Transcriptional and Posttranscriptional Regulation of Cyclooxygenase-2 Expression by Fluid Shear Stress in Vascular Endothelial Cells

Hiroyasu Inoue, Yoji Taba, Yoshikazu Miwa, Chiaki Yokota, Megumi Miyagi, Toshiyuki Sasaguri

Objective—Fluid shear stress induces cyclooxygenase (COX)-2 gene expression in vascular endothelial cells. We investigated the underlying mechanism of this induction.

Methods and Results—Exposure of human umbilical vein endothelial cells to laminar shear stress in the physiological range (1 to 30 dyne/cm²) upregulated the expression of COX-2 but not COX-1, a constitutive isozyme of COX. The expression of COX-2 mRNA began to increase within 0.5 hour after the loading of shear stress and reached a maximal level at 4 hours. Roles of the promoter region and the 3′-untranslated region in the human COX-2 gene were evaluated by the transient transfection of luciferase reporter vectors into bovine arterial endothelial cells. Shear stress elevated luciferase activity via the region between −327 and 59 bp. Mutation analysis indicated that cAMP-responsive element (−59/−53 bp) was mainly involved in this response. On the other hand, shear stress selectively stabilized COX-2 mRNA. Moreover, shear stress elevated luciferase activity when a 3′-untranslated region of COX-2 gene containing 17 copies of the AUUUA mRNA instability motif was inserted into the vector.


Key Words: shear stress - vascular endothelial cells - cyclooxygenase-2 - posttranscriptional regulation

Vascular endothelial cells are always exposed to a wide variety of biochemical and biomechanical stimuli, including fluid shear stress caused by blood flow. Shear stress modulates several endothelial functions, such as control of vascular tone, maintenance of antithrombotic surfaces, regulation of inflammation, protection against oxidative stresses, and regulation of endothelial cell proliferation and apoptosis.1

Cyclooxygenase (COX), a rate-limiting enzyme for prostaglandin (PG) biosynthesis, comprises 2 isozymes, COX-1 and COX-2.2-3 COX-1 is constitutively expressed in most cell species, whereas COX-2 is an inducible enzyme whose expression is regulated differently among cell types. Growing evidence indicates that COX-2 plays a key role in several biological processes, such as inflammation, tumorigenesis, development, and atherogenesis.4-9 Laminar shear stress upregulates COX-2 gene expression.10 COX-2 is involved in lipopolysaccharide-stimulated production of prostacyclin (PGI₂) in endothelial cells11 and is also involved in PGI₂ biosynthesis in healthy humans.12 Previously, we have reported that shear stress promotes the production of PGD₂ in endothelial cells by stimulating the expression of lipocalintype PGD₂ synthase (L-PGDS), whereas PGI₂ synthase was constitutively expressed but did not respond to shear stress.13 Therefore, the induction of COX-2 expression by shear stress may be involved in the production of PGI₂ and PGD₂ in endothelial cells.

Three cis-acting elements, namely, the nuclear factor (NF)-κB binding site, the NF–interleukin-6 (NF-IL6) binding site, and the cAMP-responsive element (CRE), reside in the region between base pairs −327 and +59 (−327/+59) in the human COX-2 gene promoter. Their involvement in COX-2 gene transcription varies among cell species.14-24 Recently, the COX-2 gene has been reported to be posttranscriptionally regulated through its 3′-untranslated region (3′-UTR) containing 17 copies of the AUUUA motif, which is assumed to promote mRNA degradation.25-30 However, the mechanism underlying shear stress–induced COX-2 gene expression remains to be elucidated, although it has been recently reported that shear stress stimulates the transcription of the COX-2 gene in murine osteoblastic MC3T3-E1 cells that produce PGE₂ but not PGI₂ or PGD₂.24

In the present study, we investigated the molecular mechanism for the shear stress–induced expression of COX-2 in vascular endothelial cells. The gene expression of COX-2 was more sensitive to shear strength than that of L-PGDS. We found that shear stress induces COX-2 expression not only at.

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the transcriptional level but also at the posttranscriptional level through the 3′-UTR, which would make it possible to rapidly and persistently induce COX-2 expression in response to shear stress.

Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described. Bovine arterial endothelial cells (BAECs) were grown in DMEM supplemented with 10% FCS (Flow), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Shear Stress Apparatus
HUVECs and BAECs were plated on a gelatin-coated polyester sheet (Plastic Suppliers), and flow experiments were performed in a parallel-plate flow chamber as described.

Western blot Analysis and Electrophoretic Mobility Shift Assay
Western blot analysis and electrophoretic mobility assay were performed as described.

RNA Analysis
Total RNA was isolated using the acid guanidinium thiocyanate procedure. RNAs were then subjected to electrophoresis. The cDNA probes for COX-1, COX-2, and GAPDH have been described previously. The levels of mRNA and 28S rRNA were calculated on the basis of hybridization signals and ethidium bromide intensities as measured with the imaging analyzers Fujix BAS 2500 and FLA 2000, respectively (Fuji Photo Film Co).

Plasmid Construction
The following control vectors were used: for luciferase (Luc), pGV-C (Toyobo); for β-galactosidase (β-gal), pCMV-βgal; and for green fluorescent protein (GFP), pEGFP-N1 (Clontech). The human COX-2 genomic clone hPESII95 was digested by EcoRI, and these fragments were subcloned into pBluescript II SK(−). Digestion of the subclone 7k1-2 with MroI and EcoRI yielded a fragment containing part of the coding region (57 bp) and the full-length 3′-UTR, which contains 17 copies of the ATTTA motif. This fragment was blunt-end ligated into the blunted PflM I site of phPES2 (Fig 1A), a plasmid that expresses firefly Luc under the control of the human COX-2 promoter, we used phPES2 (−327/+59), a plasmid that expresses firefly Luc under the control of the human COX-2 gene promoter (−327/+59). Because DNA transfection efficiency was much higher in BAECs than in HUVECs, phPES2 (−327/+59) was transfected into BAECs. Online Figure IIA (available at http://atvb.ahajournals.org) demonstrates BAECs transfected with pEGFP-N1, a GFP expression plasmid. The transfection efficiency seemed to be sufficient, and shear stress induced cell elongation along with the flow direction, which indicated that shear stress was successfully loaded on the cells. Moreover, that bovine COX-2 mRNA expression is also inducible in response to shear stress was confirmed by Northern blotting, as shown in online Figure IIB. Bovine COX-2 mRNA was induced by shear stress after 5 and 17 hours in duplicate experiments with independent materials. However, bovine COX-1 mRNA was not induced, again similar to human COX-1. As shown in online Figure IIC, the promoter activity of phPES2 (−327/+59) was markedly elevated by shear stress loaded for 5 and 17 hours.

Transcription Assays
Transfection of BAECs with plasmids has been described previously. In this experiment, 200 μL DMEM containing 2 μg COX-2 Luc reporter vector, 0.2 μg pCMV-βgal, and 0.1 μg pEGFP-N1 was mixed with 200 μL DMEM containing 10 μL Trans IT-LT-1 (Pan Vera) and incubated at room temperature for 15 minutes. This DNA/reagent complex was added to semiconfluent BAECs growing in a 90-mm dish containing 8 mL complete growth medium that was changed 1 day before the transfection. After 5 hours of transfection, cells were incubated with new complete growth medium for 19 hours and placed in the flow shear stress chamber. After incubation in the chamber for 24 hours, cells were subjected to fluid shear stress for 5 or 17 hours by using the apparatus described above. The cells were lysed in Reporter lysis buffer (Promega) to release the Luc and β-gal for their activity assays. The numerical readings from the Luc assay were normalized to those of the β-gal assay.

Statistical Analysis
Results are expressed as mean±SD. Statistical significance was assessed by the Student t test.

Results

Laminar Shear Stress Induces COX-2 Expression in HUVECs
To determine the effect of shear stress on the expression of COX genes, we performed Western and Northern blot analyses with the use of protein and RNA extracted from HUVECs. COX-2 protein was expressed after the loading of shear stress (15 dyne/cm²) for 24 hours but not in cells cultured under static conditions (please see online Figure IA, available at http://atvb.ahajournals.org). As shown in Figure IA, the expression levels of COX-2 mRNA were very low under static conditions. Exposure of the cells to shear stress (15 dyne/cm²) for the periods indicated led to an increase in the amount of COX-2 mRNA. On the other hand, COX-1 mRNA was constitutively expressed independently of shear stress. Because the expression level of COX-2 mRNA reached an almost maximal value at 6 hours, we further examined the time course of COX-2 induction immediately after loading shear stress (Figure 1B and online Figure IB). The level of COX-2 mRNA began to increase within 0.5 hours and reached a plateau at 4 hours.

Figure 2 shows the dependence of the expression of COX-2 mRNA on the strength of shear stress. The expression of COX-2 mRNA was maximally upregulated even at 5 dyne/cm², which corresponds to the level of shear stress in veins. Therefore, we performed an additional experiment to determine whether shear stress <5 dyne/cm² is able to stimulate COX-2 expression. As shown in Figure 2A, shear stress as low as 1 dyne/cm² significantly upregulated the expression.

Laminar Shear Stress Activates COX-2 Promoter
To examine whether shear stress stimulates the human COX-2 promoter, we used phPES2 (−327/+59), a plasmid that expresses firefly Luc under the control of the human COX-2 gene promoter (−327/+59). Because DNA transfection efficiency was much higher in BAECs than in HUVECs, phPES2 (−327/+59) was transfected into BAECs. Online Figure IIA (available at http://atvb.ahajournals.org) demonstrates BAECs transfected with pEGFP-N1, a GFP expression plasmid. The transfection efficiency seemed to be sufficient, and shear stress induced cell elongation along with the flow direction, which indicated that shear stress was successfully loaded on the cells. Moreover, that bovine COX-2 mRNA expression is also inducible in response to shear stress was confirmed by Northern blotting, as shown in online Figure IIB. Bovine COX-2 mRNA was induced by shear stress after 5 and 17 hours in duplicate experiments with independent materials. However, bovine COX-1 mRNA was not induced, again similar to human COX-1. As shown in online Figure IIC, the promoter activity of phPES2 (−327/+59) was markedly elevated by shear stress loaded for 5 and 17 hours.

The human COX-2 promoter region (−327/+59) contains 3 cis-acting elements, namely, an NF-kB binding site, an NF-IL6 binding site, and a CRE, all of which have been
shown to be involved in the regulation of COX-2 gene transcription. When we destroyed all of the 3 consensus sequences as shown in Figure 3A, the shear stress–induced elevation of the promoter activity completely disappeared, suggesting that the elements responsive to shear stress are in these 3 consensus sites. To identify the elements, we then destroyed each of the 3 motifs as shown in Figure 3B. A mutation in CRE (−59/−53) markedly reduced the basal and shear stress–induced promoter activities. Mutations at the NF-κB site (−223/−214) or NF-IL6 site (−132/−124) did not affect the response to shear stress compared with effects of the CRE, although the basal promoter activity was significantly reduced by the NF-κB mutation. Electrophoretic mobility shift assay showed that proteins specifically binding to the CRE (−59/−53) were enhanced by the shear stress (see online Figure III, available at http://atvb.ahajournals.org). These data suggest that the CRE (−59/−53) is mainly involved in the shear stress–increased COX-2 promoter activity.

Laminar Shear Stress Stabilizes COX-2 mRNA Through 3′-UTR

To examine whether posttranscriptional mechanisms are involved in shear stress–induced COX-2 expression, we chased the decay of COX-2 mRNA after the addition of actinomycin D, an inhibitor of transcription. As shown in Figure 4, COX-2 mRNA increased by shear stress loaded for 2 hours was almost completely degraded 2 hours after the addition of actinomycin D in the absence of stress. However, when shear stress was loaded simultaneously with actinomycin D, the decay of COX-2 mRNA was significantly delayed, and the enhanced expression was still retained at 4 hours after the addition of actinomycin D.

The entire 3′-UTR of the human COX-2 gene contains 17 copies of the ATTTA motif, which is found in many immediate-early genes and has been shown to promote mRNA degradation.34,35 To determine whether the 3′-UTR of COX-2 mediates shear stress–induced mRNA stabilization, we examined the effect of the insertion of the COX-2 3′-UTR into the Luc expression vector. As shown in Figure 5, transfection of pG-3UCOX2 resulted in a 50% reduction in the promoter activity compared with that of pGV-C in cells cultured under static conditions, suggesting that the 3′-UTR of COX-2 destabilizes the Luc mRNA. Shear stress did not influence the Luc activity in cells transfected with pGV-C, whereas it markedly elevated the activity by 2.8-fold in cells transfected with pG-3UCOX2. This suggested that exposure to laminar shear stress prevented Luc mRNA breakdown through the 3′-UTR of the COX-2 gene.

Discussion

The present study demonstrated for the first time that laminar shear stress within the physiological range induces COX-2 gene expression through transcriptional and posttranscriptional mechanisms in vascular endothelial cells. Shear stress as low as 1 dyne/cm² was able to induce COX-2 mRNA expression in HUVECs, as in murine osteoblastic MC3T3-E1 cells.34 However, it was not able to stimulate COX-1 mRNA expression in either HUVECs or BAECs. A similar result, ie, that laminar shear stress induced COX-2 but not COX-1 expression, has been reported in a study using HUVECs, although higher shear stress (24 dyne/cm²) has been reported to induce COX-1 mRNA in HUVECs and mechanical cyclic strain also has been shown to induce COX-1 in human
vascular smooth muscle cells. COX-2 has been reported to have a higher affinity for arachidonate than COX-1, and moreover, COX-2 preferentially cooperates more with PGI2 synthase than with COX-1. When these findings are taken together, COX-2 but not COX-1 appears to be mainly involved in PGI2 formation in blood vessels loaded with laminar shear stress in the physiological range. This assumption is consistent with recent studies reporting that selective COX-2 inhibitors suppress the systemic biosynthesis of PGI2 in healthy humans and in patients with atherosclerosis. There has been a report that glucocorticoids do not depress the excretion of urinary PGI2 metabolite. We found that COX-2 expression was not suppressed by dexamethasone, especially in vascular endothelial cells, because of the lower expression of the glucocorticoid receptor. There are variable reports of COX-2 expression ex vivo in endothelial cells. It will be difficult to evaluate the precise expression levels of COX-2 mRNA and protein are very unstable.

We reported that laminar shear stress also induces the expression of L-PGDS. However, the induction of gene expression by shear stress was more rapid and sensitive for COX-2 than for L-PGDS, whose mRNA expression increases depending on shear stress between 5 and 30 dyne/cm². PGI2 synthase is constitutively expressed in endothelial cells, and its expression level is not influenced by shear stress. Therefore, we hypothesize that there are 2 steps in COX-2-mediated arachidonate metabolism in endothelial cells. Step 1, which operates under low shear stress conditions, predominantly produces PGI2, and step 2, which operates under high shear stress conditions, produces not only PGI2 but also PGD2. In this context, PGD2 may also play a role in preventing the formation of atherosclerotic lesions by being converted to 15-deoxy-Δ12,14-PGJ2, which has been reported to display several antiatherogenic effects on cultured vascular cells.

In the mean time, macrophages were reported to express augmented levels of COX-2 in atherosclerotic lesions. This abnormally elevated COX-2 expression in macrophages may be related to inflammation in the lesions. Because the PGs produced in macrophages are different from those produced in endothelial cells, the regulation of COX-2 and downstream enzymes should be very different between endothelial cells and macrophages. In this context, we have recently reported that COX-2 expression is negatively regulated by nuclear receptor peroxisome proliferator-activated receptor-γ and its ligand candidate 15-deoxy-Δ12,14-PGJ2 in macrophages but not in endothelial cells. Whether changes...
in the pattern of blood flow, such as turbulence, influence the PG species produced in endothelial cells should be examined.

In the present study, mutation analysis and gel-retardation assay indicated that the shear stress responsiveness of the COX-2 promoter was mainly dependent on CRE (−59/−53). The promoter activity of the fragment −1432/+59 was similar to that of the fragment −327/+59 induced by shear stress (data not shown), as reported in our previous studies using different stimuli. Previous reports have indicated that CRE, CCAAT/enhancer binding protein-β (same as NF-IL6), and activator protein-1 motifs play major roles in the shear stress induction of the COX-2 gene in osteoblasts, which is consistent with our results because of multiple binding activity of the CRE for CRE binding protein, CCAAT/enhancer binding proteins, and activator protein-

Figure 4. Effect of shear stress (SS) on COX-2 mRNA stability. HUVECs were exposed to SS of 15 dyne/cm² for 2 hours and then treated with actinomycin D (5 μg/mL) for a further 2 and 4 hours under static conditions (SC) or SS. Extracted RNAs (10 μg per lane) were analyzed by RNA blot analysis. Expression levels of COX-2 mRNAs were normalized with amounts of GAPDH mRNA and standardized to the value obtained at 2 hours of SS. Similar results were obtained by 2 additional experiments.

Figure 5. Involvement of the 3′-UTR of the COX-2 gene in response to shear stress. BAECs were transfected with Luc control vector pG-C or pG-3UCOX2 containing 3′-UTR of the human COX-2 gene along with pCMV-βgal and pEGFP-N1. The transfected cells were subjected or not to shear stress at 15 dynes/cm² for 5 hours and then assayed for Luc and β-gal activities. The Luc activity was normalized to the β-gal activity and presented as the fold increase against the value obtained without shear stress of pG-C. Data are mean±SD of 4 experiments. **P<0.01 vs static control.

Figure 6. Shear stress regulates COX-2 expression in BAECs. BAECs were transfected with Luc reporter gene constructs. The promoter activity of the fragment 59 was induced by shear stress (data not shown), as reported in our previous studies.


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A

Strength 0 0 15 (dyne/cm²)

COX-2 protein
actin

B

1 2 4 6 (h)

SC SS SC SSSC SS SC SS

COX-2
GAPDH

Online Fig. I. Inoue et al.
**A**

Control  

Shear 17 h

**B**

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<th>Time</th>
<th>COX-2</th>
<th>COX-1</th>
<th>GAPDH</th>
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**C**

Relative Luciferase Activity of phPES2(-327/+59)

<table>
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<th>Activity</th>
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<td>5 h</td>
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<td>17 h</td>
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Online Fig. II, Inoue et al.
A

PES2CRE: AAACAGTCATTTTCGTACATGGGCTTG
PES2CRM: AAACAGTCATTTTgagCtCATGGGCTTG

B

Probe: PES2CRE

Competitor: __ __ PES2CRE PES2CRM

SC SS SS SS

Online Fig. III. Inoue et al.