Sp1-mediated downregulation of P2X<sub>4</sub> receptor gene transcription in endothelial cells exposed to shear stress

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Korenaga, Risa, Kimiko Yamamoto, Norihiko Ohura, Takaaki Sokabe, Akira Kamiya, and Joji Ando. Sp1-mediated downregulation of P2X<sub>4</sub> receptor gene transcription in endothelial cells exposed to shear stress. Am J Physiol Heart Circ Physiol 280: H2214–H2221, 2001.—Endothelial purinoceptors play an important role in vascular responses to extracellular adenine nucleotides and hemodynamic forces. Here we report that P2X<sub>4</sub> purinoceptor expression in human umbilical vein endothelial cells is transcriptionally downregulated by fluid shear stress. When human umbilical vein endothelial cells were subjected to a laminar shear stress of 15 dyn/cm<sup>2</sup>, P2X<sub>4</sub> mRNA levels began to decrease within 1 h and further decreased with time, reaching 60% at 24 h. Functional analysis of the 1.9-kb P2X<sub>4</sub> 5' -promoter indicated that a 131-bp segment (−112 to +19 bp relative to the transcription start site) containing a consensus binding site for the Sp1 transcription factor was critical for the shear stress responsiveness. Mutations of the Sp1 site decreased the basal level of transcription and abolished the response of the P2X<sub>4</sub> promoter to shear stress. Electrophoretic mobility shift assays showed a marked decrease in binding of Sp1 to the Sp1 consensus element in shear-stressed cells, suggesting that Sp1 mediates the shear stress-induced downregulation of P2X<sub>4</sub> gene transcription.

EXTRACELLULAR ADENINE NUCLEOTIDES such as ATP and ADP play an important role in the regulation of many biological processes, including vascular tone, platelet aggregation, muscle contraction, neurotransmission (peripheral and central), and cardiac functions (4, 10). They exert their effects through binding P2 purinoceptors on the target cells and subsequent signal transduction (12, 26). There are two types of P2 receptors: G protein-coupling receptors (P2Y) and ligand-gated cation channels (P2X). The binding of adenine nucleotides to P2Y activates phospholipase C via G protein and increases inositol trisphosphate, which induces Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores, such as the endoplasmic reticulum, followed by Ca<sup>2+</sup> entry. P2X receptors provide a pathway for the influx of extracellular Ca<sup>2+</sup> into cells on binding to adenine nucleotides.

When ATP is administered into blood vessels, an endothelium-dependent vasodilation occurs. This is due to ATP-induced production of nitric oxide (NO) and prostacyclin in endothelial cells (ECs) (8, 24), which is believed to be triggered by an increase in intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) (20). ECs show a peak and sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to ATP (11, 25). The peak increase is assumed to be P2Y-mediated Ca<sup>2+</sup> release, and the sustained increase is thought to be Ca<sup>2+</sup> influx through P2X or store-operated channels (22). The production of prostacyclin is mainly dependent on the peak increase in [Ca<sup>2+</sup>]<sub>i</sub>, whereas NO production is dependent on a sustained rise in [Ca<sup>2+</sup>]<sub>i</sub> (23). However, it was unclear which subtypes of P2X or P2Y receptors are involved in the ATP-induced Ca<sup>2+</sup> responses in ECs.

Recently, we found that a subtype of P2X receptor, P2X<sub>4</sub> (9), is predominantly expressed in human vascular ECs and plays a central role in ATP-induced Ca<sup>2+</sup> influx (30). Furthermore, we observed that, in the presence of relatively low levels of extracellular ATP, fluid shear stress activates P2X<sub>4</sub> receptors, causing a dose-dependent Ca<sup>2+</sup> influx into ECs (29). This suggests that P2X<sub>4</sub> receptors recognize shear stress directly or indirectly and transmit the signal into the cell interior via Ca<sup>2+</sup> signaling. Shear stress, a mechanical force generated by flowing blood, can modulate endothelial morphology, function, and gene expression (1, 7). These EC responses to shear stress are not only essential for the maintenance of vascular homeostasis but are also related to blood flow-dependent phenomena, such as angiogenesis, vascular development and remodeling, and atherogenesis. Thus the expression level of P2X<sub>4</sub> receptor in ECs may be an important determinant of the vascular sensitivity to adenine nucleotides or shear stress, which play a crucial role in the control of a variety of cardiovascular functions.

In the present study, we investigated the effect of shear stress on P2X<sub>4</sub> receptor expression in ECs. Cultured human umbilical vein ECs (HUVECs) were ex-
posed to physiological levels of laminar shear stress in a flow-loading apparatus, and changes in the P2X$_4$ protein and mRNA levels were measured. Luciferase assay was used to examine whether shear stress can affect P2X$_4$ gene transcription via a shear stress-responsive element (SSRE) in the P2X$_4$ gene promoter. Electrophoretic mobility shift assay (EMSA) was used to determine the effect of shear stress on the binding of transcription factor to the SSRE.

**MATERIALS AND METHODS**

**Cell Culture**

Primary cultures of HUVECs were prepared from human umbilical cord vein by collagenase treatment and grown on a 1% gelatin-coated flask in medium 199 containing 15% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were routinely passaged by trypsinization in a 0.05% trypsin-2 mM EDTA solution. At each passage, cell number was counted with a Coulter counter (Coulter Electronics), and cumulative population doubling was determined. Cells with $8 – 14$ cumulative population doublings ($\text{passages } 4 – 7$) were grown to confluence on gelatin-coated glass plates ($55 \times 70$ mm) for use in the experiments. The purity of the cultures was confirmed by immunofluorescent staining of factor VIII-related antigen and by the uptake of fluorescence-labeled acetylated low-density lipoprotein.

Bovine ECs were isolated from the descending thoracic aorta of a bovine fetus by brief collagenase digestion and cultured in medium 199 containing 15% FBS, 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Cells at passages 4 – 7 were used for transfection experiments.

**Flow-Loading Apparatus**

To apply controlled levels of laminar shear stress to cultured cells, we used the same parallel plate type of flow chamber described previously (3). One side of the chamber was formed by a coverslip on which ECs were cultured. The other side was machined from a polymethacrylate plate. These two flat surfaces were held ~ 200 μm apart by a silicone rubber gasket. The chamber had an entrance and an exit for the medium, and the entrance was connected to a flow-loading apparatus, and changes in the P2X$_4$ gene transcription via a shear stress-responsive element (SSRE) in the P2X$_4$ gene promoter. Electrophoretic mobility shift assay (EMSA) was used to determine the effect of shear stress on the binding of transcription factor to the SSRE.

**RT-PCR Analysis**

RT-PCR was performed to quantify the P2X$_4$ mRNA levels, as previously described (16). Briefly, total RNA was isolated from the cells by the acid guanidine thiocyanate-phenol-chloroform extraction method. Reverse transcription of RNA was carried out in 20 μL of reaction mixture containing 1.0 μg of total RNA, 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO), 0.5 μg of oligo(dT)$_{12-18}$ (Perkin Elmer), 40 units of RNase inhibitor (Perkin-Elmer), 2.5 mmol of each dNTP mixture, and 10 mmol of dithiothreitol in a first-strand buffer. The mixture was incubated at 37°C for 1 h, heated at 99°C for 5 min, and chilled at 4°C for 5 min. The cDNA samples were then amplified by PCR with sense and antisense primer pairs for each P2X$_4$ (5'-AAGTCGTGTCGCTGTTGAAGTC-3' and 5'-AGTGTTGTGCTACTCTCACC-3') and glyceroldehyde 3-phosphate dehydrogenase (GAPDH; 5'-ACATACATCCGCTGCCTACTG-3' and 5'-AGTGGTGTCGCTGTGAAGTC-3'). A solution containing 0.25 units of Ex Taq DNA polymerase (Takara), 370 kBq of [α-32P]dCTP, and each primer was added to each sample. Each cycle consisted of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Amplified product was sampled every other cycle and resolved by electrophoresis with a 5% polyacrylamide gel.

For quantification of PCR products, the radioactivity of each band was measured with an imaging system (model GS363, Bio-Rad) and plotted against the number of PCR cycles on a semilogarithmic scale, forming a sigmoidal curve. From the curve, the cycle in which the operating range of the PCR was linear was selected, and the ratio of radioactivity of P2X$_4$ to GAPDH in the cycle was calculated as a measure of relative P2X$_4$ mRNA levels.

The sequences of the P2X$_4$ and GAPDH PCR products were verified by cloning into the pCR2.1 vector and cycle sequencing using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

**Northern Blot Analysis**

For Northern blot analysis, 2 μg of mRNA were fractionated on a 1% agarose-formaldehyde gel with MOPS buffer containing 0.02 M MOPS (Sigma Chemical), 5 mM sodium acetate, and 1 mM EDTA, capillary transferred to noncharged nylon membranes, and ultraviolet cross-linked. After 30 min of prehybridization at 68°C in ExpressHyb hybridization solution (Clontech), the membrane was hybridized with an [α-32P]dCTP scrambled-primed P2X$_4$ DNA probe, which was obtained from the cloning previously described (30). The blot was washed with 2 × saline-sodium citrate and 0.05% SDS and visualized with a molecular imaging system (model GS363, Bio-Rad).

**Western Blot Analysis**

HUVECs were washed with cold PBS and solubilized in 500 μL of RIPA buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 0.1% SDS, 0.2 mM Na$_2$MoO$_4$, 10 mM Na$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 0.2 μM aprotonin). Lysates were centrifuged at 26,000 g for 30 min. The supernatants were mixed with SDS sample buffer (0.2 M Tris-HCl, pH 6.8, 18% glycerol, 4% SDS, 0.01% bromophenol blue, and 10% β-mercaptoethanol) for SDS-PAGE. Gels were transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked in Tris-buffered saline with 5% skim milk and 0.1% Tween 20 and then incubated for 1 h with the anti-P2X$_4$ antisera (30 μg/ml) (30) or anti-intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody (2 μg/ml; Becton-Dickinson). ICAM-1 has been shown to be sensitive to shear stress (21). Membranes were washed in PBS and incubated with IgG horseradish peroxidase-conjugated antibody (Amersham). The blots were developed using an enhanced chemiluminescence kit (Amersham) and analyzed by a molecular imaging system (model GS363, Bio-Rad).
Cloning and Sequencing of Human P2X4 Receptor

A 150-mer synthetic oligonucleotide encoding exon 1 of the human P2X4 gene was radiolabeled with [α-32P]dCTP using a random primer labeling kit (Takara). A human genomic library (Clontech) was screened by lifting 1.2 × 10^6 phages onto Hybond-N nylon membrane (Amersham). After a 12-h prehybridization in 5× saline-sodium phosphate-EDTA, 5× Denhardt solution, 0.5% SDS, 10% dextran sulfate, and 0.25 mg/ml salmon testis DNA at 65°C, hybridization was carried out in the same solution with radiolabeled P2X4 probe for 20 h at 65°C. The positive clones were identified by autoradiography on X-ray film. The DNA from the positive clones was isolated, digested with EcoRI (−6.2 kb), and subcloned into pBluescript II KS(+) vector (Stratagene). The complete nucleotide sequence was determined using a DNA sequencer (373S-36, Applied Biosystems) and deposited in Genbank (accession number AF191093).

Construction of Reporter Plasmids and Luciferase Assay

To determine the transcriptional activity of the P2X4 gene, we used reporter plasmids containing the human P2X4 promoter linked to the luciferase gene (pGL3-enhancer vector; Promega). The following deletion constructs were generated.

−1.9-kb luc. 1.9 kb (−1924 to +19) of the P2X4 upstream region was removed from pBluescript II KS(+) plasmid containing a 6.2-kb P2X4 fragment by digestion with XbaI-BglI and cloned into the pGL3 enhancer.

−1.7-kb luc. 1.7 kb (−1684 to +19) of the P2X4 upstream region was cut from pBluescript II KS(+) plasmid containing a 6.2-kb P2X4 fragment by digestion with PstI-BglI and cloned into the pGL3 enhancer.

−145 luc. 164 bp (−145 to +19) of the P2X4 upstream region was removed from pBluescript II KS(+) plasmid containing a 6.2-kb P2X4 fragment by digestion with SmaI-BglI and cloned into the pGL3 enhancer.

−112 luc. 131 bp (−112 to +19) of the P2X4 upstream region was removed from pBluescript II KS(+) containing a 6.2-kb P2X4 fragment by digestion with ApaI-BglI and cloned into the pGL3 enhancer.

These constructs were transfected into cultured bovine ECs using LipofectAMINE PLUS (GIBCO). The pB5V-β-galactosidase vector (Promega) was cotransfected to monitor transfection efficiency. A mixture of 2 µg of plasmid DNA, 8 µl of PLUS reagent, and 250 µl of medium 199 was added to 12 µl of LipofectAMINE reagent in 2 ml of medium 199. The solution was put onto bovine ECs, and after 3 h of incubation at 37°C, the solution was replaced with fresh medium 199 +15% FBS. After 18 h, the cells were incubated under static conditions or exposed to a shear stress of 15 dyn/cm² for 24 h. The cells were washed twice with PBS, and 250 µl of lysis buffer (included in Promega’s luciferase assay kit) were added. The lysates were centrifuged at 10,000 g for 2 min. Luciferase activity was determined using 20 µl of the clarified lysate and 100 µl of luciferase assay substrate (Promega) in a luminometer (model LB9501, Berthold Lumat). The level of β-galactosidase was assayed in parallel by addition of o-nitrophenyl-β-d-galactopyranoside to the cell lysate and incubation at 37°C for 1 h. The absorbance at 420 nm was measured by microplate reader (model 3550, Bio-Rad). The luciferase activity of each sample was normalized to that of β-galactosidase before results were calculated.

Site-Directed Mutagenesis

Mutations were generated at Sp1-binding sites in the P2X4 gene promoter using a Quick change site-directed mutagenesis kit (Stratagene). Briefly, PCR was performed with the P2X4 (−112 bp) plasmids as template and two synthetic oligonucleotide primers containing specific mutations (5’-CAGGCGAGGCGGACTGGTGCGGCTCCGAGCGCGG-3’ and 5’-CCCCGTCTGGAGCCCCAGAACGTCCGCCTGGCGTCG-3’) using PfuTurbo DNA polymerase. After the products were treated with DpnI, the DNA was transformed into Epicurian Coli XL1-Blue supercompetent cells. The mutation was confirmed by DNA sequencing. Luciferase assay was carried out in the bovine ECs that were transfected with this mutated DNA and were cultured under static conditions or exposed to shear stress.

EMSA

Nuclear extracts from HUVECs cultured under static conditions or subjected to a shear stress of 15 dyn/cm² for 24 h were prepared as previously described (15). A 30-mer oligonucleotide containing Sp1 with a sequence of 5’-CCAGGCGGAGGCGGACTGGTGCGGCTCCGAGCGCGGG-3’ was labeled using T4 polynucleotide kinase and [γ-32P]ATP. The binding reactions between radiolabeled oligonucleotides and nuclear extracts were allowed to proceed, and the reaction mixtures were separated by 4.7% PAGE using reagents supplied with the Stratagene Gelshift assay kit. The protein-DNA complexes on the gel were analyzed on a molecular imager system (model GS363, Bio-Rad). In supershift assay, 2 µg of antibody against Sp1 (Santa Cruz) were added to the binding reaction, and the mixture was incubated for 30 min at room temperature before addition of the labeled oligonucleotide.

Statistical Analysis

Values are means ± SD. The mean values obtained in the control and experimental groups were analyzed for significant differences by ANOVA and the Bonferroni modification of the t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Laminar Flow Decreases P2X4 mRNA and Protein Levels in HUVECs

To examine the effect of shear stress on P2X4 gene expression, HUVECs were exposed to laminar flow with a shear stress of 15 dyn/cm² for 1, 3, 6, 12, and 24 h, and P2X4 mRNA levels were determined by RT-PCR. The P2X4 mRNA levels began to decrease as early as 1 h after the onset of flow and decreased progressively with time, reaching ~59% of the static control at 24 h (Fig. 1). The decrease in the P2X4 mRNA levels by shear stress was confirmed by Northern blotting.

Western blotting showed that the P2X4 protein levels also decreased significantly in HUVECs subjected to a shear stress of 15 dyn/cm² for 6 or 24 h (Fig. 2). In contrast, the ICAM-1 protein used as a positive control increased in response to shear stress.
Flow-Induced Decrease in P2X4 mRNA Levels Is Shear Stress, Rather Than Shear Rate, Dependent

To determine whether the flow-induced decrease in P2X4 mRNA levels is shear stress dependent, HUVECs were exposed to flow with two perfusates of different viscosities, and changes in the mRNA levels were measured. The P2X4 mRNA level decreased as shear rate increased but to a greater extent with the flow of high-viscosity perfusate (Fig. 3, A). However, when changes in the mRNA levels were plotted against shear stress, they formed almost a single curve irrespective of viscosity (Fig. 3, B). These results indicate that the flow-induced decrease in P2X4 mRNA levels is shear stress, rather than shear rate, dependent.

Shear Stress Downregulates P2X4 Gene Transcription

To examine whether the flow-induced decrease in P2X4 mRNA levels is a transcriptional event, luciferase assay was performed using reporter plasmids containing the human P2X4 promoter region (−1924 to +1) linked to the luciferase gene. When bovine ECs transfected with the reporter plasmids were exposed to a shear stress of 15 dyn/cm² for 24 h, the P2X4 promoter activity decreased by 57.6 ± 5.5% (mean ± SD, n = 6) of that of static control cells (Fig. 4; −1.9-kb luc). This means that laminar shear stress downregulates the transcription of the P2X4 gene.

A Region Containing an Sp1 Binding Site Is Essential for P2X4 Gene Response to Shear Stress

Deletion analysis was performed to determine the cis-element in the P2X4 gene promoter essential for shear stress-mediated downregulation of P2X4 gene transcription. Bovine ECs transfected with reporter plasmids containing sequentially deleted segments of the P2X4 promoter region were exposed to a shear stress of 15 dyn/cm² for 24 h, and changes in the luciferase activity were measured. A decrease in P2X4 gene transcription occurred in response to shear stress, even when the promoter length was reduced to −112 bp, indicating that the putative cis-element is located within 112 bp of the start site (Fig. 4; −112 luc).
On examining the sequence of the P2X4 gene promoter, we found a consensus binding site (TGG- GCGGGGC) for a known transcription factor, Sp1, between bp -67 and -58 from the transcription start site. A chimeric gene generated by site-specific mutagenesis at the Sp1-binding site (TGGGCGGGGC replaced by TGTTCGGGGC) was constructed and tested by luciferase assay. Transfection of bovine ECs with the chimeric gene decreased the basal P2X4 gene transcription to the same low level as that of cells with empty vector (pGL3) and abolished the responsiveness to shear stress (Fig. 4; -112 mut). These data suggest that the Sp1-binding site plays a major role in the control of basal transcription of the P2X4 gene.

**Transcription Factor Sp1 Is Involved in the Shear Stress-Induced Downregulation of P2X4 Gene Transcription**

EMSA was performed to examine which transcription factors are involved in shear-induced downregulation of the P2X4 gene transcription. Incubation of a 30-mer oligonucleotide containing the Sp1 binding site sequence with nuclear extracts obtained from HUVECs placed under static conditions or subjected to a laminar shear stress of 15 dyn/cm² for 24 h formed a DNA-protein complex (Fig. 5). The DNA-protein complex was significantly smaller in the nuclear extracts from shear-stressed cells than in those from static control cells (lane 2 vs. lane 7). Addition of unlabeled oligonucleotide in 25-fold (lanes 3 and 8) and 100-fold excess (lanes 4 and 9), but not of nonspecific DNA (lanes 5 and 10), completely blocked the formation of the DNA-protein complex, indicating specificity of the observed gel shift. Addition of antibody against Sp1 markedly suppressed the formation of the DNA-protein complex (lanes 6 and 11). Furthermore, oligonucleotides with a mutated Sp1 consensus sequence did not form the DNA-protein complex seen in oligonucle-
otides containing a native Sp1 consensus sequence (lanes 13 and 14). With all these findings together taken into consideration, it appears that laminar shear stress decreases the amount of Sp1, which binds to the Sp1 consensus sequence in the P2X4 gene promoter, resulting in the downregulation of P2X4 gene transcription.

Reactive Oxygen Species Modulate P2X4 Gene Response to Shear Stress

To test whether reactive oxygen species (ROS) are involved in shear stress-induced downregulation of P2X4 gene expression (Fig. 6), experiments were performed in HUVECs treated with superoxide dismutase (SOD; 100 μg/ml), a peroxide-scavenging enzyme, catalase (3.5 × 105 U/l), the nitric oxide synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA; 200 μM), or H2O2 (100 μM). SOD or catalase significantly inhibited the shear stress-induced decrease in the P2X4 mRNA levels. L-NMMA slightly lowered the basal P2X4 mRNA levels but did not affect the response to shear stress. H2O2 augmented the downregulation of P2X4 gene expression by shear stress. These results indicate that ROS play a role, at least partially, in shear stress-mediated regulation of P2X4 gene expression in ECs.

DISCUSSION

This study presents the first evidence that physiologically relevant levels of laminar shear stress can regulate the expression of purinoceptors in vascular ECs. When HUVECs were exposed to an arterial level of shear stress (15 dyn/cm²), the P2X4 mRNA levels began to decrease within 1 h and decreased progressively with time, reaching ~60% of the control level at 24 h. The decrease in the mRNA levels was due to downregulation of P2X4 gene transcription by shear stress, in which binding of the transcription factor Sp1 to the P2X4 gene promoter played a crucial role. Shear stress significantly reduced the binding of Sp1 to its target site. These results indicate that laminar shear stress decreases the binding of Sp1 to its consensus element in the P2X4 gene promoter, which downregulates gene transcription, leading to a decrease in P2X4 receptor expression in ECs.

Flow exerts two effects on ECs. One effect is flow velocity-dependent changes in mass transport (5). If a
certain cell-activating substance exists in the perfusate, its diffusion to the cell surface increases as flow velocity increases, which stimulates cells further. Another effect is mechanical cell deformation induced by shear stress. Flow-loading experiments using two perfusates with different viscosity allow us to discriminate these two effects (2). Because shear stress is the product of shear rate (flow velocity gradient) and viscosity, cells can be subjected to different levels of shear stress at the same shear rate when exposed to flows of different viscosity. If the flow effect is shear rate dependent, the response may be similar regardless of difference in viscosity, or the response may be greater in low viscosity than in high viscosity, because diffusion is restricted with increasing viscosity. In contrast, if it is shear stress dependent, the response would be greater in high viscosity than in low viscosity. The present results coincided with the latter assumption; the extent of flow-induced decrease in the P2X4 mRNA levels was always greater in high viscosity than in low viscosity at the same shear rate. This means that the P2X4 gene response was shear stress, rather than shear rate, dependent.

A number of recent studies have shown that many other endothelial genes are also responsive to shear stress (1). In most cases, expression is regulated transcriptionally, but as shown in our previous study (16), expression of the granulocyte/macrophage colony-stimulating factor gene is regulated by shear stress posttranscriptionally via mRNA stabilization. Resnick et al. (27) demonstrated that a GAGACC sequence in the platelet-derived growth factor B-chain gene promoter contains an Sp1 consensus sequence at 2654, and three sequences complementary to GAGACC at 2544, and 252 bp in the gene promoter functioned as positive SSREs in the genes encoding monocyte chemotactic protein-1 (28), tissue factor (TF) (19), and platelet-derived growth factor A-chain (13), respectively. We showed that double activator protein-1 binding sites are a negative regulatory element involved in shear stress-induced downregulation of vascular cell adhesion molecule-1 gene transcription (15). The P2X4 gene promoter contains an Sp1 consensus sequence at −67 bp and three sequences complementary to GAGACC at −635, −544, and −342 bp. None of these GGTCTC sequences were involved in the P2X4 gene response to shear stress. This suggests that SSREs do not necessarily function wherever they are present.

Lin et al. (19) first demonstrated that shear stress upregulates the transcription of the TF gene in HUVECs, in which a Sp1-binding site (GGGGCGGGCGG) at −96 to −65 bp in the gene promoter functions as a positive regulatory element. In this case, shear stress did not change the amount of Sp1 but activated Sp1 by phosphorylation, leading to augmentation of TF gene transcription. The phosphorylation of Sp1 occurred transiently as early as 1 h after shear stress. In contrast, we observed a significant decrease in the amount of Sp1 binding to its consensus element in HUVECs exposed to shear stress for 24 h. This suggests that Sp1 can mediate gene responses to shear stress via changes not only in its activity but also its amount. We also observed that shear stress decreased the Sp1 mRNA levels in HUVECs; i.e., the mRNA levels decreased to ~72% of the static control after exposure to a shear stress of 15 dyn/cm² for 24 h (data not shown). Thus the decrease in the production of Sp1 may be, at least partially, involved in the shear stress-induced decrease of Sp1 binding to its target sequence.

Recently, ROS have been shown to be produced in ECs exposed to shear stress and to be involved in shear stress-induced signal transduction and EC responses. Laurindo et al. (18) showed in ex vivo perfusion experiments using the rabbit aorta that an increase in flow rate caused free radical release, which disappeared in the endothelium-denuded aorta. Chiu et al. (6) reported that ROS-mediated activation of NF-κB is involved in shear stress-induced upregulation of ICAM-1 gene expression. In addition to NF-κB, the transcription factors activator protein-1 and Sp1 have also been shown to be activated by shear stress (17). In the present study, SOD and catalase inhibited the downregulation of P2X4 gene expression by shear stress, indicating the involvement of ROS in the P2X4 gene response. However, it is not clear at which point ROS act in the signal transduction pathway from the sensing of shear stress to the decrease in P2X4 mRNA levels. Because Sp1 had a negative regulatory effect on P2X4 gene transcription, Sp1 is unlikely to be activated by ROS.

Our previous study showed that human vascular ECs predominantly express P2X4 receptors, which mediate Ca²⁺ influx on ATP stimulation (30). We also observed that, at a certain level of extracellular ATP, ECs show stepwise increases in Ca²⁺ influx in response to stepwise increases in shear stress and that the inhibition of P2X4 expression by antisense oligonucleotides abolished the shear stress-dependent Ca²⁺ influx (29). These data suggest that P2X4 receptors play an important role in shear stress-induced Ca²⁺ signaling. Thus the level of P2X4 receptor expression may have a great influence on the sensitivity of ECs to ATP and shear stress. Actually, we observed that ECs in which P2X4 receptor expression was decreased by shear stress showed less sensitivity to ATP; i.e., control HUVECs showed a marked increase in [Ca²⁺]; in response to ATP at lo as low as 2 μM, whereas HUVECs exposed to shear stress (15 dyn/cm² for 24 h) required >10 μM ATP to show a similar Ca²⁺ response (data not shown). However, further studies are needed to clarify the molecular events that lead to the weaker response of shear-stressed ECs to ATP. EC responses to adenine nucleotides or shear stress are strongly associated with diverse vascular functions, including short-term control of vessel tone and long-term control of vascular cell differentiation, growth, and death. Thus there may be a feedback system in our body that controls the sensitivity of ECs to adenine nucleotides or shear stress via
blood flow-mediated attenuation of purinoreceptor expression to maintain vascular homeostasis.

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REFERENCES