein levels. In panel B, the transfected cells were incubated in a growth medium for 48 h before stimulation with TNF-α for 4 h. Endothelial cells were then harvested for Western blotting or co-incubated with monocytes (THP-1 cells) for 1 h, followed by quantification of monocytes adhered on the endothelial cells. Western blot images are representative of three experiments. Fold changes are over control cells transfected with pcDNA only. Bar graphs represent Mean ± SEM (n=3). Data not sharing the same letter are significantly different from each other (p < 0.05).

Fig. 2. NOS3 is required for the inhibitory action of ASS1 on monocyte adhesion to HUVECs stimulated by TNF-α. HUVECs were transfected with a plasmid construct encoding ASS1 (pcDNA-ASS1) or an empty vector (pcDNA), in combination with ASS1 siRNA or NOS3 siRNA or a negative control oligoribonucleotide duplex (NC). The transfected cells were incubated in a growth medium for 48 h and stimulated with TNF-α for 4 h. Endothelial cells were then harvested for Western blotting of ASS1, NOS3 and β-actin, or co-incubated with monocytes (THP-1 cells) for 1 h, followed by quantification of monocytes adhered on the endothelial cells. Western blot images are representative of three experiments. Fold changes are over control cells transfected with pcDNA + NC. Bar graphs represent Mean ± SEM (n=3). Data not sharing the same letter are significantly different from each other (p < 0.05).

Fig. 3. Not only NOS3 but also ASS1 regulate the NO production stimulated by LSS in HAECs. Cells transfected with ASS1 siRNA or NOS3 siRNA or a negative control oligoribonucleotide duplex (NC) were cultured in a growth medium for 24 h and then exposed to LSS at 12 dyn·cm\textsuperscript{-2} for 24 h in DMEM. Control cells were kept under static conditions. In panel A, the mRNA levels of ASS1, NOS3, CAT1 and GAPDH were monitored by RT-PCR analysis using the gene-specific primers. In panel B, the protein levels of ASS1, NOS3, Ser\textsuperscript{1177}-phosphorylated NOS3, CAT1, β-actin and NOS2 were monitored by Western blotting of cell lysates. For NOS2, the lysates of THP-1 cells treated with TNF-α or not were used as positive and negative controls, respectively. In panel C, NO production was determined by measuring nitrite plus nitrate accumulated in the conditioned medium. Fold changes are over static control cells transfected with NC only. Bar graphs represent means ± SEM (n=3). Data not sharing the same letter are significantly different from each other (p < 0.05).

Fig. 4. Both ASS1 and NOS3 are required for the inhibitory action of LSS on monocyte adhesion and the cell surface expression of VCAM-1 in HAECs. Cells transfected with ASS1 siRNA or NOS3 siRNA or a negative control oligoribonucleotide duplex (NC) were incubated in a growth medium for 24 h and then exposed to LSS at 12 dyn·cm\textsuperscript{-2} for 24 h or kept under static conditions. Cells were stimulated with TNF-α for 4 h and then co-incubated with monocytes (THP-1 cells) for 1 h, followed by quantification of monocytes adhered on the endothelial cells. In panels A and B, monocytes adhered on the endothelial cells were observed under a microscope (A) and quantified fluorometrically (B). In panel C, cell surface expression of VCAM-1 was determined by ELISA. Fold changes are over static control cells transfected with NC only. Bar graphs represent Mean ± SEM (n=3). Data not sharing the same letter are significantly different from each other (p < 0.05).
Mun et al. Fig. 1.

A

**NO production (nmol protein mg⁻¹ 24 h⁻¹)**

- ASS1
- NOS3
- pSer^{1177}-NOS3
- β-actin

B

**Monocyte adhesion (Fold)**

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By guest on November 15, 2017 http://www.jbc.org/ Downloaded from
Mun et al. Fig. 2.

**Graph:**
- Y-axis: Monocyte adhesion (Fold)
- X-axis: TNF-α, NC, ASS1-siRNA, NOS3-siRNA, pcDNA, pcDNA-ASS1

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Mun et al. Fig. 3.

A  mRNA analysis

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B  Protein analysis

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C  NO production (nmol•protein mg⁻¹•24 h⁻¹)

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Different letters indicate significant differences. A: mRNA analysis; B: Protein analysis; C: NO production; NC: Negative control; TNF-α: Tumor necrosis factor-α; THP-1 cells: Human monocyte-like cell line.
Mun et al. Fig. 4.

**A**

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**B**

- Monocyte binding (Fold)

**C**

- VCAM-1 expression (Fold)
Endothelial argininosuccinate synthetase 1 regulates nitric oxide production and monocyte adhesion under static and laminar shear stress conditions
Gyeong In Mun, In-San Kim, Byung-Heon Lee and Yong Chool Boo

J. Biol. Chem. published online November 24, 2010

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