Activator Protein-1 Mediates Shear Stress–Induced Prostaglandin D Synthase Gene Expression in Vascular Endothelial Cells

Megumi Miyagi, Yoshikazu Miwa, Fumi Takahashi-Yanaga, Sachio Morimoto, Toshiyuki Sasaguri

Objective.—We attempted to determine the molecular mechanism of fluid shear stress–induced lipocalin-type prostaglandin D synthase (l-PGDS) expression in vascular endothelial cells.

Methods and Results.—We examined the promoter region of the l-PGDS gene by loading laminar shear stress (20 dyne/cm²), using a parallel-plate flow chamber, on endothelial cells transfected with luciferase reporter vectors containing the 5'-flanking regions of the human l-PGDS gene. A deletion mutant analysis revealed that a shear stress–responsive element resided in the region between −2607 and −2523 bp. A mutation introduced into the putative binding site for activator protein-1 (AP-1) within this region eliminated the response to shear stress. In an electrophoretic mobility shift assay, shear stress stimulated nuclear protein binding to the AP-1 binding site, which was supershifted by antibodies to c-Fos and c-Jun. Shear stress elevated the c-Jun phosphorylation level in a time-dependent manner, similar to that of l-PGDS gene expression. SP600125, a c-Jun N-terminal kinase inhibitor, decreased the c-Jun phosphorylation, DNA binding of AP-1, and l-PGDS expression induced by shear stress. Additionally, an mRNA chase experiment using actinomycin D demonstrated that shear stress did not stabilize l-PGDS mRNA.

Conclusions.—Shear stress induces l-PGDS expression by transcriptional activation through the AP-1 binding site. (Arterioscler Thromb Vasc Biol. 2005;25:970-975.)

Key Words: shear stress ■ vascular endothelial cells ■ PGD synthase ■ AP-1 ■ JNK

In vascular cells and tissues, prostaglandin D₂ (PGD₂) applied in vitro prevents platelet aggregation and induces endothelium-dependent arterial relaxation. PGD₂ is metabolized to the PGJ₂ family, which includes PGJ₂, Δ²⁵-PGJ₂, and 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (15d-PGJ₂), without the requirement of specific enzymes. The PGJ₂ family, particularly 15d-PGJ₂, exhibits strong anti-inflammatory effects by inhibiting nuclear factor-κB and IkB kinase or through the activation of a peroxisome proliferator-activated receptor-γ. In macrophages, 15d-PGJ₂ inhibits inflammatory cytokine production, metalloproteinase-9 activation, and inducible NO synthase expression. The PGJ₂ family strongly inhibits vascular smooth muscle cell proliferation and promotes smooth muscle cell differentiation. In vascular endothelial cells, 15d-PGJ₂ inhibits apoptotic cell death by upregulating the caspase inhibitor cellular inhibitor of apoptosis protein 1 (c-IAP1). The majority of the effects induced by PGD₂ and the PGJ₂ family members appear to be anti-inflammatory, and therefore atheroprotective, because atherosclerosis is considered to be a chronic inflammation in vascular cells and tissues.

Lipocalin-type PGD synthase (l-PGDS) catalyzes the isomeric conversion of PGH₂ to PGD₂. In human cardiovascular tissues, the l-PGDS mRNA is most strongly expressed in the heart, where immunoreactivity of the enzyme is localized in the myocardial and endocardial cells. Human aortic endothelial cells and intimal smooth muscle cells in vivo also stain positive for the l-PGDS mRNA by in situ hybridization. Recent studies have suggested relationships between l-PGDS and cardiovascular diseases. For example, l-PGDS is secreted into the coronary circulation in angina patients. Elevated serum l-PGDS levels after coronary angioplasty correlate with a decreased occurrence of restenosis. The l-PGDS expression levels in macrophages of human atherosclerotic plaques correlate with plaque stability. Serum and urinary levels of l-PGDS are significantly elevated in patients with essential hypertension or renal dysfunction. Therefore, increased l-PGDS levels in vivo may play a protective role in cardiovascular diseases and may also serve as a sensitive disease marker.

We reported previously that endothelial cells cultured under static conditions express very low amounts of l-PGDS, whereas the l-PGDS mRNA expression is markedly increased when loaded with arterial levels of laminar fluid shear stress. However, the mechanism of the shear stress–induced l-PGDS gene expression remains to be determined.
In the present study, we investigated the molecular mechanism of the shear stress–induced \(\tau\)-PGDS expression in vascular endothelial cells. We report that the \(\tau\)-PGDS expression induced by shear stress is mediated by activator protein-1 (AP-1) binding to the 5′-flanking region of the \(\tau\)-PGDS gene.

Materials and Methods

Chemicals

SP600125, a specific c-Jun N-terminal kinase (JNK) inhibitor, was purchased from Biomol Research Laboratories.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously.\(^{20}\) Bovine arterial endothelial cells (BAECs) were grown on a 37°C polyester sheet and subconfluent (50%–70%) was incubated at room temperature for 15 minutes. The DNA/transfection reagent (Mirus Corporation) was added, and the mixture was incubated at room temperature for 20 minutes, before the addition of the labeled probe. Anti-rabbit IgG was used as an irrelevant control.

Shear Stress Apparatus

A confluent monolayer of endothelial cells on a polyester sheet (Plastic Suppliers) was placed in a parallel-plate flow chamber and subjected to steady laminar shear stress as described previously.\(^{21}\) A confluent monolayer of endothelial cells on a polyester sheet (Plastic Suppliers) was placed in a parallel-plate flow chamber and subjected to steady laminar shear stress as described previously.\(^{21}\)

Western Blot Analysis

Western blot analysis was performed as described previously using the polyclonal anti–phospho-c-Jun antibody (Cell Signaling Technology), the polyclonal anti–c-Jun antibody (Santa Cruz Biotechnology), and the polyclonal anti–\(\tau\)-PGDS antibody (Maruha Corporation).

Luciferase Reporter Assay

The 5′-flanking regions of the human \(\tau\)-PGDS gene (\(\tau\)-PGDS expression analysis. As demonstrated in Figure 1, shear stress (20 dyne/cm\(^2\)) loaded for 24 hours elevated the \(\tau\)-PGDS protein

Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from HUVECs cultured on a 37×75 mm polyester sheet using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). A double-stranded oligonucleotide probe containing the putative AP-1 binding site (\(5′\)-TTTGCTCGACAGGAGCTG-3′) or its mutant (\(5′\)-TTTGCTCGACAGGAGCTG-3′) was labeled at the 3′-end with biotin using the Biotin 3′End DNA Labeling Kit (Pierce), as instructed by the manufacturer. Incubation for the DNA binding reaction was performed using the LightShift Chemiluminescent EMSA (electrophoretic mobility shift assay) Kit (Pierce). A 100- to 200-fold molar excess of the unlabeled oligonucleotide was simultaneously added as a competitor, with the labeled probe. To identify DNA binding proteins, nuclear extracts were incubated with 3 \(\mu\)g of antibodies to c-Jun, c-Fos (Santa Cruz Biotechnology), or rabbit IgG at room temperature for 20 minutes, before the addition of the labeled probe. Anti-rabbit IgG was used as an irrelevant control.

Reverse Transcription–PCR

Total cellular RNA was extracted with Isogen (Nippon Genl). Using 1 \(\mu\)g of this RNA, the \(\tau\)-PGDS mRNA expression was analyzed by RT-PCR using the Ready-To-Go RT-PCR Beads (Amerham Biosciences), and PCR primers were synthesized on the basis of the information obtained from the GenBank database.

Statistical Analysis

The results are expressed as mean±SE. Statistical significance was assessed by Student’s \(t\) test.

Results

Vascular Endothelial Cells Express \(\tau\)-PGDS in Response to Shear Stress

Exposure to arterial levels (15 to 30 dyne/cm\(^2\)) of laminar fluid shear stress markedly elevates the \(\tau\)-PGDS gene expression in HUVECs.\(^{16}\) Using an antibody specific to \(\tau\)-PGDS, we examined the effect of shear stress on the \(\tau\)-PGDS protein expression. As demonstrated in Figure 1, shear stress (20 dyne/cm\(^2\)) loaded for 24 hours elevated the \(\tau\)-PGDS protein expression in HUVECs. 16 Using an antibody specific to \(\tau\)-PGDS, the \(\tau\)-PGDS expression was analyzed by Western blotting. A representative result from 3 independent experiments is shown. B, Data obtained in A was quantified. The \(\tau\)-PGDS expression levels normalized to those of \(\beta\)-actin are shown as percentages of the value obtained at time 0. * \(P<0.05\) vs static control.

Figure 1. Effect of shear stress on \(\tau\)-PGDS protein expression. A, Confluent HUVECs were cultured under static conditions or exposed to shear stress (20 dyne/cm\(^2\)) for 24 hours. \(\tau\)-PGDS expression was analyzed by Western blotting. A representative result from 3 independent experiments is shown. B, Data obtained in A was quantified. The \(\tau\)-PGDS expression levels normalized to those of \(\beta\)-actin are shown as percentages of the value obtained at time 0. * \(P<0.05\) vs static control.
expression level by 1.9-fold compared with the static control. This result was consistent with the effect on the mRNA.

**Analysis of the 5′-Flanking Region of the l-PGDS Gene**

We attempted to determine the precise mechanism of the shear stress–induced l-PGDS expression by analyzing the l-PGDS gene construct (Figure 2A; also see Figure I, available online at http://atvb.ahajournals.org). Luciferase reporter constructs driven by the 5′-flanking regions of the human l-PGDS gene (−2707/+41, −2660/+41, −2607/+41, −2523/+41, −2426/+41, −2092/+41, −1574/+41, −1148/+41, and −681/+41 bp) were transfected into BAECs. The promoter activities were measured after exposure to laminar shear stress (20 dyne/cm²) for 24 hours because l-PGDS mRNA was maximally upregulated at 24 hours after the initiation of shear stress loading.16 The reason for using BAECs in this experiment was that the efficiency of DNA transfection was much higher in BAECs (60% to 70%) than in HUVECs (10% to 20%). Shear stress induced 1.9-, 2.9-, and 1.6-fold increases in luciferase activity on transfection of the −2707/+41, −2660/+41, and −2607/+41 bp constructs, respectively. However, shear stress did not increase the activity when the −2523/+41 bp construct was used. Therefore, it is possible that the response element to shear stress was located between −2607 and −2523 bp in the 5′-flanking region, where the putative binding sites for GATA-1/2 (−2580/−2571), TR (−2575/−2561),24 and AP-1 (−2530/−2522) are located.

**Figure 2. Analysis of the 5′-flanking region of the l-PGDS gene.**

To identify the shear stress–responsive element residing between −2607 and −2523 bp of the l-PGDS gene, we introduced mutations into the 3 putative binding sites (GATA-1/2, TR, and AP-1) to destroy the consensus sequences, as described in Materials and Methods. The mutations in the consensus sequences for GATA-1/2 (−2580/−2571) or TR (−2575/−2561) had no effect on the shear stress response (Figure 2B). In contrast, the mutation introduced into the putative AP-1 binding site (−2530/−2522) completely eliminated the response to shear stress, suggesting the possibility that this site is involved in the response.

In addition, destruction of the proximal AP-1 consensus sites (−2203/−2195 and −1830/−1820) had no effect on the response (Figure II, available online at http://atvb.ahajournals.org).

**Shear Stress Stimulates AP-1 Binding to the l-PGDS Gene**

We attempted to identify the transcription factor involved in the shear stress–induced transactivation of the l-PGDS gene by EMSA using a DNA oligonucleotide containing the putative AP-1 binding site (−2532/−2513). Shear stress loaded on HUVECs for 24 hours stimulated the binding of a nuclear protein to the oligonucleotide (Figure 3A, lane 3). The addition of a 100- to 200-fold molar excess of the unlabeled oligonucleotide (wild type [WT]) resulted in a marked reduction of the shifted band (Figure 3A and 3B). In contrast, a mutant oligonucleotide (mAP-1), in which the same mutation as that used for the luciferase reporter assay was introduced, failed to compete for binding to AP-1 (Figure 3B, lane 4).

Subsequently, we examined the effect of anti–c-Jun and anti–c-Fos antibodies because the AP-1 complex may comprise either a c-Jun/c-Fos heterodimer or a c-Jun/c-Jun homodimer. Supershifted complexes were observed in the presence of the anti–c-Jun or anti–c-Fos antibodies (Figure 3C, lanes 2 and 3, top arrow). Therefore, the involvement of AP-1, at least in part including c-Jun/c-Fos heterodimers, was suggested in the shear stress–induced DNA–protein binding.

In addition, to investigate the involvement of JNK, we used its specific inhibitor SP600125. As shown in Figure 3D, SP600125 inhibited the DNA–protein complex formation, suggesting that the l-PGDS gene transcription is regulated by AP-1 through JNK activation.
To elucidate the mechanism by which shear stress stimulates AP-1 activation, we examined whether shear stress elevates the c-Jun or c-Fos expression levels or activates JNK, which activates c-Jun by phosphorylation. As shown in Figure 4A, the c-Jun level was not increased by shear stress. However, the c-Jun phosphorylation level was slowly elevated by loading shear stress, and it was maximal at 24 hours (Figure 4A and 4B). Importantly, this time course was very similar to the time course of L-PGDS induction by shear stress. On the other hand, c-Fos, the other component of AP-1, was persistently expressed, regardless of the loading shear stress (Figure III, available online at http://atvb.ahajournals.org).

To investigate whether JNK mediates the shear stress–dependent L-PGDS induction, HUVECs were exposed to shear stress in the presence of SP600125. SP600125 significantly attenuated the shear stress–dependent c-Jun phosphorylation (Figure IV, available online at http://atvb.ahajournals.org) and L-PGDS mRNA induction (Figure 5), thereby suggesting the involvement of JNK.

Shear Stress Does Not Stabilize L-PGDS mRNA
To examine whether post-transcriptional mechanisms are involved in the shear stress–induced L-PGDS expression, we followed the decay of the L-PGDS mRNA after the addition of transcriptional inhibitor actinomycin D. The L-PGDS mRNA expressed in HUVECs pre-exposed to shear stress for 24 hours was slowly degraded after the cessation of shear stress.
stress in the presence of actinomycin D (Figure V, available online at http://atvb.ahajournals.org). The degradation of the L-PGDS mRNA did not significantly slow down even when shear stress was continuously loaded after the addition of actinomycin D. Therefore, shear stress appeared to elevate the L-PGDS mRNA expression level by activating the transcription and not by stabilizing the mRNA.

Discussion

We reported previously that vascular endothelial cells express the L-PGDS mRNA in vitro and in vivo, and laminar shear stress stimulates its expression and PGD2 production in vitro.16 As first shown in this article, we confirmed that the L-PGDS protein expression level also increases in response to shear stress. However, the manner in which shear stress induces the L-PGDS gene expression was unknown. Therefore, we attempted to identify the mechanism by which shear stress stimulates the L-PGDS mRNA expression. Consequently, we found that AP-1 mediates the shear stress–induced L-PGDS expression. To our knowledge, this is the first report of a shear stress–responsive element in the human L-PGDS gene.

Although fluid shear stress is known to elevate the expression levels of some proteins by stabilizing their mRNAs, such as granulocyte–macrophage colony-stimulating factor,25 cyclooxygenase-2,26 and cyclin-dependent kinase inhibitor p21 (Miwa et al, unpublished data, 2004), shear stress did not prolong the half life of the L-PGDS mRNA. This suggests that shear stress stimulates transcription of the L-PGDS gene.

The deletion analysis of the 5′-flanking region of the L-PGDS gene indicated that the response to shear stress depended on the region between −2607 and −2523 bp, where the putative binding sites for GATA-1/2, TR, and AP-1 are located. Among these, only the TR consensus site has been reported to function in vitro and in vivo. The complex of T1 and TR-β stimulates the L-PGDS promoter by binding to the thyroid hormone–responsive element in COS-7 cells27 and human medulloblastoma TE671 cells.24 The L-PGDS mRNA expression level decreases in the brain of hypothyroid rats.28,29 However, in our study, deletion or mutation of the TR or GATA-1/2 consensus sites had no effect on the shear stress–induced increase in luciferase activity. Only the mutation introduced into the AP-1 binding site abolished the shear stress response. Although there are 2 other AP-1 consensus sites in the proximal region (−2203/−2195 and −1830/−1820), destruction of them had no influence on the shear stress response. Therefore, the distal AP-1 site (−2530/−2522), and not the TR, GATA-1/2, or proximal AP-1 sites, seemed to be required for the shear stress–induced L-PGDS gene expression. In addition, deleting the region between −2707 and −2660 bp, which did not contain any known consensus sequence, enhanced the shear stress response, suggesting the possibility of suppressor elements in this region.

The involvement of AP-1 was confirmed by the subsequently performed EMSA. Exposure to shear stress increased nuclear protein binding to the oligonucleotide, including the AP-1 consensus site, primarily identified as the 12-O-tetradecanoyl phorbol 13-acetate–responsive element (TRE).30 The anti–c-Jun and anti–c-Fos antibodies partially supershifted the protein–DNA complex, suggesting that AP-1, at least in part, including c-Jun/c-Fos heterodimers, is involved in the response. The c-Jun/c-Fos heterodimers form more stable complexes with TRE, thereby exhibiting a stronger transactivation than the c-Jun/c-Jun homodimers.31

Previous studies have suggested the involvement of AP-1 in the shear stress–induced gene expression of some endothelial cell proteins, such as monocyte chemoattractant protein-1 (MCP-1)32 and vascular cell adhesion molecule-1 (VCAM-1).33 AP-1 upregulates the MCP-1 gene expression by stimulating TRE in the MCP-1 gene promoter.32 On the other hand, AP-1 downregulates VCAM-1 through 2 consensus AP-1 binding sites.33

Shear stress has been reported to increase the amounts of c-Jun and c-Fos at the mRNA and the protein levels in human endothelial cells.34,35 However, in this study, shear stress had no significant influence on c-Jun or c-Fos expression levels. AP-1 expression is induced by multiple stimuli, including serum and growth factors.36 In this experiment, c-Fos and c-Jun proteins might have already been expressed at time 0 because HUVECs were cultured in DMEM supplemented with 20% FBS and basic fibroblast growth factor.

Phosphorylation is required for the activation of AP-1 family members. With regard to Jun proteins, the activation domain is regulated to a large extent by the JNK family of mitogen-activated protein kinases.30 JNK phosphorylates c-Jun at Ser-63, resulting in the binding of c-Jun to the CREB-binding protein/p300 family of transcriptional coactivators.30 Regarding Fos proteins, the N-terminal and C-terminal domains flanking the basic leucine zipper domain require phosphorylation for their biological activity. Kinases responsible for the phosphorylation of Fos proteins remain to be determined.30,37 In the present study, the phosphorylation level of c-Jun was slowly but significantly elevated after loading shear stress. This finding is consistent with a previous report38 that the shear stress–induced phosphorylation of c-Jun takes several hours to reach a maximal level. Furthermore, SP600125 inhibited the shear stress–induced DNA–protein complex formation and L-PGDS mRNA expression. Therefore, induction of the L-PGDS gene by shear stress appeared to be mediated by AP-1, which is activated on phosphorylation by JNK.

Vascular endothelium plays an essential role as a physiological barrier to protect vascular walls from undergoing atherosclerotic changes. The PGD2, and PGJ2 family, produced by endothelial cells in response to shear stress, might be involved in the endothelial cell functions. In particular, the PGJ2 family members are unique in nature; they can strongly repress inflammatory processes and inhibit apoptosis in vascular endothelial cells. We hypothesize that the PGJ2 family produced by endothelial cells maintains vascular homeostasis by functioning as an antiatherogenic factor, analogous to NO, PGH2, and other endothelium-derived substances. Substances that modify PGD2 metabolism might provide novel preventive and therapeutic strategies for the treatment of atherosclerotic vascular diseases.

In conclusion, our present study has demonstrated that AP-1 activated by c-Jun phosphorylation mediates the lami-
nal fluid shear stress–induced transcriptional activation of the human 1- PGDS gene in vascular endothelial cells. Further study is required to elucidate the physiological and pathological roles of this mechanism.

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Figure I

-2758 GAGCTCAGCT CCACCAGTCA CTGGGACAGC CTCGGCCCTC AAGGCGAAAC CATTGGTGGG GATGCTGGCC

-2660

-2707 GATA-1

-2688 GGGGCCAGG GTGGAGACAG CTTCCCTCCA GGAAAGGTT GGGTCAGTT CTGGGAGCTT AGGCCCAGGC

-2607 TR

-2618 CACCAGGCTT GTCCATGACC TGCCTGGGCC GCAGGCAGGG GGATGGCC

-2607

-2523

-2618

-2548 GCCAAGTCTT TGCATTTTGTT GACTCAAAGA GACTGGTTCC CAGAGAGCAG GGTGGGGCGG CCAGCCTCCT

-2523

-2453 AP-1

-2478 TCCTGCCGAG CCCTCCGCCA GGCTCCACTA AGGTCCTGCC TGGCACCCAA GCTCAGAGCA AAGGGAAGGG

-2426
Figure II

Relative luciferase activity (%)

0 100 200 300

Wild type  mAP-1 (-2203/-2195)  mAP-1 (-1830/-1820)

-2707 TATA

LUC

0 100 200 300

LUC

LUC

GATA-1/2 Sp1

TR CREB

AP-1 NF-κB

Static control Shear stress

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*

*
Figure III

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Figure IV

A

Shear (24 h) - + +
SP600125 - - +
p-c-Jun □
c-Jun □

B

Shear (24 h) - + +
SP600125 - - +
c-Jun phosphorylation (%)

Bar graph showing c-Jun phosphorylation levels with different conditions.
Figure V

![Graph showing L-PGDS/GAPDH (%) over time with static control and shear stress lines. The x-axis represents time in hours (0 to 10), and the y-axis represents L-PGDS/GAPDH in percentage. The graph displays a decrease in L-PGDS/GAPDH over time for both static control and shear stress conditions.](image-url)