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 regulates several endothelial functions, such as control of 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 regulates several endothelial functions, such as control of 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

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 AUUUUA mRNA instability motif was inserted into the vector.


Key Words: shear stress • vascular endothelial cells • cyclooxygenase-2 • posttranscriptional regulation
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 by 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–staining 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 (Toyob); for β-galactosidase (β-gal), pCMV-βgal; and for green fluorescent protein (GFP), pEGFP-N1 (Clontech). The human COX-2 genomic clone hPESI95 was digested by EcoRI, and then these fragments were subcloned into pBluescript II SK(+) plasmid. Digestion of the subclone 7kI-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 followed by 3 copies of the polyadenylation signal (AATAAA). This fragment was blunt-end ligated into the blunt-end–PstI M1 site of pGV-C, which is located downstream from the Luc coding region. In this clone, designated pG-3UCOX2, Luc mRNA is expressed under the control of the SV40 enhancer/promoter and the 3′-UTR of the human COX-2 gene. Construction of other COX-2 reporters has been described previously.

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 (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 I). 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 (59), a plasmid that expresses firefly Luc under the control of the human COX-2 gene promoter (59). Because DNA transfection efficiency was much higher in BAECs than in HUVECs, phPES2 (59) was transfected into BAECs. Online Figure IIA demonstrates the secretion of COX-2 mRNA after shear stress in BAECs. 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 (59) was markedly elevated by shear stress loaded for 5 and 17 hours.

The human COX-2 promoter region (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 ATTTC motif, which is found in many immediate-early genes and has been shown to promote mRNA degradation. 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 destabilized 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. 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 strength 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 expression of COX-2 in macrophages are related to the atherosclerotic process needs further investigation.

Figure 2. Effect of shear strength on induction of COX-2 mRNA expression in HUVECs. A, HUVECs were exposed to various levels of shear stress (0 to 30 dyne/cm²) for 24 hours. Total cellular RNAs (10 μg per lane) were analyzed by RNA blot analysis for COX-2 and GAPDH. Imaging-exposure times for the left and right parts were 1 day and 5 days, respectively. B, Data obtained in panel A was quantified. Expression levels of COX-2 normalized to those of GAPDH mRNA are shown as the fold increase against the value obtained at time 0. Similar results were obtained by 2 additional experiments.

Figure 3. Site-specific mutation of the COX-2 promoter region in response to shear stress. The human COX-2 gene promoter region (−327/+59) was mutated at each putative transcriptional regulatory element. Lowercase letters in the upper sequence of each promoter indicate mutated bases, and the lower sequence shows wild-type bases. BAECs were transiently transfected with the wild-type and mutated constructs along with pCMV-βgal and pEGFP-N1. The transfected cells were subjected or not to shear stress at 15 dynes/cm² for 17 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 phPES2 (−327/+59). Data are mean±SD of 3 or 4 experiments. *P<0.05 vs static control; **P<0.01 vs static control.
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.11,16 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-

1.6.20 However, a triple mutation assay in Figure 3A suggested that other cis-acting elements also play a role in the COX-2 promoter activity.

Actinomycin D chase experiments have indicated that shear stress stabilizes COX-2 mRNA levels in HUVECs. Similar experiments have also shown granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA also to be stabilized by shear stress,45 although there was no induction of the promoter activity of the GM-CSF gene. The COX-2 and GM-CSF genes contain 17 and 8 copies, respectively, of the AUUUA motif in their 3'-UTRs.33,34 Conserved AUUUA sequences have been identified within the 3'-UTR of many short-lived mRNAs and regulate their stability. The 3'-UTR of the COX-2 gene contains multiple copies of the AUUUA motif implicated in posttranscriptional regulation. Several investigators have shown that insertion of the 3'-UTR of the COX-2 gene into the 3'-UTR of the reporter gene alters its expression.25–30

The present study demonstrated for the first time that shear stress stabilizes COX-2 mRNA through the 3'-UTR of the COX-2 gene. Recently, it has been reported that the 3'-UTR of the murine COX-2 gene contains multiple regulatory elements that alter mRNA stability and translational efficiency even in the steady state.30 On the other hand, the AUUUA motif found in GM-CSF mRNA has been considered not to function as a shear stress–responsive element, because protein binding to this motif was not influenced by shear stress.45 Further study is necessary to resolve these issues.

Acknowledgments

This study was partly supported by grants from the Ministry of Education, Science, Sports, and Culture, Japan (grants-in-aid for scientific research Nos. 11838021 and 13670110); from the Ministry of Health and Welfare, Japan (research grants for cardiovascular diseases Nos. 11C-1 and 12C-3); and from the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

References


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 and Toshiyuki Sasaguri

*Arterioscler Thromb Vasc Biol.* 2002;22:1415-1420; originally published online July 8, 2002; doi: 10.1161/01.ATV.0000028816.13582.13

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2002 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/22/9/1415

Data Supplement (unedited) at:

http://atvb.ahajournals.org/content/suppl/2002/09/16/22.9.1415.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:

http://atvb.ahajournals.org//subscriptions/
Online Fig. I. Inoue et al.
Online Fig. II, Inoue et al.
A

PES2CRE: AAACAGTCATTTTCGTCACATGGGCTTG
PES2CRM: AAACAGTCATTTTgagCtCATGGGCTTG

B

Probe: PES2CRE

Competitor: SC SS SS SS

Online Fig. III. Inoue et al.