**Effect of Shear Stress on Asymmetric Dimethylarginine Release From Vascular Endothelial Cells**

Tomohiro Osanai, Masayuki Saitoh, Satoko Sasaki, Hirofumi Tomita, Toshiro Matsunaga, Ken Okumura

**Abstract**—We demonstrated recently that plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthase, are increased by high salt intake concomitantly with a decrease in plasma levels of NO in human hypertension. We investigated the effect of shear stress on ADMA release in 2 types of cells: transformed human umbilical vein endothelial cells (HUVECs; cell line ECV-304) and HUVECs. Exposure of ECV-304 cells and HUVECs to shear stress with the use of a cone-plate viscometer enhanced gene expression of protein arginine methyltransferase (PRMT-1), ADMA synthase. In HUVECs, the ratio of PRMT-1 to glyceraldehyde 3-phosphate dehydrogenase mRNA was increased by 2-fold by a shear stress of ≥15 dyne/cm². A dominant-negative mutant of IκB kinase α and troglitazone at 8 μmol/L, an activator of peroxisome proliferator–activated receptor γ, abolished the shear stress–induced increase in PRMT-1 gene expression in parallel with the blockade of nuclear factor (NF)-κB translocation into the nucleus. The activity of dimethylarginine dimethylaminohydrolase, the degradation enzyme of ADMA, was unchanged after shear stress ≥15 dyne/cm² and was enhanced by 1.48±0.06-fold (P<0.05) by shear stress at 25 dyne/cm². The release of ADMA was increased by 1.64±0.10-fold (P<0.05) by shear stress at 15 dyne/cm² but was not affected by shear stress at 25 dyne/cm². These results indicate that shear stress enhances gene expression of PRMT-1 and ADMA release via activation of the NF-κB pathway. Shear stress at higher magnitudes facilitates the degradation of ADMA, thus returning ADMA release levels to baseline. *(Hypertension. 2003;42:985-990.)*

**Key Words:** stress, mechanical ■ endothelium ■ gene expression ■ arginine ■ nitric oxide ■ nitric oxide synthase

Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle tone and growth, platelet aggregation, and leukocyte adhesion to the endothelium.¹ Altered biosynthesis of NO has been implicated in the pathogenesis of atherosclerosis, and it is possible that accumulation of endogenous asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of NO synthase (NOS), underlies the reduced NO generation. We demonstrated recently that a high salt intake decreases the plasma concentrations of nitrite and nitrate (NOx) concomitantly with an increase in plasma concentrations of ADMA in patients with essential hypertension.² A high salt intake causes a number of physiologic and pathologic effects, such as hemodynamic modulation, increased activity of the renin-angiotensin-aldosterone system, and activation of the sympathetic nerve system. High salt intake–induced hemodynamic modulation includes augmentation of shear stress to the vascular wall, especially to endothelial cells, and we hypothesized that a high salt intake–induced decrease in plasma NOx levels might be caused by a shear stress–induced elevation of circulating ADMA.

Vascular endothelial cells are capable of synthesizing ADMA, which is derived from the catabolism of proteins containing methylated arginine residues.³ There are 2 types of enzymes that methylate arginine residues. Type I arginine methyltransferase (PRMT-1) monomethylates and asymmetrically dimethylates arginine residues within the human heterogeneous ribonucleoprotein A1 and other proteins.⁴ In contrast, type II PRMT monomethylates and symmetrically dimethylates myelin basic protein in the brain and therefore is unlikely to be involved in the generation of ADMA.⁵ There are 4 different isoforms of type I PRMT, with specificity for different proteins.⁶ Human PRMT-1 was identified as a protein that interacts with the intracellular domain of the interferon-α,β receptor,⁷ and this enzyme is the most predominant form of type I PRMT in mammalian cells.⁸ PRMT-2 was identified as a distinct human gene, *HRMT1L1,* whereas PRMT-3 is another enzyme of this family with distinct properties.⁹ *HRMT1L2* was detected in a yeast 2-hybrid screen.¹⁰ We thus hypothesized that PRMT-1 might be involved in the regulation of ADMA release under shear stress in vascular endothelial cells, thereby providing a novel mechanism for the regulation of circulating ADMA. Shear stress induces sustained activation of a key transcriptional regulator, nuclear factor-κB (NF-κB),¹² that upregulates the expression of redox-sensitive genes encoding endothelial

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From the Second Department of Internal Medicine, Hirosaki University School of Medicine, Hirosaki, Japan.
Correspondence to Tomohiro Osanai, MD, Second Department of Internal Medicine, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8562 Japan. E-mail osanait@cc.hirosaki-u.ac.jp
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985
adhesion molecules. On the other hand, peroxisome proliferator-activated receptor γ (PPARγ) inhibits NF-κB–dependent gene expression through suppression of IkB kinase (IKK) and modification of the DNA-binding domains of NF-κB subunits. The present study was designed to investigate the effect of shear stress on ADMA release in vascular endothelial cells and the involvement of PRMT-1 and NF-κB pathways in it.

Methods

Materials

Medium 199, fetal bovine serum (FBS), Petri dishes, and phosphate-buffered saline (PBS) were purchased from Gibco. HuMedia was purchased from Kurabo Co, Ltd. An expression vector encoding the dominant-negative mutants IKKα (K44M) was kindly provided by Tanabe Seiyaku Co, Ltd (Osaka, Japan). A Bond Elut PRS column was purchased from Varian Associates Inc. ADMA and all other reagents were of the finest grade available from Sigma Chemical Co.

Cell Culture

ECV-304 cells (spontaneously transformed human umbilical vein endothelial cells [HUVECs]; American Type Culture Collection, Manassas, Va) were cultured in medium 199 containing 10% FBS, streptomycin, and penicillin. The medium was replaced with 6 mL serum-free medium 199 or HuMedia. ECV-304 cells were exposed to shear stress at 15 dyne/cm² for 3 hours without affecting GAPDH content of the dishes and staining with trypan blue.

Flow Methods

Subconfluent ECV-304 cells and HUVECs were exposed to fluid shear stress with the use of a cone-plate viscometer specifically designed to accept standard tissue-culture plates. Viability of the cells exposed to shear stress was assessed by measurement of protein content of the dishes and staining with trypan blue.

Protocol

Subconfluent monolayers of ECV-304 cells and HUVECs on 100-mm Petri dishes were gently washed 3 times with PBS, and the medium was replaced with 6 mL serum-free medium 199 or HuMedia. ECV-304 cells were exposed to shear stress at 15 dyne/cm² (moderate magnitude) for 6 hours, except that one monolayer of the Petri dish was kept in a static condition (control). The medium was taken for the measurement of ADMA at 3 and 6 hours after shear stress, and the cells were scraped for the measurement of PRMT-1 gene expression, dimethylarginine dimethylaminohydrolase (DDAH) activity, and protein. The effects of different magnitudes of shear stress at 5 and 25 dyne/cm² on PRMT-1 gene expression, DDAH activity, and protein. To examine the reproducibility of shear-induced release of ADMA, ECV-304 cells already exposed to shear stress at 15 dyne/cm² for 3 hours were further subjected to the same shear stress for another 3-hour period after the 30-minute static interval. HUVECs were exposed to shear stress at 15 dyne/cm² for 3 hours. The medium was taken for the measurement of ADMA, and the cells were scraped for the measurement of PRMT-1 gene expression, DDAH activity, and protein. In some experiments, the effects of pretreatment with the PPARγ activator troglitazone at 8 μmol/L and the stable expression of a dominant-negative mutant of IKKα on PRMT-1 gene expression and ADMA release were examined. In addition, the effect of shear stress on the translocation of NF-κB and the effect of troglitazone and a dominant-negative mutant of IKKα on shear stress–induced translocation of NF-κB were examined after exposure of HUVECs to 30 minutes of shear stress at 15 dyne/cm². Expression Vector and Transfection

HUVECs were transfected with expression vectors encoding the dominant-negative mutant of IKKα with the use of Effectene transfection reagent (Qiagen). Cells were maintained in selective medium containing 200 μg/ml genetin until colonies appeared.

Determination of PRMT-1 Gene Expression

Total RNA was prepared from ECV-304 cells and HUVECs, and cDNA was amplified by using oligonucleotide primers designed against the N-terminal region of PRMT-1: forward primer, 5′-AGTGGAGAGGTGACATCATCAT-3′ and reverse primer, 5′-CTCGGGAAGGAGGAGAAGG-3′. All PCR procedures were performed as follows: 25 cycles for PRMT-1 and GAPDH (45 seconds at 94°C, 45 seconds at 62°C, and 1 minute at 72°C) and a final elongation step (5 minutes at 72°C). The efficiency of amplification of each primer pair was calculated beforehand from the slope of the semilogarithmic relation between cycles of amplification (20 to 30) and amplification products. All reverse transcription–PCR studies were performed in duplicate in all specimens investigated.

Immunostaining and Fluorescence Microscopy

Subconfluent monolayers of HUVECs were fixed in methanol and incubated with 100% goat serum at 4°C overnight. The specimens were incubated in PBS containing 1% bovine serum albumin, 0.2% Triton X-100, and polyclonal anti-NF-κB p65 antibody (Santa Cruz Biototechnology) for 2 hours at 37°C and then incubated with fluorescein-conjugated anti-rabbit IgG for 2 hours at room temperature. The immunostaining was observed under an epifluorescence microscope.

Measurement of DDAH Activity

DDAH activity was assayed by determining l-citrulline formation from ADMA in cell lysates.16 As negative controls, the cell lysates were boiled for 10 minutes to inactivate the enzyme. Background values obtained were subtracted from the experimental data to provide true DDAH activity.

Measurement of ADMA

Concentration of ADMA in the conditioned medium was measured by high-performance liquid chromatography, as previously described. The minimum detection level of ADMA was 1 pmol/tube (0.02 μmol/L) in our assay system, and the ADMA level in the medium after cell incubation was ±10 pmol/assay tube (±0.2 μmol/L) in all cases. The intra-assay and interassay coefficients of variance were 9.0% and 10.6%, respectively.

Statistical Analysis

Data are expressed as mean±SE. Comparison of multiple means was made by ANOVA. Comparisons of 2 groups were made by Student unpaired t test. A value of P<0.05 was accepted as statistically significant.

Results

PRMT-1 Gene Expression

Figure 1A shows the cycle-dependent amplification of 750-bp and 195-bp products corresponding to mRNA for PRMT-1 and GAPDH, respectively. The graph indicates that linear amplification occurred between 20 and 30 cycles. As illustrated in Figure 1B, exposure of ECV-304 cells to shear stress at 15 dyne/cm² enhanced the gene expression of PRMT-1 mRNA at ≥3 hours without affecting GAPDH expression. The ratio of PRMT-1 to GAPDH mRNA was significantly higher, by 1.8±0.2-fold, at 3 hours and by
2.0±0.3-fold at 6 hours after shear stress at 15 dyne/cm². Exposure of ECV-304 cells to shear stress at 5 dyne/cm² for 3 hours did not affect gene expression of PRMT-1 mRNA, whereas shear stress at ≥15 dyne/cm² significantly enhanced that of PRMT-1 mRNA without affecting GAPDH expression (Figure 1C). The ratio of PRMT-1 to GAPDH mRNA was significantly higher after 3-hour exposure to shear stress at 15 (2.0±0.1-fold) and 25 (1.8±0.2-fold) dyne/cm² compared with that in a static condition (both P<0.05, n=4).

As shown in Figure 1D, exposure of HUVECs to shear stress at 15 dyne/cm² for 3 hours enhanced the ratio of PRMT-1 to GAPDH mRNA by 2.0±0.1-fold compared with that in a static condition (P<0.05). Pretreatment with troglitazone at 8 μmol/L, PPARγ activator, and the stable expression of the dominant-negative mutant of IKKα blocked the increase in the ratio of PRMT-1 to GAPDH mRNA after 3 hours of shear stress at 15 dyne/cm². The number of trypan blue–positive cells was undetectable after exposure to shear stress at each magnitude in both ECV-304 cells and HUVECs.

**Immunostaining of the p65 Subunit of NF-κB**

In static HUVECs, NF-κB was mainly distributed in the cytoplasm (Figure 2A), whereas at 30 minutes after shearing at 15 dyne/cm², NF-κB was mainly localized in the nucleus (Figure 2B). Antibody specificity was verified by the absence of NF-κB immunostaining in control experiments, in which nonimmune serum was used instead of the primary antibody. Shear stress induced the translocation of NF-κB from the cytoplasm to the nucleus in HUVECs.

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**Figure 1.** Effect of shear stress on gene expression of PRMT-1 in cultured ECV-304 cells and HUVECs. A, Amplification of PRMT-1 and GAPDH in ECV-304 cells in a static condition. B, Time dependence of shear (15 dyne/cm²)-induced PRMT-1 gene expression in ECV-304 cells. C, Magnitude dependence of shear (3 hours)-induced PRMT-1 gene expression in ECV-304 cells. D, Effect of troglitazone and dominant-negative mutant of IKKα on shear (15 dyne/cm², 3 hours)-induced PRMT-1 gene expression in HUVECs. *P<0.05 by 1-way ANOVA.

**Figure 2.** Immunostaining of the p65 subunit of NF-κB in HUVECs. A, Static condition; B, shear stress at 15 dyne/cm² for 30 minutes; C, shear stress at 15 dyne/cm² for 30 minutes under dominant-negative mutant of IKKα; and D, shear stress at 15 dyne/cm² for 30 minutes under troglitazone.
cytoplasm to the nucleus in the expression vector–transfected cells only. In contrast, the transfection of IKKα (Figure 2C) and pretreatment with troglitazone (Figure 2D) blocked the NF-κB translocation induced by shear stress.

**DDAH Activity**
DDAH activities in ECV-304 cells and HUVECs were not affected after 6-hour shear stress at 15 dyne/cm². As shown in Figure 3, shear stress at ≤15 dyne/cm² did not affect DDAH activity in ECV-304 cells, whereas shear stress at 25 dyne/cm² enhanced its activity by 148±6% compared with that in a static condition (n=4, P<0.05). Pretreatment of HUVECs with troglitazone at 8 μmol/L did not affect DDAH activities in a static condition (101±2%) and at 3 hours after shear stress at 15 dyne/cm² (105±5%). The increase in DDAH activity after shear stress at 25 dyne/cm² was blocked by troglitazone, to 107±3% (P<0.05).

**ADMA Release**
As shown in Figure 4A, in a static condition, the concentration of ADMA in serum-free conditioned medium of ECV-304 cells increased gradually with time, to a level of 60±14 pmol/mg protein at 3 hours and of 102±11 pmol/mg protein at 6 hours (n=8). Exposure of the cells to shear stress at 15 dyne/cm² enhanced the release of ADMA, to a level of 93±15 pmol/mg protein at 3 hours and of 183±22 pmol/mg protein at 6 hours (P<0.05 vs static condition, 2-way ANOVA). Compared with the effect of initial shear stress at 15 dyne/cm², an additional 3-hour exposure of the cells to the same shear after a 30-minute static interval caused a similar increase in ADMA release (1.77±0.15-fold increase after initial shear and 1.67±0.16-fold increase after second shear; n=3). As shown in Figure 4B, ADMA release was increased by 1.64±0.10-fold after shear stress at 15 dyne/cm² (P<0.05), whereas it was not affected at 3 hours after shear stress at 5 and 25 dyne/cm².

As shown in Figure 4C, the release of ADMA was increased, from a level of 72±17 pmol/mg protein to 106±22 pmol/mg protein after 3-hour shear stress at 15 dyne/cm² in HUVECs (P<0.05). Pretreatment with troglitazone at 8 μmol/L and stable expression of a dominant-negative mutant of IKKα blocked the shear stress–induced increase in ADMA release. Pretreatment of HUVECs with troglitazone at 8 μmol/L did not affect ADMA release in a static condition (102±4%) and at 3 hours after shear stress at 25 dyne/cm² (107±13%).

**Discussion**
The present study showed that shear stress at a moderate magnitude, which is comparable to mechanical forces usually exerted on the arterial wall under physiologic conditions, enhanced the expression of PRMT-1 mRNA via activation of the NF-κB pathway in HUVECs. Shear stress at a higher magnitude facilitated the degradation of ADMA via enhancement of DDAH activity and returned ADMA release levels to baseline. Protein arginine N-methylation has been recognized in a number of eukaryotic proteins, but its functional significance is not fully understood. Postulated roles for these posttranslational modifications include signal transduction, nuclear mRNA transport, and modulation of protein–nucleic acid interactions. Type I PRMT activity has been found in various tissues and cell types. It catalyzes asymmetric dimethylation and monomethylation of arginine residues in various proteins that are involved in signal transduction events and in mRNA processing, splicing, and transport into the cytoplasm.
contrast, type II PRMT symmetrically dimethylates and monomethylates arginine residues in myelin basic protein in cerebral tissue and therefore is unlikely to be involved in the effects observed in the present study.

It was recently reported that type I PRMT is expressed in human endothelial cells under baseline conditions and that gene expression of PRMT is upregulated in the presence of oxidized low-density lipoproteins (oxLDLs). Our present data further extend the role of PRMT by showing that the stimulatory effect of shear stress on ADMA release is accompanied by the upregulation of gene expression of PRMT-1 and is completely abrogated in the presence of a dominant-negative mutant of IKKβ and troglitazone. Therefore, modulation of methyltransferase activity might be involved in the stimulation of endothelial cell ADMA release by shear stress. It has been noted that methylation of protein-bound arginine residues is not sufficient for inhibition of NOS activity and that the active, free form of ADMA is derived from the catabolism of proteins containing asymmetric methylated arginine residues. Thus, modulation of protein catabolism also might be associated with the regulation of ADMA release by shear stress. In a static condition, a significant amount of ADMA was released, as under shear stress. Therefore, the PRMT-1 pathway might be constitutively active and serve to regulate NO production.

A metabolic pathway selective for degradation of ADMA has recently been characterized. The enzyme DDAH selectively hydrolyzes ADMA to form l-citrulline and dimethylamine. To date, at least 2 DDAH isoforms have been identified, and their tissue distributions are markedly different. DDAH type I is found in tissues that express predominantly neuronal NOS, whereas DDAH type II is found in tissues with high levels of endothelial NOS. Reduced metabolism might therefore be an alternative explanation for the selective modulation of ADMA release by endothelial cells. Oxidative stress induced by tumor necrosis factor-α and oxLDL was shown to enhance the release of ADMA into the conditioned medium without any apparent changes in DDAH type I protein in human vascular endothelial cells. However, it is now explained by the downregulation of DDAH type II activity in these cells. We showed that shear stress at 25 dyne/cm² (higher magnitude) facilitated the degradation of ADMA because of enhanced activity of DDAH, whereas at 15 dyne/cm² (moderate or low magnitude) did not affect DDAH activity. Thus, under a physiologic (moderate) magnitude of shear stress, the PRMT-1 pathway rather than DDAH activity seems to be an important determinant of circulating ADMA.

The posttranslational modification of arginine residues to ADMA is mainly carried out by PRMT-1, with S-adenosyl-L-methionine as a methyl donor. The present study showed that PRMT-1 gene expression was significantly enhanced after shear stress ≥15 dyne/cm². A 6-bp sequence, GAGACC, was demonstrated to be a shear stress–responsive element and seems to be involved in shear-induced regulation of vasoactive substances. Resnick et al showed that shear stress activates NF-κB and that this nuclear factor specifically binds to the shear stress–responsive element in endothelial cells. Thus, it is reasonable to presume that activation of this pathway might be involved in upregulation of gene expression of PRMT-1. Recently, it was shown that activation of PRMT gene expression by oxLDL was blocked by the intracellular antioxidant pyrrolidine dithiocarbamate, suggesting that redox-regulated mechanisms might underlie this effect. We investigated the effect of a dominant-negative mutant of IKKα and troglitazone on the shear stress–induced increase in gene expression of PRMT-1 in HUVECs because ECV-304 cells do not necessarily retain their original phenotype. The result demonstrated that the dominant-negative mutant of IKKα and troglitazone blocked the increase in the ratio of PRMT-1 to GAPDH mRNA by shear stress. In addition, the immunostaining study and fluorescence microscopy showed that shear stress stimulated translocation of the activated form of NF-κB into the nucleus and that its translocation was blocked after pretreatment with the dominant-negative mutant of IKKα and troglitazone. Taken together, these findings indicate that shear stress enhances gene expression of PRMT-1 and ADMA release via activation of the NF-κB pathway.

The inhibition of NOS by ADMA increases endothelial oxidative stress and upregulates the expression of redox-sensitive genes that code for endothelial adhesion molecules, comparable to what has been observed in early atherogenesis. ADMA might therefore act as an endogenous proatherogenic molecule. In humans, hypercholesterolemia, peripheral vascular disease, and hypertension are associated with elevated ADMA concentrations, which are related to impaired endothelium-dependent vasodilation, a feature that is indicative of increased cardiovascular risk. The plasma level of ADMA is also elevated in end-stage renal disease and congestive heart failure, which are both relevant to the hypervolemic state during high salt intake. In end-stage renal disease, however, the loss of renal clearance of ADMA is also an important factor for the high plasma level of ADMA. Kielstein and colleagues further observed that the plasma concentration of ADMA was higher in patients with end-stage renal disease and atherosclerotic vascular disease than in those without vascular complications.

Salt-sensitive hypertension is another hypervolemic state in which the magnitude of shear stress is elevated. We demonstrated recently that a high salt intake increases the plasma concentration of ADMA and decreases that of NO, in patients with salt-sensitive essential hypertension. We further showed that salt-sensitive hypertension manifests an absent or decreased nocturnal reduction in blood pressure, and nondippers, i.e., patients with an absent or decreased nocturnal reduction in their blood pressure, tend to have more severe target-organ damage than do dippers, i.e., those with a normal nocturnal decrease in blood pressure. Furthermore, a prospective study demonstrated that cardiovascular morbidity was higher in nondippers than in dippers, indicating that ADMA release by shear stress might contribute to the occurrence of cardiovascular events in human hypertension.

It is noted that endothelial NOS activity as well as ADMA release is stimulated by shear stress in vascular endothelial cells. Therefore, they might antagonistically regulate NO generation and systemic circulation. In the arterial tree, regions exposed to reduced shear stress (~15 dyne/cm²) such as areas of bends, branches, and bifurcations are predisposed to atherosclerosis. In these conditions, endothelial NOS activity would be overwhelmed by ADMA release, and NO generation would be suppressed.
In conclusion, the present data suggest that ADMA is formed in human vascular endothelial cells by PRMT-1 and that shear stress stimulates ADMA generation via activation of the NF-xB pathway. Although the functional significance of protein arginine N-methylation is still incompletely understood, our present data suggest an important regulatory role for this pathway for one of the products that is released during hydrolytic protein turnover. ADMA functions as an endogenous inhibitor of NOS and might thereby play an important role in endothelial function associated with the initiation and progression of vascular disease. Increased gene expression of PRMT-1 with subsequently enhanced methyltransferase activity might be a novel mechanism by which ADMA concentration is elevated during high salt intake in human hypertension.

Perspectives

Shear stress stimulates ADMA release as well as endothelial NOS activity in vascular endothelial cells. Thus, shear-induced ADMA release provides further understanding of the regulation mechanism of NO generation. The present data extend the role of PRMT by showing shear-induced ADMA release. It is noted that shear-induced ADMA release is completely abrogated in the presence of a dominant-negative mutant of IKKα and troglitazone. This might provide a therapeutic strategy aimed at blocking shear-induced ADMA release and the progression of arteriosclerosis. Future studies will be focused on assessment of the benefit of PPAR γ ligand treatment to pathologic states in which plasma ADMA levels are increased.

References


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