Inhibition of Tumor Necrosis Factor-α–Induced SHP-2 Phosphatase Activity by Shear Stress
A Mechanism to Reduce Endothelial Inflammation

Nicole Lerner-Marmarosh,* Masanori Yoshizumi,* Wenyi Che,* James Surapisitchat, Hisaaki Kawakatsu, Masashi Akaike, Bo Ding, Qunhua Huang, Chen Yan,* Bradford C. Berk, Jun-ichi Abe

Objectives—Atherosclerosis preferentially occurs in areas of turbulent flow, whereas laminar flow is atheroprotective. Inflammatory cytokines have been shown to stimulate adhesion molecule expression in endothelial cells that may promote atherosclerosis, in part, by stimulating c-Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB transcriptional activity. Methods and Results—Because Src kinase family and Src homology region 2-domain phosphatase-2 (SHP-2) may regulate JNK activation, we studied the effect of shear stress on endothelial inflammation and JNK. Human umbilical vein endothelial cells preexposed to flow showed decreased tumor necrosis factor (TNF)-α–induced c-Jun and NF-κB transcriptional activation. TNF-α–mediated JNK, c-Jun, and NF-κB activation required Src and SHP-2 activity. Shear stress significantly inhibited SHP-2 phosphatase activity without affecting TNF-α–induced Src family kinase activation. Because MEKK3 and Gab1 are critical for TNF-α–induced c-Jun and NF-κB activation, we determined the role of SHP-2 phosphatase activity in MEKK3 signaling. A catalytically inactive form of SHP-2 increased MEKK3/Gab1 interaction and inhibited MEKK3 (but not MEKK1)-mediated c-Jun and NF-κB activation. Conclusions—These results suggest that SHP-2 is a key mediator for the inhibitory effects of shear stress on TNF-α signaling in part via regulating MEKK3/Gab1 interaction, MEKK3 signaling, and subsequent adhesion molecule expression. (Arterioscler Thromb Vasc Biol. 2003;23:1775-1781.)

Key Words: pathophysiology ■ cell signaling ■ endothelium ■ mechanism of atherosclerosis

The development of atherosclerosis occurs in a highly consistent focal manner, preferentially affecting the outer edges of vessel bifurcations. In these predisposed areas, the flow pattern is turbulent, not laminar as found in protected regions. Many findings suggest that steady laminar flow in blood vessels activates signal transduction events that lead to expression of atheroprotective genes. Previously, we have found that shear stress inhibits tumor necrosis factor (TNF)-α–induced ASK1 and c-Jun N-terminal kinase (JNK) activation.1,2 There are likely many mechanisms by which flow may inhibit TNF-α signaling. An important regulator of the TNF-α pathway is the Src homology 2 domain–containing protein-tyrosine phosphatase SHP-2, whose activity is required for activation of JNK and nuclear factor (NF)-κB.3,4 The SHP-2 is an ubiquitously expressed cytosolic protein that contains 2 amino-terminal tandem SH2 domains and a carboxyl-terminal catalytic domain.5 SHP-2 associates with tyrosine-phosphorylated growth factor receptors such as epidermal growth factor receptor and platelet-derived growth factor and with cytokine receptors such as α/β interferon receptors.6–8 Given that growth factors and cytokines can potently activate SHP-2 phosphatase activity and that SHP-2 phosphatase activity may be a key mediator to regulate mitogen-activated protein kinases,9 we hypothesized that flow-mediated inhibition of SHP-2 phosphatase activity may be a protective mechanism of laminar shear stress through inhibition of cytokine-mediated inflammatory responses such as adhesion molecule expression in endothelial cells.

Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated and maintained in 20% FBS/RPMI 1640 medium as previously described.1 At least 4 different HUVEC preparations were used for the experiments shown.

Flow Experiments
Short-flow experiments (<200 minutes) were performed as previously described.1 For long-flow experiments (>6 hours), large react angular parallel flow chamber (5×14 cm, Glyco Tech) was used for transfection and stimulation of the cells by a constant shear stress (please see the online supplement at http://atvb.ahajournals.org).
Plasmids and Transfection
pcDNA3 HA-Gab1 was a gift from Dr Hirano (Osaka University, Osaka, Japan), and we subcloned it into pcDNA3.1/His vector (Invitrogen) to add Xpress and His tag. pRK5-MEKK3 (wild-type [WT]) or pRK5-CA-MEKK3 (constitutive active form [CA]) by deleting the first 11 amino acids) plasmids are subcloned into pCMV-tag2 (Stratagene) to add Flag tag, pFR-Luc, pFA2-c-Jun, pFC-cdb, pNF-κB-Luc, and pFC-MEKK1 were from Stratagene. pJ3-hSHP-2 WT and C459S (SHP-2 [C/S]), which abolishes SHP-2 phosphatase activity, were a gift from Dr Neel (Harvard Medical School, Boston, Mass) and were also subcloned into pcDNA3.1/His vector. The phosphatase domain deletion mutant of SHP-2 (SHP-2 [1-411]) was created by polymerase chain reaction from SHP-2 WT and subcloning into pcDNA3.1/hisC vector (Invitrogen). Kinase-negative forms of Src were cloned into pcDNA3.1 vector. All plasmid constructs of Src were cloned into pcDNA3.1 vector.12,13 All plasmid constructs were verified by DNA sequencing. For transient expression experiments, HUVECs were transfected with Lipofectamine Plus (Gibco BRL) as described previously.14

Immunoprecipitation, Western Blot Analysis, Kinases, and SHP-2 Phosphatase Activity
After treatment with reagents, the cells were washed with PBS and harvested in 0.5 mL of lysis buffer as described previously.14 Immunoprecipitation was performed as described previously with mouse anti-Flag (Sigma).15 Western blot analysis was performed as previously described.14 In brief, the blots were incubated for 4 hours at room temperature with the anti-activated Src antibody clone 28,16 anti-SHP-2 (Santa Cruz), Xpress (Invitrogen), or Flag (Sigma) antibody, followed by incubation with horseradish peroxidase–conjugated secondary antibody (Amersham). Antibodies for assaying NF-κB activation, anti-IκBα, and anti-IκB kinase (IKK) α antibody were from Santa Cruz, and the phospho-IKKα/β and phospho-IκBα (ser32) antibodies were from Cell Signaling.

JNK activity was measured with a commercially available kit based on phosphorylation of recombinant c-Jun (Cell Signaling), as described previously.14 SHP-2 phosphatase activity was measured as previously described17,18 (see the online data supplement).

PathDetect in Vivo Signal Transduction Pathway Reporting System
c-Jun and NF-κB activity was assayed using PathDetect Signal Transduction Pathway trans-Reporting Systems (Stratagene) as described previously19 (please see the online data supplement).

Using pRK5-MEKK3, pcDNA3.1 His-Gab1, and WT and phosphatase-negative form of SHP-2 (SHP-2[C/S]), recombinant proteins were expressed by the TNT (Coupled Reticulocyte Lysate System) incorporating [35S] methionine, as previously described.20

Cell-Surface Immunoassay
To detect the expression of adhesion molecules, ELISA was performed as previously described21 (please see the online data supplement).

Statistical Analysis
Data are reported as mean±SD. Statistical analysis was performed with the StatView 4.0 package (ABACUS Concepts). Differences were analyzed with 1-way or 2-way repeated-measures ANOVA, as appropriate, followed by Scheffe’s correction. P<0.05 is indicated by an asterisk and P<0.01 by 2 asterisks.

Results
Flow Inhibits TNF-α–Mediated c-Jun and NF-κB Transcriptional Activity in Endothelial Cells
The present study, we first analyzed the effect of flow on TNF-α–mediated c-Jun and NF-κB transcriptional activation in endothelial cells. We transiently transfected HUVECs with c-Jun and NF-κB reporter genes. After 24 hours of transfection, cells were exposed to flow (shear stress=5 dyne/cm²) for 20 minutes or maintained in static conditions for 20 minutes. We chose 5 dyne/cm² of flow instead of 12 dyne/cm² in the long-term flow regimen, because 5 and 12 dyne/cm² of laminar flow have similar effects on its downstream events, including ERK5 activation,22 and cell viability and attachment in 5 dyne/cm² of flow is better than in 12 dyne/cm² after 6 hours of exposure. Cells were then kept in static or flow condition for 6 hours with TNF-α (20 ng/mL) or vehicle. c-Jun and NF-κB transcriptional activity were measured using a luciferase reporter assay system.23 As shown in Figures 1A and 1B, flow alone caused no significant increase in c-Jun and NF-κB transcriptional activity. TNF-α alone significantly increased c-Jun (Figure 1A) and NF-κB (Figure 1B) transcriptional activity after 6 hours of stimulation. We found that exposure to flow significantly inhibited TNF-α–mediated activation of c-Jun and NF-κB transcriptional activity. We found a similar inhibitory effect of flow when cells were preexposed to shear stress for 60 minutes before TNF-α stimulation (data not shown). These observations are consistent with our previous work1,2 and suggest that the inhibitory effect of flow is mediated by events that are relatively long lived.

Flow Inhibited the TNF-α–Induced Late Phase of IκB Phosphorylation But Not the Early Phase of IKKα/β and IκB Phosphorylation
NF-κB activation is initiated by the degradation of IκB inhibitor protein leading to the nuclear import of NF-κB and culminating with the resynthesis of IκBα. The IκB degradation is mediated through the IKK activation, which functions to phosphorylate 2 serine residues on IκBα proteins.24 To detect the effect of flow on acute-phase events of TNF-α–induced NF-κB signaling, we investigated IKKα/β and IκB phosphorylation mediated by TNF-α. As shown in Figure 1A (available online at http://atvb.ahajournals.org), IKKα/β is phosphorylated after ~5 to 10 minutes of TNF-α stimulation. Analysis of HUVEC extracts using IκB–specific antibodies showed that stimulation of the cells with TNF-α caused rapid degradation of IκB. Protein levels of IκB decreased by >80% after 30 minutes; thereafter, IκB levels increased gradually up for to 240 minutes of incubation, attributable to NF-κB–induced de novo synthesis of IκB, as previously described.24 IκB phosphorylation was rapidly increased after 5 minutes of TNF-α stimulation (Figure 1C, top). Because the phosphorylated IκB protein level was decreased by degradation, the level of IκB was also decreased from ~10 to 30 minutes after TNF-α stimulation, but whereas IκB levels increased, phosphorylation of IκB could be detected again ~30 to 240 minutes after TNF-α stimulation. To investigate the effect of flow on these rapid events in NF-κB signaling, cells were exposed to flow (shear stress=12 dyne/cm²) or maintained in static conditions for 20 minutes. Cells were then kept in static or flow conditions for 10 (Figure 1B, available online), 5, or 120 minutes (Figure 1D) with TNF-α (20 ng/mL) or vehicle to detect IKKα/β and

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phosphorylation after 120 minutes of TNF-α stimulation was completely inhibited by flow. These data suggest that shear stress does not inhibit IKK-IκB phosphorylation induced after ∼5 to 10 minutes of TNF-α stimulation. However, shear stress inhibited the late phase (after 120 minutes of TNF-α stimulation) of IκB phosphorylation and the subsequent NF-κB transactivation activity (Figure 1B).

**TNF-α Stimulates JNK or c-Jun Activation Via a Src Kinase Family–Dependent Mechanism**

Because several investigators, including our group, have reported the involvement of Src family kinases in JNK signaling, we determined whether TNF-α induced Src kinase activation in HUVECs. As shown in Figure IIA (available online), Src family kinase was activated by TNF-α. TNF-α increased JNK activity at 15 minutes, and PP2, a specific inhibitor for Src family kinase, inhibited TNF-α–mediated JNK activation in HUVECs in a dose-dependent manner (Figure IIB, available online). Src kinase inactive form (Src-KN) significantly inhibited TNF-α–induced c-Jun transactivation activity in a dose-dependent manner (Figure IIC, available online), additionally indicating that TNF-α–mediated activation of JNK and c-Jun is dependent on Src kinase family activation.

**TNF-α Stimulates JNK, c-Jun, and NF-κB Transcriptional Activity Via a SHP-2 Phosphatase Activity–Dependent Mechanism**

We examined the role of SHP-2 tyrosine phosphatase activity in TNF-α–mediated signaling events in HUVECs. As shown in Figure III (available online), TNF-α stimulated SHP-2 phosphorylation (2.3±0.4-fold, P<0.05). To inhibit SHP-2 function, we cotransfected HUVECs with Flag-tagged JNK and a phosphatase inactive mutant of SHP-2 (SHP-2aa1–411, as we have described previously). As shown in Figure 2A, transfection of WT SHP-2 (SHP-2WT) had no significant effect on TNF-α–induced JNK activation. However, transfection of SHP-2aa1–411 inhibited JNK activation by TNF-α. In addition, SHP-2aa1–411, but not SHP-2WT, significantly inhibited TNF-α–induced c-Jun and NF-κB transcriptional activity in a dose-dependent manner, suggesting an essential role of SHP-2 kinase activity in TNF-α–mediated JNK, c-Jun, and NF-κB transcriptional activation in HUVECs (Figures 2B and 2C). To confirm the importance of SHP-2 phosphatase activity in regulating TNF-α–induced signaling, we used another SHP-2 phosphatase-negative form, a deletion mutant of C-terminal phosphatase domain of SHP-2 (SHP-2aa1–411), as we have described previously. As shown in Figure IV (available online), we found that this phosphatase-negative form of SHP-2 (SHP-2aa1–411) also inhibited TNF-α–induced c-Jun and NF-κB transcriptional activation. These data also suggested the critical role of SHP-2 phosphatase activity on TNF-α–induced c-Jun and NF-κB transcriptional activation.

**Shear Stress Did Not Inhibit TNF-α–Induced Src Kinase Family Activation But Inhibited SHP-2 Tyrosine Phosphatase Activity**

To determine the role of Src kinase in the regulation of SHP-2 phosphatase activity and flow-mediated inhibition of TNF-
α–induced JNK activation, we first determined the effect of PP2 on TNF-α–induced SHP-2 phosphatase activity. As shown in Figure III (available online), TNF-α–induced SHP-2 phosphatase activity was significantly inhibited by PP2 in a dose-dependent manner. Flow and TNF-α significantly increased Src family kinase activity in HUVECs (Figures IID and V, available online). In contrast to JNK activation, flow did not change Src family kinase activation by TNF-α in HUVECs (Figure V, available online). These results suggest that Src family kinase activity is not responsible for flow-mediated inhibition of TNF-α–induced c-Jun and NF-κB transcriptional activation.

Next, to determine whether SHP-2 phosphatase activity is a key target of flow-mediated inhibitory effect on TNF-α signaling, we examined the effect of 20- or 60-minute pretreatment of flow on TNF-α–induced SHP-2 phosphatase activity. As shown in Figure 3A (left), 20-minute preexposure to flow significantly inhibits TNF-α–induced SHP-2 phosphatase activity. We also found a similar inhibitory effect after 60 minutes of preexposure to flow on TNF-α–induced SHP-2 phosphatase activity (Figure 3A, right). These results suggest that SHP-2 is a key target for the inhibitory effect of flow on TNF-α–induced c-Jun and NF-κB transcriptional activation in HUVECs.

Shp-2 Phosphatase Activity Regulates MEKK3-Mediated But Not MEKK1-Mediated c-Jun and NF-κB Activation in HUVECs

Previously, we found that MEKK3 was required for c-Jun and NF-κB transcriptional activity induced by TNF-α in endothelial cells.23 Because MEKK1 also participates in the JNK and NF-κB activation by directly stimulating the IKK,25 we investigated the role of SHP-2 phosphatase activity in regulation of MEKK3 and MEKK1 signaling. We transfected HUVECs with SHP-2C/S and a constitutively active form of MEKK3 (CA-MEKK3). As shown in Figures 3B and 3C, CA-MEKK3 stimulated c-Jun and NF-κB transcriptional activity; SHP-2C/S, but not SHP-2WT, inhibited CA-MEKK3–induced c-Jun and NF-κB transcriptional activation in a dose-dependent manner. In contrast to CA-MEKK3, SHP-2C/S did not significantly inhibit CA-MEKK1–induced c-Jun and NF-κB transcriptional activity (Figures VIA and VIB, available online). These data indicate that SHP-2C/S specifically inhibits MEKK3- but not MEKK1–induced c-Jun and NF-κB transcriptional activity.

Shp-2 Phosphatase Activity Regulates MEKK3 and Gab1 Association and Subsequent c-Jun and NF-κB Transcriptional Activity in HUVECs

Gab1 associates with SHP-2 and regulates SHP-2–dependent signal transduction.26 Previously, we have shown that Gab1 associates with MEKK3 and inhibits MEKK3 (but not MEKK1)–induced c-Jun and NF-κB transcriptional activity.23
Therefore, we evaluated whether SHP-2 phosphatase activity regulates the association of Gab1 with MEKK3. As shown in Figures 4A and 4B, the direct interaction between Gab1/MEKK3 in vitro was increased in the presence of SHP-2C/S compared with SHP-2WT. As shown in Figures 4C and 4D, cotransfection of SHP-2WT did not change association of Gab1/MEKK3, but compared with SHP-2WT transfection, the association of Gab1/MEKK3 was increased by cotransfection of SHP-2C/S in vivo. No differences in SHP-2 and Gab1 protein expression were observed in lysates from SHP-2WT–transfected and SHP-2C/S–transfected cells, as determined by immunoprecipitation and Western blot analysis with anti-Flag (Figure 4C, middle) and anti-SHP-2 antibody (Figure 4C, bottom). These data suggest that SHP-2 phosphatase activity regulates Gab1 and MEKK3 association and consequently TNF-α signaling in endothelial cells.

**SHP-2 Phosphatase Activity Regulates CA-MEKK3–Mediated Intracellular Adhesion Molecule-1, Vascular Cellular Adhesion Molecule-1, and E-Selectin Expression in HUVECs**

Both c-Jun and NF-κB are important mediators of adhesion molecule gene expression in HUVECs. Therefore, we evaluated whether SHP-2 phosphatase activity regulates the association of Gab1 with MEKK3. As shown in Figures 4A and 4B, the direct interaction between Gab1/MEKK3 in vitro was increased in the presence of SHP-2C/S compared with SHP-2WT. As shown in Figures 4C and 4D, cotransfection of SHP-2WT did not change association of Gab1/MEKK3, but compared with SHP-2WT transfection, the association of Gab1/MEKK3 was increased by cotransfection of SHP-2C/S in vivo. No differences in SHP-2 and Gab1 protein expression were observed in lysates from SHP-2WT–transfected and SHP-2C/S–transfected cells, as determined by immunoprecipitation and Western blot analysis with anti-Flag (Figure 4C, middle) and anti-SHP-2 (Figure 4C, bottom) antibody. These data suggest that SHP-2 phosphatase activity regulates Gab1 and MEKK3 association and consequently TNF-α signaling in endothelial cells.

**Discussion**

The major finding of the present study is that SHP-2 functions as a molecular switch for the inhibition of JNK, c-Jun, and NF-κB activity by shear stress. We propose a scheme (Figure 5) for shear stress–mediated signal transduction leading to inhibition of TNF-α–induced c-Jun and NF-κB transcriptional activation based on previous work from our laboratory and other investigators. A novel aspect of this model is the role of SHP-2 as the target for shear stress effects on TNF-α signaling pathway.

Because shear stress did not inhibit TNF-α–induced Src activity, Src kinase members are not the mediators by which
shear stress inhibits the TNF-α signaling. Instead, we found that flow significantly inhibited TNF-α-induced SHP-2 phosphatase activity, as shown in Figure 3A. SHP-2 is a non-transmembrane-type protein-tyrosine phosphatase that contains 2 SH2 domains. In the present study, we found that SHP-2 phosphatase activity regulates MEKK3 and Gab1 interaction and regulates TNF-α and MEKK3 signaling pathway (Figure 5). Previously, Yang et al. and we found that MEKK3 is one of the critical mediators to regulate TNF-α-induced c-Jun and NF-κB transcriptional activity and Gab1 has an inhibitory effect on TNF-α-induced and MEKK3-induced c-Jun and NF-κB transcriptional activity. The inhibitory effect of SHP-2/Gab1 interaction and MEKK3-induced c-Jun and NF-κB transcriptional activity suggests the critical role of SHP-2/Gab1/MEKK3 complex in regulating TNF-α signaling pathway (Figure 5).

The results of the present study differ from those of Liu et al., who demonstrated that shear stress did not inhibit NF-κB DNA binding activity after 15 minutes of TNF-α stimulation by using EMSA. We found significant shear stress-mediated inhibition of TNF-α-induced NF-κB transcriptional activity by using a reporter gene assay. As shown in Figures 1 and I (available online), shear stress did not inhibit the rapid (5-10-minute) phosphorylation of IKKα/β and IκB induced by TNF-α. However, shear stress completely inhibited the slower phase of IκB phosphorylation after 2 hours of TNF-α stimulation as well as the total sum of NF-κB transcriptional activity, which was evaluated by luciferase activity by NF-κB reporter gene after 6 hours of stimulation. These data suggest that flow-mediated inhibition of NF-κB transcriptional activation occurred at the late phase (after 120 minutes of stimulation) of NF-κB signaling activation. Prolonged exposure to certain stimuli, such as TNF-α and LPS, leads to long-term induction of NF-κB activation, and it has been suggested that the late phase of IκB phosphorylation is important in controlling persistent activation of NF-κB. IKKs have 3 components, IKKα, IKKβ, and IKKγ, and IKKβ and IKKγ are required for activation of NF-κB in response to TNF-α and several cytokines, as determined by studies in IKKβ- and IKKγ-deficient cells. We did not find any inhibitory effect of flow on TNF-α-induced IκB phosphorylation (Figure IB, available online). Therefore, it is possible that another IKK, including IKKγ, is responsible for this late phase of IκB phosphorylation. Additional studies need to describe this late-phase IκB phosphorylation induced by flow.

Acknowledgments

This work was supported by grants NIH/NHLB RO1 HL61319 (to J.A.) and NIH/NHLB RO1 HL64839 (to B.C.B.).

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Arterioscler Thromb Vasc Biol. 2003;23:1775-1781; originally published online August 28, 2003;
doi: 10.1161/01.ATV.0000094432.98445.36
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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