Original Article

Laminar Shear Stress Up-Regulates Inducible Nitric Oxide Synthase in the Endothelium

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Shear stress caused by blood flow is a potent physiological stimulus for the generation of nitric oxide (NO) in endothelial cells, which is believed to derive from the up-regulation and post-transcriptional activation of endothelial constitutive NO synthase (ecNOS). However, it has yet to be demonstrated that inducible NO synthase (iNOS) plays a significant role in shear stress-induced NO production from endothelial cells. We used parallel plate-type flow chambers that detect fluid shear stress to determine that shear stress, as quantified by a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), increased iNOS gene transcripts in cultured endothelial cells, which resulted in increased NO production. Shear stress-induced iNOS expression was inhibited by pyrrolidine dithiocarbamate (PDTC), an antioxidant and nuclear factor κ B (NF- κ B) blocker, and by MG132, an aldehyde peptide proteasome inhibitor that antagonizes I κ B-kinase. Laminar shear stress increased the transcriptional activity of NF- κ B, whereas over-expression of an I κ B- mutant that inhibits the activation of NF- κ B in a dominant-negative fashion was found to attenuate the induction of endothelial iNOS by shear stress. The present results demonstrate that shear stress induces iNOS in the endothelium, mainly *via* the activation of NF- κ B. (*Hypertens Res* 2004; 27: 93–99)

Key Words: nitric oxide, inducible nitric oxide synthase, shear stress, nuclear factor κ B, endothelium

Introduction

Cells *in situ* are influenced by various mechanical forces such as gravitational force, mechanical stretch, and shear stress. Vascular endothelial cells are constantly exposed to shear stress due to blood flow, and increased shear stress regulates a number of endothelial factors resulting in vasodilation; nitric oxide (NO) is known to play the most dominant role in this context. Endothelial constitutive NO synthase (ecNOS), initially described in the 1980s (1, 2) has been considered to be solely responsible for shear stress-induced NO generation in endothelial cells (3–5). Laminar shear stress up-regulates ecNOS expression in a magnitude-dependent fashion, whereas turbulent flow does not up-regulate ei-

ther NO production or ecNOS gene expression (5–7). Although ecNOS activation usually requires calcium-activated calmodulin, the up-regulation of ecNOS by shear stress involves calcium-independent pathways (8), including changes in cytosomal pH, and structural changes in the cytoskeleton (9).

While ecNOS is regarded as constitutive, inducible NO synthase (iNOS) is expressed only after induction by stimuli, and unlike ecNOS, it does not require intracellular calcium for activation; the activation of iNOS can result in the production of a greater amount of NO than ecNOS can provide (10, 11). Though shear stress has recently been shown to induce iNOS in vascular smooth muscle cells (12), it is generally thought that iNOS does not play a significant role in shear stress-induced NO production in endothelial cells.

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Nuclear factor- κ B (NF- κ B) is a transcription factor and plays a pivotal role in the induction of iNOS (13, 14). NF- κ B is activated by various extracellular stimuli (*e.g.*, tumor necrosis factor (TNF)- α , interleukin-1 β , free radicals, and hypoxia); in the cytoplasm, NF- κ B is tightly associated with inhibitory I κ B proteins. The activation of I κ B kinase and the subsequent phosphorylation of I κ B allow the translocation of NF- κ B into the nucleus to transactivate its target genes. There is evidence that shear stress is an inducer of NF- κ B activation in endothelial cells (15, 16).

These observations prompted us to investigate whether shear stress induces iNOS gene expression in endothelial cells *via* the activation of NF- κ B.

Methods

Materials

Pyrrolidine dithiocarbamate (PDTC), an NF- κ B blocker and antioxidant, was purchased from Sigma Chemical Co. (St. Louis, USA). MG132, a proteasome inhibitor, was purchased from the Peptide Institute (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and Nutrient Mixture F-12 Ham was obtained from Flow Laboratories (Irvine, Scotland), and fetal bovine serum (FBS) was purchased from Cell Culture Laboratories (Cleveland, USA). The polymerase chain reaction (PCR) primers were synthesized by Sawady, Inc. (Tokyo, Japan).

Cell Culture

A vascular endothelial cell line originally established from rat lung (TRLEC) (17, 18) was kindly donated by the Institute of Cytosignal Research Inc. (Tokyo, Japan), and was cultured in DMEM containing 10% FBS. Human adult dermal microvascular endothelial cells (hADM) and human aortic smooth muscle cells (hASM) were purchased from Cell Systems (Kirkland, USA), and were cultured in a half-Nutrient Mixture F-12 Ham/half-DMEM mixture containing endothelial cell growth supplement, heparin, glutamine, and 10% FBS. The cells were maintained in a 5% CO₂ atmosphere at 37°C.

Flow Stimulation

Confluent monolayers of cells on glass slides were subjected to laminar shear flow using a parallel plate flow chamber (Yasuhisa Koki, Ltd., Tokyo, Japan). The glass slide and the polymethacrylate plate that formed the other side of the chamber were held $\sim 200 \mu\text{m}$ apart by a silicone rubber gasket. The chamber had an entrance connected to a reservoir by a silicone tube and an exit for the medium, which was circulated by a roller/tube pump. The entire circuit was placed in an automatic CO₂ incubator, and flow-loading experiments were performed in a 5% CO₂ atmosphere at 37°C. The

intensity of wall shear stress (τ , in dyn/cm²) on the monolayer cells was calculated by the following formula: $\tau = 6\mu Q/a^2b$, where μ is the viscosity of the perfusate (in P), Q is flow volume (in ml/s), and a and b are cross-sectional dimensions of the flow path. Since the maximum Reynolds number corresponding to the highest flow rate used in this study was ~ 40 , we assumed that the flow was laminar.

Determination of Nitrite/Nitrate (NO_x)

Rat endothelial cells on glass slides were exposed to a shear stress of 16 dyn/cm² for 3 h, replaced into 6-cm dishes, and were further incubated without shear stress for 16 h. NO_x concentrations in the conditioned media were measured by an autoanalyzer (TCI-NOX 100, Tokyo Kasei Kogyo, Tokyo, Japan), as previously described (19). In brief, samples premixed with a carrier solution (0.07% EDTA and 0.3% NH₄Cl) were passed through a copperized cadmium reduction column to reduce NO₃⁻ to NO₂⁻, which reacts with Griess reagent (1% sulfonamide/0.1% *N*-1-naphthylethylenediamine dihydrochloride/5% HCl). Absorbance at 540 nm was measured by a flow-through visible spectrophotometer. NO₃⁻ was used as a standard.

Real-Time Quantitative Reverse Transcription (RT)-PCR

Total RNA was extracted from cells on single glass slides using RNeasy[®] (GIBCO/BRL, Gaithersburg, USA). First-strand cDNA from each mRNA was generated by a reverse transcription method using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, USA) (20). Rat and human iNOS mRNA was amplified using synthetic oligomers as probes (rat: forward 5'-GAAGGCTGGAACCTA ACTGC-3', complement 5'-TGCCTTGCTGAATGAACTT G-3'; human, forward 5'-TACTCCACCAACAATGGCAA-3', complement 5'-GATGAGCTGAGCATTCCACA-3'), detected and quantified in real time using a Light Cycler[™] (Roche Diagnostics, Basel, Switzerland), as previously described (21, 22). The amplification mixture contained 50 nmol/l template cDNA and 500 $\mu\text{mol/l}$ primer DNA in a DNA amplification kit (DNA Master SYBR Green I: Roche Diagnostics, Mannheim, Germany). Denaturation was carried out at 95°C, annealing at 52°C for 5 s, and extension at 72°C for 22 s. The reaction produced 377 bp and 98 bp PCR products; these results were confirmed by agarose gel electrophoresis. The quantification of the cDNA copy number was performed using control samples according to the manufacturer's protocol.

Western Blot Analysis

Rat endothelial cells cultured on glass slides were exposed to a shear stress of 16 dyn/cm² for 3 h, replaced in 6-cm dishes, and were further incubated without shear stress for 12 h. The cells were then lysed in cell lysis buffer and the extracted

Fig. 1. Effects of laminar shear stress on iNOS expression and NO production in rat lung endothelial cells. (A) Endothelial cells were exposed to 16 dyn/cm² shear stress for 3 to 6 h in a flow chamber, and total RNAs were extracted and subjected to real-time quantitative RT-PCR for the quantification of iNOS mRNA. Control cells were cultured under the same conditions without insertion in the flow chamber. (B) Endothelial cells exposed to 16 dyn/cm² shear stress for 3 h were incubated for 16 h, and the concentrations of NO_x released into the media were measured. Each column with a bar shows the mean $\pm$ SEM (n = 6). *p<0.005, **p<0.001, vs. control.

protein was subjected to Western blotting using monoclonal antibody raised against murine iNOS, as previously described (23, 24). Cells serving as the negative control were incubated for 3 h in a flow chamber without being subjected to shear stress, and then the cells were placed in 6-cm dishes for an additional 12 h.

Transient Transfection

The involvement of NF- κ B was assessed utilizing the pNF- κ B-Luc plasmid (BD Biosciences Clontech, Palo Alto, USA), which contains four NF- κ B consensus elements (GGGAATTTCC) upstream of the luciferase reporter gene. The mediatory role of I κ B- α was assessed using a MercuryTM I κ B- α Dominant-Negative Vector Set (BD Biosciences Clontech) containing both I κ B- α expressing cDNA and I κ B- α mutant (I κ B- α M). Proteins encoded by I κ B- α M are not subjected to degradation in response to the activation of I κ B kinase (IKK), thereby acting as a dominant-interfering form of NF- κ B. Transient transfections of pNF- κ B-Luc, I κ B- α and I κ B- α M were performed with high efficiency by a modified transferrin receptor-operated transfer method, as previously described (25, 26).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Differences between groups were examined for statistical significance using Wilcoxon's *t* test or analysis of variance.

Results

Shear Stress Induces iNOS Expression

To examine the effects of shear stress on iNOS gene expression, rat endothelial cells were exposed to laminar shear stress for 3 or 6 h, and iNOS mRNA levels were quantified. The expression of iNOS was induced by 16 dyn/cm² shear stress after 3 h (Fig. 1A). Six hours of 5 dyn/cm²-shear stress was insufficient to induce iNOS expression.

NO_x levels in the conditioned media of cells after exposure to 16 dyn/cm² shear stress for 3 h markedly increased compared to the control cells, which had been incubated in the same chamber without being subjected to fluid shear stress (Fig. 1B). Western blot analysis revealed the expression of 130 kDa iNOS protein in the endothelial cells exposed to 16 dyn/cm² shear stress, but this result was not observed among the control cells that had not been exposed to shear stress (Fig. 2).

We then determined whether shear stress induces iNOS genes in human vascular cells. Shear stress (16 dyn/cm² for 3 h) markedly increased iNOS mRNA levels in hADM and hASM (Fig. 3).

PDTC and MG132 Block Shear Stress-Induced iNOS Expression

To elucidate the possible involvement of NF- κ B in shear stress-induced iNOS expression, rat endothelial cells were pretreated with either an NF- κ B blocker (PDTC) or a proteasome inhibitor (MG132), and the effect of shear stress was

Fig. 2. Effect of shear stress on iNOS protein expression in endothelial cells. Endothelial cells were exposed to 16 dyn/cm² shear stress for 3 h in a flow chamber, and the cell lysates were subjected to Western blot analysis for the detection of iNOS protein. Cells incubated in the flow chamber for 3 h that were not exposed to shear stress served as the control. The arrow indicates the position of the 130 kDa-iNOS protein.

determined. PDTC completely blocked the increase of iNOS mRNA induced by shear stress (3 h, 16 dyn/cm²) (Fig. 4A). MG132 also suppressed the induction of shear stress-induced iNOS (Fig. 4B). These results indicate that a proteasome-dependent transcription factor, NF- κ B, is very likely to be involved in the induction of iNOS by shear stress in endothelial cells.

Shear Stress Activates NF- κ B

We then determined whether or not shear stress was able to induce genes through the transcriptional activation of NF- κ B consensus sequences. Shear stress (3 h, 16 dyn/cm²) significantly increased the luciferase activity of pNF- κ B-Luc, which had been transiently overexpressed in endothelial cells (Fig. 5).

Suppression of iNOS mRNA by Blocking NF- κ B

Overexpression of the I κ B- α M construct in endothelial cells significantly attenuated the shear stress-induced iNOS expression, compared to that of control cells transfected with I κ B- α expressing-cDNA (Fig. 6). These results demonstrate that I κ B- α -dependent NF- κ B activation played a mediatory role in shear stress-induced iNOS expression, although other mechanisms may also have been involved.

Discussion

The present study demonstrated that shear stress up-regulates iNOS gene expression, not only in rat lung endothelial cells,

Fig. 3. Effect of shear stress on iNOS expression in human vascular cells. Human microvascular endothelial cells (hADM) and aortic smooth muscle cells (hASM) were exposed to 16 dyn/cm² shear stress for the indicated amount of time, and iNOS mRNA levels were quantified. Control cells were cultured under the same conditions without being placed in the flow chamber. Each column with a bar shows the mean $\pm$ SEM (n = 6). *p < 0.001, vs. control.

but also in primary human microvascular endothelial cells and human aortic smooth muscle cells; these results therefore suggest the possibility that this phenomenon may be universal among vascular cells. Although the exact mechanotransduction pathway is not clear, our results strongly indicate the involvement of NF- κ B in shear stress-induced iNOS expression. Shear stress is known to increase NO production by endothelial cells (3, 4), but this has long been ascribed to eNOS up-regulation due to blood flow (5–7). It was recently reported that shear stress-induced iNOS expression in cultured aortic smooth muscle cells, whereas it led to a decrease in brain NOS mRNA levels (12). The iNOS gene up-regulation was shown to occur only after prolonged exposure to 20 dyn/cm² shear stress of up to 24 h, but it did not occur within 6 h; in that previous study, iNOS gene up-regulation appeared to be mediated by oxidative stress-induced NF- κ B activation, resulting in significant NO production (12). In that study, the increased NO production was considered to be derived solely from the up-regulated iNOS gene (12), since shear stress suppressed brain NOS mRNA. However, in the present study, we demonstrated for the first time that shear stress markedly induced the iNOS gene and subsequent NO generation in vascular endothelial cells. Here, the exposure of cells to shear stress for 3 h resulted in a remarkable increase in iNOS mRNA levels, iNOS protein expression, and subsequent NO_x accumulation in the conditioned

Fig. 4. Effect of NF- κ B inhibition on shear stress-induced iNOS expression. Endothelial cells pretreated with or without (A) PDTC or (B) MG132 were stimulated by 16 dyn/cm² shear stress for 3 h, and the iNOS mRNA levels were quantified. Each column with a bar shows the mean $\pm$ SEM (n = 6). * p<0.01, ** p<0.005.

Fig. 5. Activation of NF- κ B by shear stress. Rat endothelial cells transiently expressing the pNF- κ B-Luc construct were exposed to 16 dyn/cm² shear stress for 3 h, and the luciferase activities were determined. Control cells were cultured under the same conditions, but were not placed in the flow chamber. Each column with a bar shows the mean $\pm$ SEM (n = 8). * p<0.001, vs. control.

Fig. 6. Effects of I κ B- α blockade on shear stress-induced iNOS expression. Endothelial cells transfected with either I κ B- α or I κ B- α mutant (I κ B- α M)-expressing construct were stimulated with 16 dyn/cm² shear stress for 3 h, and iNOS mRNA levels were quantified. Each column with a bar shows the mean $\pm$ SEM (n = 8). * p<0.005, ** p<0.001, vs. control.

media. In contrast, the control cells that were unexposed to shear stress did not show such effects. The marked increase in NO production during the 16 h-post shear stress interval may be attributed to the increased expression of iNOS, but the contribution of eNOS remains unclear. These results suggest the possibility that iNOS may be playing a more im-

portant role in shear stress-induced endothelial NO production than previously thought.

NF- κ B plays a key role in iNOS induction by cytokines (27), and shear stress can activate NF- κ B in endothelial cells

(15, 16); such findings have led to the hypothesis that endothelial iNOS induction by shear stress is dependent on NF- κ B activation. The present study demonstrated that PDTC, an NF- κ B blocker and antioxidant, and MG132, a cell-permeable proteasome inhibitor, blocked the shear stress-induced up-regulation of iNOS. Specifically, PDTC has been shown to inhibit NF- κ B, *via* interference with reactive oxygen metabolism, chelation of divalent metal ions, and changes in intracellular thiol levels (28–30), whereas MG132 inhibits proteasome activity, thus interfering with the I κ B degradation following its phosphorylation (31). The present results, which demonstrated that both reagents blocked shear stress-induced iNOS expression, are suggestive of the involvement of NF- κ B in flow-instigated endothelial iNOS induction.

The over-expression of I κ B- α M, a component that works as a dominant-negative construct against I κ B- α -dependent NF- κ B, also attenuated shear stress-induced iNOS expression. Inactivated NF- κ B is associated with I κ B proteins such as I κ B- α , whereas the phosphorylation of I κ B proteins by IKK is necessary for NF- κ B activation. The degradation of I κ B by proteasomes following its phosphorylation leads to the dissociation of the I κ B–NF- κ B complex and to the translocation of NF- κ B to the nucleus. The I κ B- α mutant we used in the present study cannot be phosphorylated by I κ B kinase, and thus prevents the fraction of NF- κ B from being activated. Our data shows that the effect of I κ B- α M in suppressing iNOS expression was significant, but not complete. The results imply the presence of an as of yet unclarified proteasome-mediated mechanism apart from the I κ B- α -dependent NF- κ B activation pathway in shear stress-induced iNOS expression. It has already been recognized that modulatory phosphorylations of the DNA-binding subunits can control DNA binding and transactivation properties, as well as interactions between the transcription factor and regulatory proteins (32). Furthermore, it has been shown that other transcription factors and intracellular signal transduction pathways linked to the cellular stress response act in concert with NF- κ B to regulate the expression of specific genes (33, 34). In the cytoplasm of most cells, unstimulated NF- κ B is considered to be closely associated with the inhibitory I κ B protein (35, 36). Various stimuli are known to lead to the phosphorylation of I κ B by IKK, which leads to both ubiquitination and the degradation of I κ B. Shear stress has been reported to activate IKK through an integrin-dependent pathway (37). Although the entire pathway by which shear stress activates NF- κ B has not been completely elucidated, our data suggest that the activation of IKK, as well as the subsequent activation of NF- κ B, are most likely to be responsible for shear stress-induced iNOS expression.

In conclusion, we have demonstrated that physiological levels of shear stress up-regulate iNOS expression *via* the activation of NF- κ B, resulting in markedly enhanced NO production in endothelial cells. Our results suggest that iNOS may be constantly expressed in the vascular endothelium,

because it is continuously exposed to flow shear stress. Since the induction of iNOS is known to cause the production of much larger amounts of NO than eNOS can produce, iNOS might play a more important role in the production of vascular NO than presently believed. This possible role played by endothelial iNOS has been overlooked in the extensive exploration of the importance of NO in the endothelium.

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