THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 22, Issue of May 30, pp. 19702–19708, 2003
Printed in U.S.A.

Shear Flow Attenuates Serum-induced STAT3 Activation in Endothelial Cells*

Received for publication, January 27, 2003
Published, JBC Papers in Press, March 11, 2003, DOI 10.1074/jbc.M300893200

Chih-Wen Ni, Hsyue-Jen Hsieh‡, Yuen-Jen Chao, and Danny Ling Wang§

From the Cardiovascular Division, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan 11529

Vascular endothelial cells (ECs) are constantly exposed to flow-induced shear stress. Shear stress is known to induce signaling cascades, including the extracellular signal-regulated protein kinase (ERK) pathway. STAT3 transcription factor plays a key role in cytokine stimulation. Recent studies indicate that STAT3 is involved in growth factor-induced cell cycle. In the present study, we have examined STAT3 activation of ECs under conditions of shear flow. Bovine aortic ECs cultured with serum at static state show a serum concentration-dependent phosphorylation at Tyr-705 of STAT3, whereas there is a constant basal phosphorylation at Ser-727. In ECs subjected to shear flow, a shear dose-dependent phosphorylation of Ser-727 and ERK1/2 was observed. In contrast, a concomitantly shear dose-dependent inhibition of phosphorylation at Tyr-705 was exhibited. Shear stress on ECs increased the association of ERK1/2 to STAT3. ECs treated with MEK inhibitor (U0126 or PD98059) consistently and significantly reduced the shear-induced ERK1/2 and Ser-727 phosphorylation, indicating that ERK1/2 is upstream of Ser-727 phosphorylation. Interestingly, shear-induced inhibition in Tyr-705 phosphorylation was abolished in these same inhibitor-treated ECs. Similarly, ECs transfected with a dominant positive mutant of MEK1 enhanced the phosphorylation of Ser-727 with the attenuation of the Tyr-705 phosphorylation. In contrast, when ECs were transfected with dominant positive mutant of MEKK1, JNK upstream, no change in the phosphorylation of Ser-727 and Tyr-705 was observed. These results indicate that shear flow induces the phosphorylation of Ser-727 via ERK1/2 pathway, and this Ser-727 phosphorylation inhibits Tyr-705 phosphorylation in STAT3. As a result, shear flow reduced the translocation of STAT3 into nucleus. This study shows for the first time that shear flow may play a significant role by attenuating STAT3 activation and thus may reduce inflammatory responses and/or serum-induced endothelial proliferation.

Endothelial cells (ECs)[1] lining the vascular wall are constantly under the influence of hemodynamic forces, including flow-induced shear stress and pressure-induced cyclic strain. Studies have examined how the signals are involved in transmitting mechanical forces into second messengers and, subsequently, gene expression (1, 2). Studies have shown that shear flow on ECs stimulates the signals involved in the extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways that lead to gene induction (3–5). ERK1/2 is involved in shear stress- and cyclic strain-induced early growth response-1 expression (5, 6). We have shown that genes such as monocyte chemotactic protein-1 (*MCP-1*), intercellular adhesion molecule (*ICAM-1*), and *c-fos* can be induced by hemodynamic forces (7–9). The induction of these genes appears to be a redox-sensitive mechanism because an increased reactive oxygen species is required for this gene induction (7–9). Recent studies indicate that ECs under conditions of shear flow inhibit tumor necrosis factor- and H_2O_2-induced responses (10–12). A recent study (13) has nicely demonstrated an induction of antioxidant response element-mediated genes in ECs exposed to laminar flow. Furthermore, shear flow has been shown to inhibit endothelial proliferation via the induction of growth arrest proteins GADD45 and p21, as well as a decrease in phosphorylation of Rb protein (14). Thus, laminar shear flow appears to play an essential role by protecting ECs from inflammatory responses and endothelial proliferation. However, the detailed mechanisms involved in this shear flow-induced protection remain unclear.

Signal transducers and activators of transcriptions (STAT) are a family of functionally related proteins that play key roles in a variety of biological activities. Among these STATs, STAT3 is preferentially activated by interleukin-6 (IL-6) or other related cytokines and is required for gp130-mediated cell-survival signals (15, 16). STAT3 has been intensively studied for its role in cell growth, differentiation, apoptosis, transformation, inflammation, and immune response. STAT3 is activated by tyrosine phosphorylation at a single site (Tyr-705) and by serine phosphorylation at 727 (Ser-727). Tyrosine phosphorylation of STAT3 in response to cytokine stimulation is mediated by a Janus kinase, and Src family members have been widely studied (17–19). Tyrosine phosphorylation is required for STAT3 dimerization, nuclear translocation, and DNA binding. For phosphorylation at Ser-727, there is still controversy in identifying the serine kinases. Activation of kinases, including ERK, JNK, and P38, leading to serine phosphorylation in STAT3 has been documented (16). Ser-727 phosphorylation is involved in STAT3 transcriptional activation. However, there is evidence for a negative role for serine phosphorylation. It has been shown that Ser-727 phosphorylation negatively modulates tyrosine phosphorylation (16, 20, 21). In addition to this cytokine-induced STAT activation, the receptor protein-tyrosine kinase-mediated activation of STAT remains elusive. Recent studies suggest that STAT3 in ECs can be activated by growth factors such as vascular endothelial growth factor (22), basic fibroblast growth factor (23), granulocyte-macrophase

* This work was supported in part by National Science Council Grant NSC90-2320-B-001-004 and by Department of Education Program for Promoting Academic Excellence of Universities Grant 91-B-SA09-2-4, Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Chemical Engineering, National Taiwan University, Taipei, Taiwan 106.

§ To whom correspondence should be addressed. Tel.: 886-2-2652-3907; Fax: 886-2-2782-9143; E-mail: lingwang@ibms.sinica.edu.tw.

[1] The abbreviations used are: EC, endothelial cell; STAT, signal transducer and activator of transcription; IL-6, interleukin-6; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MEKK1, MEK kinase 1; HA, hemagglutinin; FBS, fetal bovine serum; JNK, c-Jun NH_2-terminal kinase.

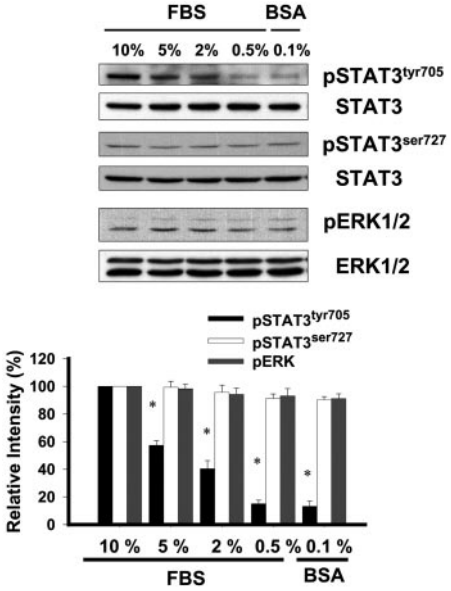

FIG. 1. **Phosphorylation of Tyr-705 in STAT3 is serum concentration-dependent.** ECs were cultured either in medium containing only bovine serum albumin (*BSA*, 0.1%) or exposed to fetal bovine serum (*FBS*) at various concentrations for 48 h. Total cell lysates were subjected to Western blot analysis with antibody to phosphorylated Tyr-705 ($pSTAT3^{Tyr\text{-}705}$), Ser-727 ($pSTAT3^{Ser\text{-}727}$), or ERK1/2 (*pERK1/2*). STAT3 (*STAT3*) or ERK (ERK1/2) detected by antibody is shown to indicate that an equal amount of protein was applied to each lane. Data were presented as means ± S.E. from at least three independent experiments. *, $p < 0.01$ *versus* ECs cultured in FBS.

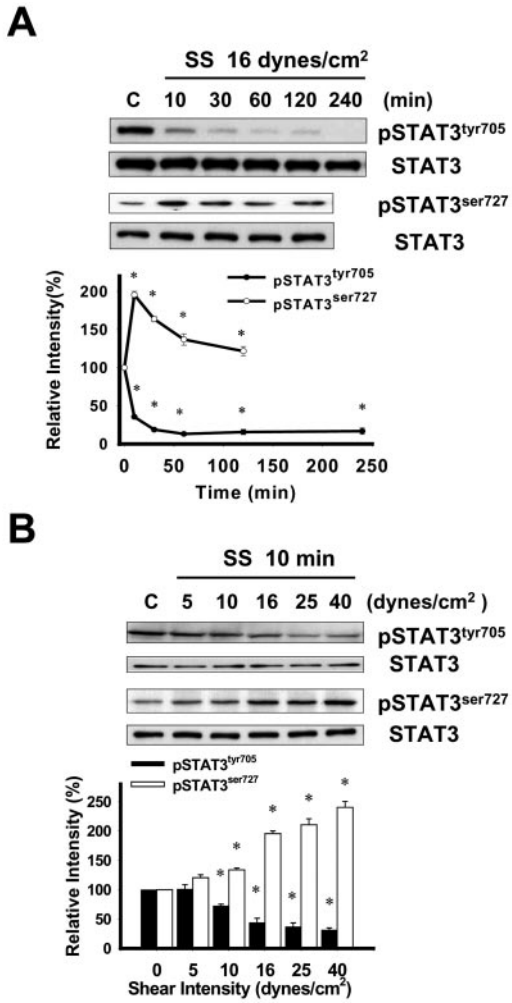

FIG. 2. **Shear flow attenuates STAT3 activation via the inhibition of phosphorylation at Tyr-705 with a time- and shear dose-dependent manner.** *A*, ECs were cultured in medium containing FBS (0.5%) for 16 h. ECs were either kept in static incubation as controls (*C*) or exposed to shear flow (*16 dynes/cm²*) for different time intervals as indicated. The phosphorylation of STAT3 was analyzed by Western blot using antibody to phosphorylated Tyr-705-STAT3 ($pSTAT3^{Tyr\text{-}705}$) or Ser-727-STAT3 ($pSTAT3^{Ser\text{-}727}$). *B*, ECs were cultured in medium containing 0.5% FBS for 16 h. ECs were either kept as static control (*C*) or exposed to shear flow for 10 min with various strengths of shear force as indicated. The phosphorylation of STAT3 was analyzed by Western blot. Data were presented as mean ± S.E. from three independent experiments. Antibody to STAT3 was used to indicate that an equal amount of protein was applied to each lane. *, $p < 0.01$ *versus* static controls.

colony-stimulating factor (24), and insulin-like growth factor I receptor (25). Simon *et al.* (26) have demonstrated regulation of STAT3 activation by direct binding of STAT3 to Rac1 GTPase. Because Rac1 mediates generation of intracellular reactive oxygen species in response to growth factor or cytokine stimulation (27–29), the Rac1-regulated STAT3 activation may play an important role in growth factor- or cytokine-induced cellular responses.

Although STAT3 has been studied in various cells, its role in ECs is not clear. STAT3 activation has been shown to contribute to the hepatocyte growth factor-inducing DNA synthesis in ECs (30). STAT3 also participates in DNA synthesis and bFGF induction in ECs by 5-hydroxyeicosatetraenoic acid (31). Recent evidence indicates that STAT3 up-regulates vascular endothelial growth factor expression and tumor angiogenesis (32). Thus, STAT3 activation plays an important role in endothelial growth and/or angiogenesis. It has been reported that STAT3 activation up-regulates target genes such as *c-myc*, *cyclin D1*, and *Bcl-xL* that lead to cell cycle progression and/or prevention of apoptosis (33, 34). Cells, when stably transfected with a dominant active STAT3 construct, have been shown to express elevated levels of Bcl-xL and cyclin D1 and exhibit an increased proliferation associated with tumor growth (35). Because STAT3 is activated by various growth factors and plays a role during endothelial growth/proliferation, STAT3 activation in ECs under shear flow condition was examined. In the present study, we have demonstrated that STAT3 activation in cultured ECs is serum concentration-dependent. More importantly, we have shown for the first time that shear flow acts as an inhibitory regulation factor by attenuating serum-induced STAT3 activation in a shear dose-dependent manner. The results from this study imply that laminar shear flow may play a key role not only in reducing inflammatory responses but also in attenuating endothelial cell cycle. This study offers an additional insight that shear flow acts as an athero-protective mechanism to ECs and thus is important in maintaining vessel integrity.

MATERIALS AND METHODS

Materials—The HA-tagged-STAT3 (HA-STAT3) overexpression plasmid was a gift from Dr. T. Hirano, Department of Molecular Oncology, Osaka University Medical School, Japan. The overexpression plasmids of mitogen-activated protein kinase/ERK 1 (MEK1) and MEK kinase 1 (MEKK1) were obtained from Stratagene. The catalytically inactive mutant of ERK was a gift from Dr. R. J. Davis, University of Massachusetts Medical School, Worcester, MA. PD98059 and U0126 were purchased from Calbiochem. Anti-phospho-Tyr-705 STAT3 and anti-phospho-ERK were purchased from Upstate Biotechnology, Inc. Anti-phospho-Ser-727 STAT3 were obtained from Cell Signaling Technology. Anti-STAT3 and anti-ERK1 were purchased from Transduction Laboratories. Anti-hemagglutinin antigen (HA) antibodies were from Covance Research Products Inc., Anti-phospho-JNK, anti-JNK, anti-nucleolin (C23) antibodies, and protein A/G-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfections—Bovine aortic ECs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) as described elsewhere (8). The cultured medium was then

changed to Dulbecco's modified Eagle's medium containing only 0.5% FBS overnight prior to shear flow experiments. Transient transfection was performed by using the LipofectAMINE method (Invitrogen) as previously described (8).

Shear Stress Experiments—Exposure of ECs to shear flow was conducted in a parallel-plate flow chamber as previously described (8). ECs were subjected to well defined shear stress in this flow chamber system linked to a roller pump. This flow loop system was placed in an acrylic plastic enclosure maintained at 37 °C by a heater attached to a temperature controller. The pH value of the medium was constantly maintained by continuous gassing of the medium reservoir with humidified 5% CO_2. The reservoir consisted of Dulbecco's modified Eagle's medium containing 0.5% FBS.

Immunoprecipitation and Western Blotting—After each flow experiment, ECs were lysed with buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture. Cells were disrupted by repeated aspiration through a 21-gauge needle. After removing cellular debris, the same amount of protein from each sample was incubated with specific antibody. The immune complex was incubated with protein A/G-agarose beads for 1 h, and these beads were resuspended in the sample buffer after washing. This immune complex was subjected to immunoblot analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred onto a nitrocellulose membrane, antigen was analyzed by specific antibody. Antigen-antibody complexes were detected using horseradish peroxidase-labeled rabbit anti-mouse or goat anti-rabbit IgG and an ECL detection system (Pierce).

Nuclear Protein Extraction—To prepare nuclear protein extracts, ECs were washed with cold phosphate-buffered saline and then immediately removed by scraping in phosphate-buffered saline. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A containing KCl (10 mmol/liter), EDTA (0.1 mmol/liter), dithiothreitol (1 mmol/liter), and phenylmethylsulfonyl fluoride (1 mmol/liter) for 15 min. The cells were lysed by adding 10% Nonidet P-40 and then centrifuged at 6000 rpm to obtain a pellet of nuclei. The pelleted nuclei were resuspended in cold buffer B containing HEPES (20 mmol/liter), EDTA (1 mmol/liter), dithiothreitol (1 mmol/liter), and phenylmethylsulfonyl fluoride (1 mmol/liter), as well as NaCl (0.4 mmol/liter), and then vigorously agitated from time to time, followed by centrifugation. The supernatant containing the nuclear proteins was used for Western blot analysis.

Statistical Analysis—Statistical analyses were performed with Student's *t* test for experiments consisting of two groups only and with analysis of variance for experiments consisting of more than two groups. Data were presented as mean ± S.E. Statistical significance was defined as indicated.

RESULTS

Phosphorylation of Tyr-705 in STAT3 Is Serum Concentration-dependent—STAT3 activation is well known for its role in cytokine-stimulated cellular responses. The phosphorylation of Tyr-705 and Ser-727 contributes to its nuclear translocation and the STAT3 activation. The basal activation of STAT3 in cultured medium containing serum was examined. ECs were cultured either in medium containing only bovine serum albumin (BSA, 0.1%) or exposed to fetal bovine serum (FBS) at various concentrations for 48 h. As shown in Fig. 1, ECs cultured in medium containing serum showed a phosphorylation at Tyr-705 of STAT3 (Fig. 1). Compared with those in ECs cultured with bovine serum albumin only, this tyrosine phosphorylation was shown to be serum concentration-dependent because an increasing Tyr-705 phosphorylation was observed with an increased serum concentration. In contrast to the serum concentration-dependent phosphorylation of Tyr-705, the phosphorylation of Ser-727 was maintained at a constant basal level. Similarly, the activation or phosphorylation of ERK1/2 was not significantly altered by the serum concentration. This study suggests that phosphorylation of Tyr-705 is serum concentration-dependent.

Shear Flow Increases Ser-727 Phosphorylation while Concomitantly Decreasing Tyr-705 Phosphorylation—Because vascular ECs are constantly under the influence of blood flow-induced shear stress, ECs under shear flow were analyzed for the STAT3 activity by revealing STAT3 phosphorylation. ECs were subjected to shear stress in a well defined parallel plate flow chamber system. As shown in Fig. 2*A*, ECs, maintained in

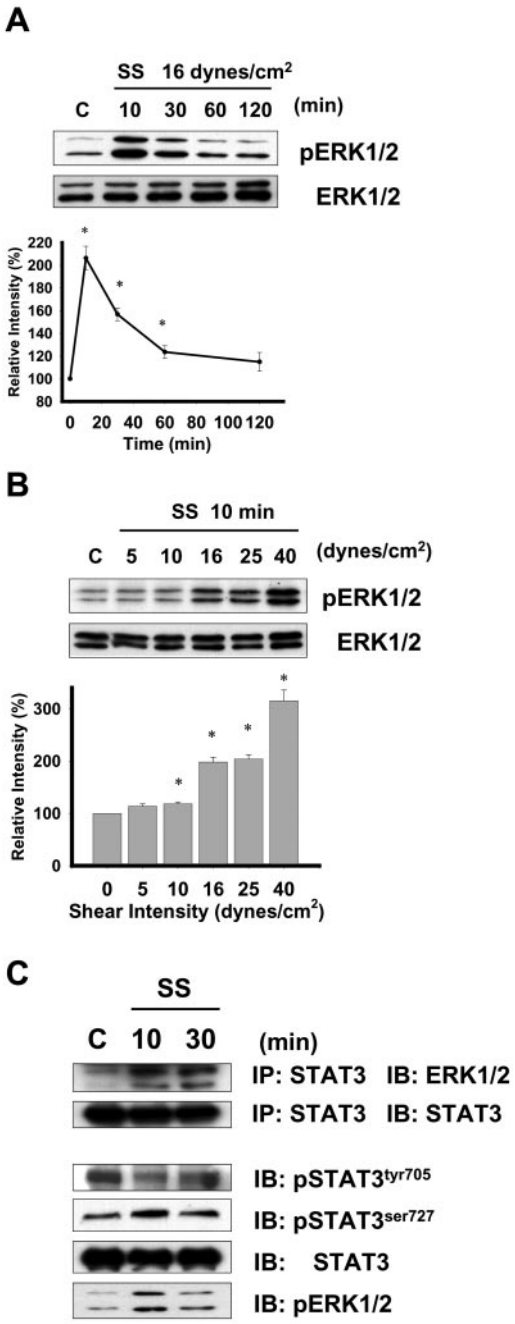

FIG. 3. **Shear flow increases ERK1/2 activation and the association of ERK/12 to STAT3.** *A*, ECs were cultured in medium containing FBS (0.5%) for 16 h. Cells were either kept as static controls (*C*) or exposed to shear flow (*16 dynes/cm²*) for various lengths of time as indicated. The phosphorylation of ERK was analyzed by Western blot using anti-phospho-ERK (*pERK1/2*) antibody. Antibody to ERK1/2 was used to indicate an equal amount of protein was applied to each lane. *B*, ECs were cultured in medium containing FBS (0.5%) for 16 h. Cells were either kept as static controls (*C*) or exposed to shear flow for 10 min with various shear forces as indicated. The phosphorylation of ERK was analyzed by Western blot using anti-phospho-ERK (*pERK1/2*) antibody. Data were presented as means ± S.E. from three independent experiments. *, $p < 0.01$ *versus* static control. *C*, ECs were cultured in medium containing FBS (0.5%) for 16 h. Cells were either kept as static controls (*C*) or exposed to shear flow (*16 dynes/cm²*) for 10 or 30 min as indicated. The cell lysates were immunoprecipitated with anti-STAT3 antibody (*IP*) and probed with anti-ERK or anti-STAT3 antibody (*IB*). Similar results were obtained from three independent experiments.

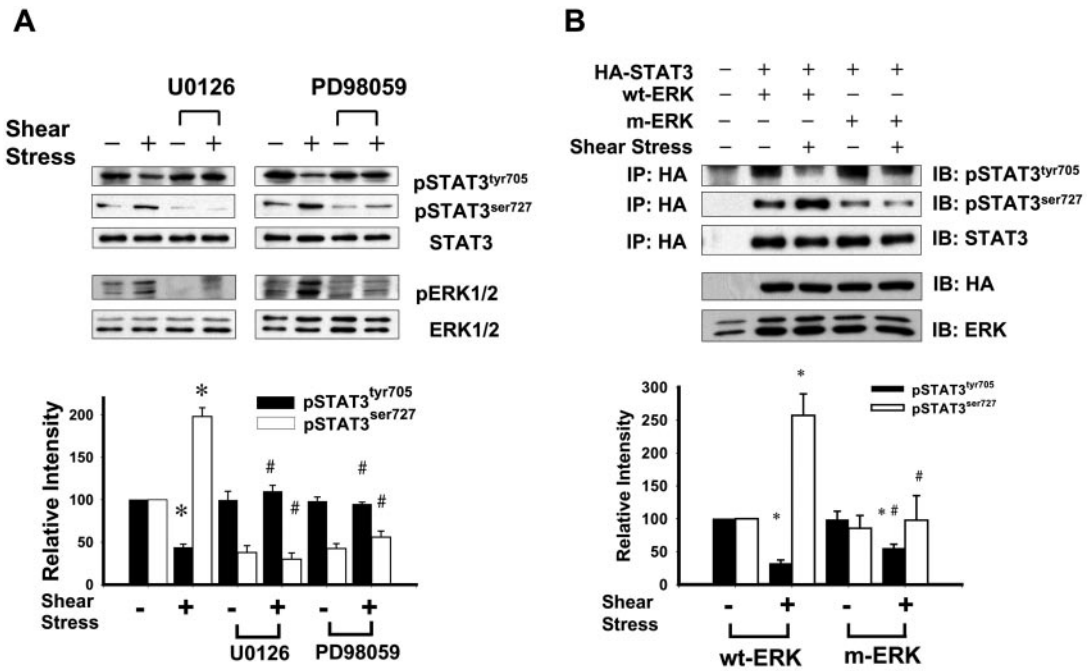

FIG. 4. **Shear flow increases phosphorylation of Ser-727 via ERK1/2, and Ser-727 phosphorylation leads to a decrease of phosphorylation in Tyr-705 in STAT3.** *A*, ECs were pretreated with a MEK-1 inhibitor, U0126 (10 μM) or PD98059 (30 μM), for 30 min and then kept as static controls or exposed to shear flow for 10 min. The phosphorylation of STAT3 was analyzed by Western blot using anti-phospho-antibody to Tyr-705-STAT3 (*pSTAT3*$^{Tyr\text{-}705}$), Ser-727-STAT3 (*pSTAT3*$^{Ser\text{-}727}$), and ERK1/2 (*pERK1/2*). Data were presented as means ± S.E. from four independent experiments. *, $p < 0.05$ *versus* static control. #, $p < 0.05$ *versus* shear flow only. *B*, ECs were transiently transfected with HA-tagged STAT3 (*HA-STAT3*), wild-type ERK (*wt-ERK*), or catalytically inactivated mutant ERK (*m-ERK*). ECs, 48 h after transfection, were maintained under static conditions as controls or exposed to shear stress (*SS*) for 10 min. Total cell lysates were subjected to immunoprecipitation (*IP*) with anti-HA antibody and probed with anti-phospho-Tyr-705-STAT3 (*pSTAT3*$^{Tyr\text{-}705}$), anti-phospho-Ser-727-STAT3 (pSTAT3$^{Ser\text{-}727}$), or anti-STAT3 (*STAT3*) antibody. Western blot analysis with anti-HA (*HA*) or anti-ERK (*ERK*) antibody was used to indicate a successful transfection. Data were presented as means ± S.E. from four independent experiments. *, $p < 0.05$ *versus* static control. #, $p < 0.05$ *versus* ECs under shear flow only.

medium containing 0.5% FBS and exposed to shear flow (16 dynes/cm^2) for various time intervals, show a rapid decline in Tyr-705 phosphorylation, whereas there is an immediate induction and an elevated and sustained phosphorylation at Ser-727 in STAT3. A rapid drop in phosphorylation of Tyr-705 was observed as early as 10 min and apparently reduced to a minimal level at 30 min subsequent to shear flow exposure. When ECs were subjected to varying degrees of shear stress for 10 min, it was noted that there is a shear force-dependent increase in Ser-727 phosphorylation. Interestingly, a concomitant inverse decrease in Tyr-705 phosphorylation was observed (Fig. 2*B*). This result clearly shows that exposure of ECs to shear flow rapidly increases Ser-727 phosphorylation, whereas a concomitant decrease occurs in Tyr-707 phosphorylation.

Shear Flow Increases ERK1/2 Association with STAT3 with a Subsequent Increase in Phosphorylation of Ser-727 in STAT3—It has been shown that ERK, subsequent to activation by growth factor, phosphorylates STAT3 (20). We have previously shown that shear flow increases the activity of ERK1/2 (5). Whether the shear flow-induced ERK1/2 contributed to Ser-727 phosphorylation was investigated. As shown in Fig. 3, *A* and *B*, shear flow induced rapid and shear force-dependent phosphorylation of ERK1/2, a phenomenon similar to the shear-induced phosphorylation of Ser-727 shown in Fig. 2. To demonstrate that ERK1/2 was involved in STAT3 phosphorylation, an immunoprecipitation assay with STAT3 antibody was performed. Shear stress increased ERK/12 association with STAT3 (Fig. 3*C*). As expected, the ERK1/2 exhibited increased phosphorylation. As a result, the STAT3 from shear stress-treated ECs was shown to have greater phosphorylation at Ser-727 but lower phosphorylation at Tyr-705. This study indicates that shear flow to ECs induces the association of ERK1/2 with STAT3 with a subsequent increase in Ser-727 phosphorylation of STAT3.

ERK1/2 Pathway Is Upstream of Flow-induced Ser-727 Phosphorylation—To evaluate whether ERK1/2 is upstream of STAT3 activation, we pretreated ECs with a MEK inhibitor (U0126 or PD98059) followed by shear flow exposure. As shown in Fig. 4*A*, U0126 or PD98059 pretreatment inhibited ERK1/2 activity in ECs under static or shear conditions. Consistently, the Ser-727 phosphorylation of STAT3 was significantly reduced. Concomitantly, the shear flow-induced inhibition of Tyr-705 phosphorylation was greatly attenuated. To further confirm that ERK is upstream of Ser-727 phosphorylation, ECs were co-transfected with HA-STAT3 and wild type or catalytically inactive mutant ERK, and the phosphorylation of HA-tagged-STAT3 was analyzed. As shown in Fig. 4*B*, ECs transfected with wild-type ERK demonstrated an increased phosphorylation of Ser-727 in HA-STAT3 following exposure to shear flow as compared with those without shear treatment. Conversely, the Tyr-705 phosphorylation in this HA-STAT3 was significantly reduced. In contrast, when ECs transfected with the catalytically inactive mutant of ERK, shear-induced Ser-727 phosphorylation was reduced compared with those ECs transfected with wild-type ERK. This reduced Ser-727 phosphorylation conversely increased Tyr-705 phosphorylation. This ERK inactive mutant also abolished the shear effects on decreasing the phosphorylation of Tyr-705. These results strongly suggest that shear flow induces ERK1/2 activity followed with Ser-727 phosphorylation in STAT3. The Ser-727 phosphorylation attenuated the phosphorylation of Tyr-705 in STAT3. To further confirm the effects of ERK1/2 on Ser-727 and Tyr-705, ECs were co-transfected with HA-tagged STAT3 and an expression plasmid encoding MEK1, upstream of ERK1/2. ECs with this MEK1 overexpression showed an increased phosphorylation of HA-STAT3 at Ser-727 (Fig. 5*A*). In contrast, this increased Ser-727 phosphorylation resulted in a

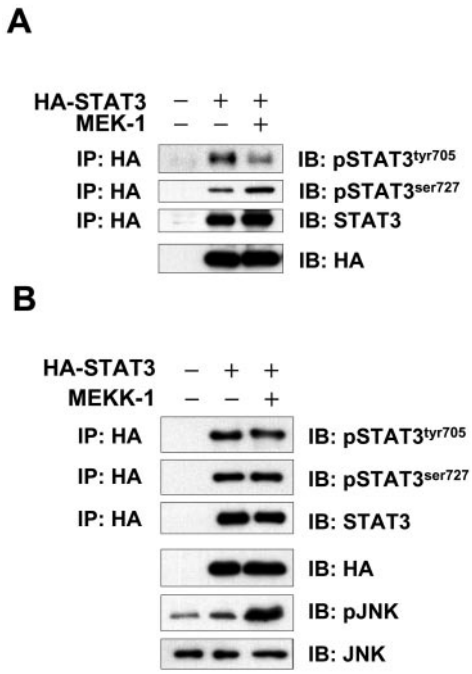

FIG. 5. **MEK1/ERK but not MEKK1/JNK pathway is involved in increased phosphorylation of Ser-727 in STAT3.** *A*, ECs were co-transfected of HA-tagged-STAT3 (*HA-STAT3*) with dominant positive mutant MEK-1 (*MEK-1*). ECs were lysed at 48 h after transfection. Total cell lysates were subjected to immunoprecipitation (*IP*) with anti-HA antibody and probed with anti-phospho-Tyr-705-STAT3 ($pSTAT3^{Tyr\text{-}705}$) or anti-phospho-Ser-727-STAT3 ($pSTAT3^{Ser\text{-}727}$). Western blot analysis with an antibody to HA (*HA*) or STAT3 (*STAT3*) was used to indicate a successful transfection. *B*, ECs were co-transfected of HA-tagged-STAT3 (*HA-STAT3*) with dominant positive mutant MEKK-1 (*MEKK-1*), JNK upstream. Total cell lysates were subjected to immunoprecipitation (*IP*) with anti-HA antibody and probed with anti-phospho-Tyr-705-STAT3 ($pSTAT3^{Tyr\text{-}705}$), anti-phospho-Ser-727-STAT3 ($pSTAT3^{Ser\text{-}727}$). Western blot analysis with anti-HA (*HA*), anti-phospho-JNK (*pJNK*), or anti-JNK (*JNK*) antibody was used to show a successful transfection.

decrease of Tyr-705 phosphorylation. Conversely, when ECs co-transfected with expression plasmids encoding MEKK1, upstream of JNK, no effect in the phosphorylation of Ser-727 and Tyr-705 was observed (Fig. 5*B*). All these results confirm that shear flow to ECs increases the phosphorylation of Ser-727 via ERK1/2 signaling pathway and that increased Ser-727 phosphorylation contributes to the decrease of Tyr-705 phosphorylation in STAT3.

Shear Flow Decreases STAT3 Transmigration into Nucleus—Tyr-705 phosphorylation is required for STAT3 nuclear translocation (16). The decrease of STAT3 activity via inhibition of Tyr-705 phosphorylation results in a reduction of intracellular STAT3 transmigration into nucleus. To clearly see the shear effects on STAT3 activity, ECs were cultured in medium containing 0.1% bovine serum albumin to minimize the STAT3 activity, followed by shear stress in the presence of 20% FBS. As shown in Fig. 6, ECs cultured in 20% serum increased STAT3 transmigration into nucleus. In contrast, nuclear proteins extracted from shear stress-treated ECs showed decreased total STAT3 content and reduced Tyr-705 phosphorylation when compared with those static-incubated ECs. These results confirm that shear flow exerts its protective mechanism by attenuating serum-induced STAT3 activation via the inhibition of Tyr-705 phosphorylation that is mediated through ERK1/2 activation on Ser-727 phosphorylation. The inhibition of STAT3 activation and its migration into nucleus by shear flow may play an important role in regulating IL-6- and/or cytokine-induced inflammatory responses. This decreased

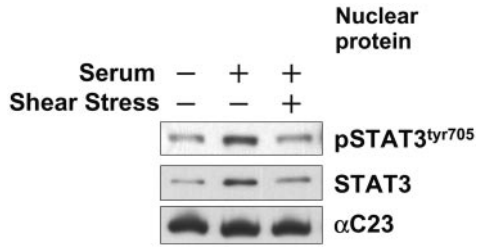

FIG. 6. **Shear flow attenuates STAT3 translocation into nucleus.** ECs were cultured in medium containing bovine serum albumin (0.1%) for 24 h. ECs were then treated with 20% fetal bovine serum (*Serum*) under static conditions or exposed to shear stress (*SS*) for 15 min. The crude nuclear extracts were prepared as described under "Materials and Methods." The nuclear extracts were subjected to Western blot analysis with anti-phospho-Tyr-705-STAT3 ($pSTAT3^{Tyr\text{-}705}$), anti-STAT3 (*STAT3*). Anti-nucleolin (*αC23*) antibody was used as a nuclear marker. Similar results were obtained from three independent experiments.

STAT3 activation may also contribute to growth arrest in ECs particularly in the region of the straight segment in the arterial tree where laminar shear stress is normally encountered.

DISCUSSION

ECs subjected to shear stress modulate gene expression that is crucial in maintaining vessel integrity. Our studies and others have shown that hemodynamic forces on ECs up-regulated expression in such genes as *ICAM-1*, *MCP-1*, and *Egr-1*. This gene induction appears to be mediated via the signaling molecules/pathway including protein kinase C, calcium, and Ras/Raf/ERK1/2 (5, 6, 36, 37). We have also demonstrated that this hemodynamic force-induced effect is a redox-sensitive process because an elevated reactive oxygen species is observed subsequent to the initiation of shear stress or cyclic strain (7, 8). Reactive oxygen species are involved in this gene induction because an antioxidant pretreatment attenuates this hemodynamic forces-induced gene expression. Shear stress has been shown to stimulate endothelial nitric oxide synthase activity (38, 39) and eNOS expression (40). Our recent studies have also demonstrated that endothelial NO plays an important role in attenuating the redox-sensitive responses (5, 41, 42). Shear stress to ECs also increases the expression of antioxidant enzymes such as Cu/Zn superoxide dismutase and heme oxygenase-1 (43). Recent studies by Chen *et al.* (13) further demonstrate that laminar shear flow induces antioxidant response element-mediated genes in ECs. Thus, shear stress exerts its protective role by inhibiting redox-induced responses. A recent study by Lin *et al.* (14) indicates that laminar shear stress to ECs inhibits the endothelial cell cycle by inducing the growth arrest proteins GADD45 and P21. Induction of antioxidant enzymes, increased NO production, and induction of growth arrest proteins may be the underlying mechanisms of the protective effects of the pre-sheared ECs to various stimuli, including cytokines.

Cytokine- and growth factor-induced STAT3 activity has been shown to be involved in cell proliferation (16). In this study, we have demonstrated that serum induced phosphorylation of Tyr-705 in STAT3 in a serum concentration-dependent manner. Furthermore, we have shown that shear flow increases Ser-727 phosphorylation in a shear force-dependent manner. Concomitantly, a decrease of Tyr-705 phosphorylation was observed. Signaling pathway Ras/Raf/ERK1/2 can be activated by hemodynamic force, including shear flow and cyclic strain as we have demonstrated (5, 6). In this study, we further demonstrate that ERK1/2 phosphorylation is shear force-dependent. Similar results have been reported by Jo *et al.* (4). Interestingly, phosphorylation of Ser-727 of STAT3 was also shown to be shear force-dependent. Our data clearly show that

shear-induced ERK1/2 pathway is upstream of Ser-727 phosphorylation. Several lines of evidence support this notion. First, a similar pattern of phosphorylation in ERK1/2 and Ser-727 was observed in shear flow-treated ECs. These include the shear dose-dependent phosphorylation of both ERK1/2 and Ser-727 in STAT3. Second, an increased association of ERK1/2 to STAT3 was observed in these shear-treated ECs. Third, a decreased activation of both ERK1/2 and Ser-727 was seen in sheared ECs pretreated with an inhibitor to MEK1. Fourth, when ECs were transfected with dominant negative mutant of ERK, the shear-induced increase in phosphorylation of Ser-727 was no longer observed. Fifth, when ECs transfected with a dominant positive mutant of MEK1, upstream of ERK1/2, increased Ser-727 phosphorylation was seen but not from those ECs transfected with MEKK1, upstream of JNK. All these data clearly show that shear flow activates ERK1/2 and subsequently phosphorylates Ser-727 in STAT3 in a shear dose-dependent manner.

More interestingly, the increased Ser-727 phosphorylation concomitantly results in a decrease of Tyr-705 phosphorylation in STAT3. This is reflected in the shear dose-dependent decrease in Tyr-705 phosphorylation. The phosphorylation of Ser-727 resulting in a decrease of Tyr-705 phosphorylation was further confirmed by the treatment of ECs with a MEK inhibitor. The shear-induced inhibition of Tyr-705 was no longer observed in those ECs. Similarly, ECs transfected with wild type of ERK1/2 or dominant positive mutant of MEK1, upstream of ERK1/2, increased Ser-727 phosphorylation with a concomitant inverse decrease in Tyr-705 phosphorylation. These confirm that an increase of ERK1/2 activation by shear flow results in an inhibition of Tyr-705 activation. As a consequence of this shear flow-induced attenuation of STAT3 activation, a decrease of STAT3 transmigration into nucleus was observed.

Earlier studies by Chung *et al.* (20) and Jain *et al*. (21) have demonstrated that growth factors stimulate STAT3 serine phosphorylation by ERK1/2-dependent pathway, which negatively modulates its tyrosine phosphorylation. The present study is consistent with their findings that shear stress-induced ERK1/2 activation results in a decrease of STAT3 tyrosine phosphorylation. Interestingly, this ERK1/2 phosphorylation by shear flow is shown to be in a shear dose-dependent manner. In the flow chamber system, ECs were maintained in a cultured medium containing 0.5% FBS, identical to static condition. Thus ERK1/2 activation in ECs by shear flow is not likely mediated by serum or growth factors contained in the serum. This shear dose-dependent ERK1/2 activation further confirms our earlier reports that shear flow and cyclic strain induces ERK1/2 signaling pathway in ECs (5, 6). However, this is the first report to show that shear flow acts on ERK1/2 and exhibits an inhibitory role in the signaling pathway that leads to STAT3 inactivation.

It is known that STAT3 is preferentially stimulated by IL-6 during inflammatory response. IL-6 is produced by various cells, including ECs and smooth muscle cells. ECs are able to secrete IL-6 upon stimulation with cytokines (44, 45), endotoxin (46), or endothelin-1 (47). ECs exposed to IL-6 may trigger inflammatory responses, including the increase of endothelial permeability (48). Shear flow-induced STAT3 inactivation may attenuate the IL-6-triggered responses. Presheared ECs have been shown to inhibit subsequent tumor necrosis factor-induced responses (10, 11). Our findings may provide new perspectives on the mechanisms of how shear flow protects ECs from cytokine-induced responses. The detailed mechanism(s) as to how the increase of Ser-727 phosphorylation results in a decrease of Tyr-705 phosphorylation remains unclear. Recent study indicates that protein tyrosine phosphatase may be involved in the shear stress-induced responses (49). ECs treated with a tyrosine phosphatase inhibitor (sodium orthovanadate) abolished the shear-induced inhibition of Tyr-705 phosphorylation.[2]

Our findings have physiological significance because STAT3 activation is found to be involved in the induction of cyclin D1, c-Myc, and Bcl-xL that participate in the regulation of cell cycle (33–35). Laminar shear flow may exert its regulatory role by inhibiting endothelial proliferation via the inhibition of STAT3 activation. Lin *et al.* (14) have indicated that shear stress induces growth arrest proteins and inhibits the endothelial cell cycle. Present results are consistent with the notion that shear flow plays a negative role in regulating cell cycle. Atherosclerotic lesions preferentially develop in areas where low shear or non-laminar flow is encountered. The shear dose-dependent inhibition of STAT3 activity may provide some insight for the athero-protective mechanism in the straight segments of the arterial tree where laminar flow and high fluid shear stress normally occur.

REFERENCES

1. Takahashi, M., and Berk, B. C. (1996) J. Clin. Invest. **98,** 2623–2631
2. Zou, Y., Hu, Y., Metzler, B., and Xu, Q. (1998) *Int. J. Mol. Med.* **1,** 827–834
3. Julali, S., Li, Y. S., Sotoudeh, M., Yuan, S., Yuan, S., Li, S., Chien, S., and Shyy, Y. J. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 227–234
4. Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J., and McDonald, J. M. (1997) *J. Biol. Chem.* **272,** 1395–1401
5. Chiu, J. J., Wung, B. S., Hsieh, H. J., Lo, L. W., and Wang, D. L. (1999) *Circ. Res.* **85,** 238–246
6. Wung, B. S., Cheng, J. J., Shyy, Y. J., Chao, Y. J., and Wang, D. L. (1999) *Circ. Res.* **84,** 804–812
7. Wung, B. S., Cheng, J. J., Chao, Y. J, and Wang, D. L. (1997) *Circ. Res.* **81,** 1–7
8. Chiu, J. J., Wung, B. S., Hsieh, H. J., and Wang, D. L. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 3570–3577
9. Hsieh, H. J., Cheng, J. J., Wu, S. T., Chiu, J. J., Wung, B. S., and Wang, D. L. (1998) *J. Cell. Physiol.* **175,** 156–162
10. Surapisitchat, J., Hoefen, R. J., Pi, X., Yoshizumi, M., Yan, C., and Berk, B. C. (2001) *Proc. Natl. Acad. Sci. (U. S. A.)* **98,** 6476–6481
11. Matsumoto, Y., Kawai, Y., Watanabe, K., Sakai, K., Murata, M. Handa, M., Nakamura, S., and Ikeda, Y. (1998) *Blood* **91,** 4164–4172
12. Hermann, C., Zeiher, A. M., and Dimmeler, S. (1997) *Arterioscler. Thromb. Vasc. Biol. (1997)* **17,** 3588–3592
13. Chen, X. L., Varner, S. E., Rao, A. S., Grey, J. Y., Thomas, S., Cook, C. K., Wasserman, M. A., Medford, R. M., Jaiswal, A. K., and Kunsch, C. (2003) *J. Biol. Chem.* **278,** 703–711
14. Lin, K., Hsu, P. P., Chen, B. P., Yuan, S., Usami, S., Shyy, J. Y-J., Li, Y-S., and Chien, S. (2000), *Proc. Natl. Acad. Sci. (U. S. A.)* **97,** 9385–9389
15. Hirano, T., Ishihara, K., and Hibi, M. (2000) *Oncogene* **19,** 2548–2556
16. Levy, D. E., and Lee, C. K. (2002), *J. Clin. Invest.* **109,** 1143–1148
17. Zhang, Y., Turkson, J., Carter-Su, C., Smithgall, T., Levitzki, A., Kraker, A., Krolewski, J. J., Medveczky, P., and Jove, R. (2000) *J. Biol. Chem.* **275,** 24935–24944
18. Wang, Y. Z., Wharton, W., Garcia, R., Kraker, A., Jove, R., and Pledger, W. J. (2000) *Oncogene* **19,** 2075–2085
19. Vignais, M-L., and Gilman, M. (1999) *Mol. Cell. Biol.* **19,** 3727–3735
20. Chung, J., Uchida, E., Grammer, T. C., and Blenis, J. (1997) *Mol. Cell. Biol.* **17,** 6508–6516
21. Jain, N., Zhang, T., Fong, S. L., Lim, C. P., and Cao, X. (1998) *Oncogene* **17,** 3157–3167
22. Bartoli, M., Gu, X., Tsai, N. T., Venema, R. C., Brooks, S. E., Marrero, M. B., and Caldwell, R. B. (2000) *J. Biol. Chem.* **275,** 33189–33192
23. Deo, D. D., Axelrad, T. M., Robert, E. G., Marcheselli, V., Bazan, N. G., and Hunt, J. D. (2002) *J. Biol. Chem.* **277,** 21237–21245
24. Valdembri, D., Serini, G., Vacca, A., Ribatti, D., and Bussolino, F. (2002) *FASEB J.* **16,** 225–227
25. Zong, C. S., Chan, J., Levy, D. E., Horvath, C., Sadowski, H. B., and Wang, L-H. (2000) *J. Biol. Chem.* **275,** 15099–15105
26. Simon, A. R., Vikis, H. G., Stewart, S., Fanburg, B. L., Cochran, B. H., and Guan, K-L. (2000) *Science* **290,** 144–147
27. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Irani, K., Goldschmidt-Clemont, P. J., and Finkel, T. (1996) *Biochem. J.* **318,** 379–382
28. Deshpande, S. S., Angkeow, P., Huang, J., Ozaki, M., and Irani, K. (2000) *FASEB J.* **14,** 1705–1714
29. Abid, M. R., Tsai, J. C., Spokes, K. C., Deshpande, S. S., Irani, K., and Aird, W. C. (2001) *FASEB J.* **15,** 2548–2550
30. Nakagami, H., Morishita, R., Yamamoto, K., Taniyama, Y., Aoki, M., Matsumoto, K., Nakamura, T., Kaneda, Y., Horiuchi, M., and Ogihara, T. (2001) *Hypertension* **37,** 581–586
31. Zeng, Z-Z., Yellaturu, C. R., Neeli, I., and Rao, G. N. (2002) *J. Biol. Chem.* **277,** 41213–41219

[2] C. W. Ni and D. L. Wang, unpublished observation.

32. Niu, G., Wright, K. L., Huang, M., Song, L., Haura, E., Turkson, J., Zhang, S., Wang, T., Sinibaldi, D., Coppola, D., Heller, R., Ellis, L. M., Karras, J., Bromberg, J., Pardoll, D., Jove, R., and Yu, H. (2002) *Oncogene* **21,** 2000–2008
33. Benekli, M., Baer, M. R., Baumann, H., and Wetzler, M. (2003) *Blood* **101,** 2940–2954
34. Masuda, M., Suzui, M., Yasumatu, R., Nakashima, T., Kuratomi, Y., Azuma, K., Tomita, K., Komiyama, S., and Weinstein, I. B. (2002) *Cancer Res.* **62,** 3351–3355
35. Kijima, T., Niwa, H., Steinman, R. A., Drenning, S. D., Gooding, W. E., Wentzel, A. L., Xi, S., and Grandis, J. R. (2002) *Cell Growth Differ*. **13,** 355–362
36. Traub, O., and Berk, B. C. (1998) *Arterioscler. Thromb. Vasc. Biol*. **18,** 677–685
37. Davies, P. F. (1995) *Physiol. Rev.* **75,** 519–560
38. Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996) *Circ. Res.* **79,** 984–991
39. Boo, Y. C., Sorescu, G., Boyd, N., Shiojima, I., Walsh, K., Du,. J., and Jo, H. (2002) *J. Biol. Chem.* **277,** 3388–3396
40. Dimmeler, S., Hermann, C., Galle, J., and Zeiher, A. M. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19,** 656–664
41. Wung, B. S., Cheng, J. J., Shyue, S. K., and Wang, D. L. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21,** 1941–1947
42. Ni, C-W., Wang, D. L., Lien, S. C., Cheng, J. J., Chao, Y. J., and Hsieh, H. J. (2003) *J. Cell. Physiol.* **195,** 428–434
43. De Keulenaer, G. W., Chappell, D. C., Ishizaka, N., Nerem, R. M., Alexander, R. W., and Griendling, K. K. (1998) *Circ. Res.* **82,** 1094–1101
44. Chen, C. C., and Manning, A. M. (1996) *Cytokine* **8,** 58–65
45. Volk, T., Hensel, M., Schuster, H., and Kox, W. J. (2000) *Mol. Cell. Biochem.* **206,** 105–112
46. Gomikiewicz, A., Sautner, T. Brostjan, C., Schmierer, B., Fugger, R., Roth, E., Muhlbacher, F., and Bergmann, M. (2000) *FASEB J.* **14,** 1093–1100
47. Stankova, J., D'Orleans-Juste, P., and Rola-Pleszczynski, M. (1996) *Am. J. Physiol.* **271,** C1073–1078
48. Ali, M. H., Schlidt, S. A., Chandel, N. S., Hynes, K. L., Schumacker, P. T., and Gewertz, B. L. (1999) *Am. J. Physiol.* **277,** L1057–1065
49. Fleming, I., Bauersachs, J., Fisslthaler, B., and Busse, R. (1998) *Circ. Res.* **82,** 686–695