Fluid Shear Stress Stimulates Big Mitogen-activated Protein Kinase 1 (BMK1) Activity in Endothelial Cells

DEPENDENCE ON TYROSINE KINASES AND INTRACELLULAR CALCIUM*

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Mitogen-activated protein (MAP) kinases including ERK1/2 and JNK play an important role in shear stress-mediated gene expression in endothelial cells (EC). A new MAP kinase termed big MAP kinase 1 (BMK1/ERK5) has been shown to phosphorylate and activate the transcription factor MEF2C, which is highly expressed in EC. To determine the effects of shear stress on BMK1, bovine aortic EC were exposed to steady laminar flow (shear stress = 12 dynes/cm²). Flow activated BMK1 within 10 min with peak activation at 60 min (7.1 ± 0.6-fold) in a force-dependent manner. Flow was the most powerful activator of BMK1, significantly greater than H2O2 or sorbitol. An important role for non-Src tyrosine kinases in flow-mediated BMK1 activation was demonstrated by inhibition with herbimycin A, but not with the Src inhibitor PP1 or overexpression of kinase-inactive c-Src. BMK1 activation was calcium-dependent as shown by inhibition with 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester or thapsigargin. As shown by specific inhibitors or activators, flow-mediated BMK1 activation was not regulated by the following: intracellular redox state; intracellular NO; protein kinase A, C, or G; calcium/calmodulin-dependent kinase; phosphatidylinositol 3-kinase; or arachidonic acid metabolism. In summary, flow potently stimulates BMK1 in EC by a mechanism dependent on a tyrosine kinase(s) and calcium mobilization, but not on c-Src, redox state, or NO production.

Changes in shear stress also cause long-term alterations in vessel structure and function by regulating protein and gene expression. For example, shear stress stimulates expression of platelet-derived growth factor A- and B-chains, tissue plasminogen activator, endothelial nitric-oxide synthase, and endothelin (1, 2).

The mechanisms by which endothelial cells sense mechanical stimuli and convert them to biochemical signals are not well characterized. Much experimental evidence indicates that the cellular response to shear stress is similar to the response to G protein-coupled receptors and growth factor receptors, which involves activation of a complex array of phosphorylation cascades. The mitogen-activated protein (MAP)1 kinases respond to diverse stimuli, including physical stress, oxidative stress, and UV light, and play pivotal roles in a variety of cell functions. Thus, MAP kinases are excellent candidates to mediate mechanotransduction in endothelial cells.

MAP kinases are serine/threonine protein kinases. Four subfamilies of MAP kinases have been identified, including the extracellular signal-regulated protein kinase (ERK1/2), c-Jun NH2-terminal kinase (JNK), p38 kinase, and big MAP kinase 1 (BMK1 or ERK5) (3). Each subfamily may be regulated by different signal transduction pathways and modulate specific cell functions (3, 4). ERK1/2 is activated by an upstream kinase (MEK1) via dual phosphorylation of the TEY motif, whereas JNK and p38 kinase are activated by MEK4 and MEK3 via TPY and TGY motifs, respectively. BMK1, a recently identified MAP kinase family member, shares the TEY activation motif with ERK1/2, but is activated by MEK5. More important, BMK1 has a unique long COOH-terminal tail, suggesting that its regulation and function may be different from other MAP kinases. BMK1 was recently shown to phosphorylate and activate the transcription factor MEF2C (5). MEF2C is highly expressed in endothelial cells (EC) and is likely to regulate EC function as shown by the abnormal blood vessel development in MEF2C knockout mice (6).

Previous studies from our laboratory (7, 8) and others (9, 10) have shown that shear stress activates ERK1/2 in EC. The upstream regulatory pathways include Gα12, Ras, a tyrosine

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1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH2-terminal kinase; BMK1, big MAP kinase 1; MEK, MAP kinase/ERK kinase; FAK, focal adhesion kinase; EC, endothelial cell(s); BAEC, bovine aortic endothelial cell(s); HUVEC, human umbilical vein endothelial cell(s); PKC, protein kinase C; PKA, protein kinase A; PKG, protein kinase G; ROS, reactive oxygen species; CaM kinase, calcium/calmodulin-dependent kinase; PI 3-kinase, phosphatidylinositol 3-kinase; BSO, N-buthyliso-thiourea-2β,3′,5′-sulfoximine; NAC, N-acetyl-l-cysteine; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester.
kinase, and PKC (7, 8, 10, 11). JNK activity is also modulated by shear stress, although the effects of shear stress on JNK are controversial. Two groups reported that JNK was activated by shear stress in BAEC via a mechanism involving Gβγ, Ras, and a tyrosine kinase(s) (10, 12). In contrast, our laboratory found that shear stress failed to activate JNK in human umbilical vein endothelial cells (HUVEC) and inhibited tumor necrosis factor-mediated JNK activation.² No studies have been published regarding changes in p38 or BMK1 activity in response to shear stress. In this study, we show that shear stress is the most potent stimulus for BMK1 activation in EC. We further investigated the roles of tyrosine kinase phosphorylation, intracellular Ca²⁺, ROS, NO, PKA, PKC, PKG, CaM kinase, PI 3-kinase, and arachidonic acid metabolism in shear stress-induced BMK1 activation.

EXPERIMENTAL PROCEDURES

Cell Culture—BAEC were harvested from fetal calf aortas by collagenase as described previously (7). Cells were grown in M199 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone Laboratories), 50 units/ml penicillin, and 0.05 mg/ml streptomycin at 37 °C in a 5% CO₂ and 95% air atmosphere. Cells used in experiments were at passages 1–3. Experiments were performed with growth-arrested cells (2 days after reaching confluence) to minimize basal BMK1 activity.

Adenoviral Transfection—BAEC were grown on 60-mm tissue culture dishes. Upon reaching 70–80% confluence, cells were incubated with 0.1 ml of M199 medium containing Ad.KI-Src or Ad.LacZ for 1 h at 37 °C in a 95% air and 5% CO₂ incubator. Cells were then infected with 5 ml of M199 medium supplemented with 10% fetal bovine serum for 2 days. The construction and preparation of Ad.KI-Src, characterization of transfection efficiency, and controls for cell toxicity have been described separately in detail.²

Flow Experiments—Two different devices were used to create fluid shear stress in vitro: the parallel plate chamber and the cone and plate viscometer (16). Cells were grown on 2 × 4-cm slides of tissue culture plastic cut from the bottom of tissue culture dishes (for the parallel plate chamber) or on 60-mm dishes (for the cone and plate viscometer). Upon reaching confluence, cells were fed fresh medium, and 2 days later, the cells were rinsed free of culture medium with Hanks’ balanced saline solution (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 5.9 mM glucose, and 7.43 mM sodium pyrophosphate) supplemented with 10 mM HEPES, pH 7.4. Cells were then infected with Ad with 0.1 ml of M199 medium containing Ad.KI-Src or Ad.LacZ for 1 h at 37 °C in a 95% air and 5% CO₂ incubator. Cells were then infected with 5 ml of M199 medium supplemented with 10% fetal bovine serum for 2 days. The construction and preparation of Ad.KI-Src, characterization of transfection efficiency, and controls for cell toxicity have been described separately in detail.²

Immunoprecipitation—Cells prepared by flush-freezing and thawing in lysis buffer (50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 mM NaPO₄, 10 mM HEPES, pH 7.4, 0.1% Triton X-100, 500 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) were mixed with 500 μl of rabbit anti-BMK1 polyclonal antibody (17, 18) overnight at 4 °C and then incubated with 30 μl of protein A-agarose (17). Immunoprecipitated proteins were eluted with 1 μl of rabbit anti-BMK1 polyclonal antibody (17, 18) overnight at 4 °C and then incubated with 30 μl of protein A-agarose (Life Technologies, Inc.) for 1 h on a roller system at 4 °C. The immune complex beads were washed twice with 1 ml of lysis buffer containing 1 mg/ml of proteinase K and 1 mg/ml of lyophilized protein A-agarose (17) before incubation with 1 ml of lysis buffer (50 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100, and 1 mM dithiothreitol), and twice with 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1% Triton X-100).

BMK1 Kinase Assay—BMK1 kinase activity was measured by auto-phosphorylation as described previously (17). In brief, immune complex kinase assays were performed at 30 °C for 20 min in a reaction mixture containing 15 μM ATP, 10 mM MgCl₂, 10 mM MnCl₂, and 3 μCi of [γ-³²P]ATP. The reaction was terminated with 8 μl of 6× electrophoresis sample buffer and boiling for 5 min. Samples were analyzed on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes (Hybond™-ECL, Amersham Pharmacia Biotech), and autoradiographed. Kinase activities were determined by densitometry of bands at the correct molecular masses in the linear range of film exposure using a scanner and NIH Image 1.6.

Western Blot Analysis—The membrane was blocked for at least 1 h at room temperature with a commercial blocking buffer (Life Technologies, Inc.). The blot was then incubated for 1 h with a 1:1000 dilution of anti-BMK1 antibody at room temperature, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech).

Determination of Endothelial Cell GSH Content—The 5,5'-dithio-bis-(2-nitrobenzoic acid) colorimetric method was used to measure glutathione (19), the predominant soluble thiol in EC (20). In brief, cells were cultured in 60-mm dishes, and post-confluent monolayers (1–10⁶ cells/dish) were preincubated in culture medium at 37 °C for 1 h in the presence of increasing concentrations of NAC. Cells were then subjected to flow for 20 min in the presence of fresh medium containing the same concentration of BSO or NAC. Cells were washed with phosphate-buffered saline three times, and 600 μl of 2% (w/v) 5-sulfosalicylic acid was added for cell lysis and deproteinization. Samples were centrifuged for 5 min at 10,000 × g, and aliquots of 500 μl were mixed with 500 μl of a Tricine solution containing 0.3 mM sodium phosphate buffer, pH 7.5, 10 mM EDTA, and 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid). After a 5-min incubation, the absorbance was read at 412 nm, and the concentration of soluble thiols was quantified by comparison with GSH standards.

RESULTS

Shear Stress Stimulates BMK1 in BAEC: Time and Force Dependence—To determine whether shear stress regulates BMK1 activity in BAEC, BAEC were exposed to steady laminar flow, and an in vitro immune complex kinase assay was performed based on BMK1 autophosphorylation, which we have shown correlates well with myelin basic protein phosphorylation (17). In the physiological range of shear stress (3.5–35 dynes/cm² for 20 min), BMK1 activity increased in a force-dependent manner that was maximal (11.1 ± 2.2-fold) at 35 dynes/cm² (Fig. 1). Flow activated BMK1 within 10 min, and the maximal activation occurred at 60 min (7.1 ± 0.6-fold) (Fig. 2). Although H₂O₂ and sorbitol also stimulated BMK1 activity in BAEC, the magnitude of BMK1 activation by flow was significantly greater than by H₂O₂ or sorbitol (Fig. 3).

Shear Stress-induced BMK1 Activation Is Dependent on a Tyrosine Kinase Other than c-Src—Tyrosine phosphorylation of multiple proteins has been demonstrated in response to flow (14, 21). Tyrosine kinases have been shown to regulate both ERK1/2 and JNK activities in response to flow (22). We showed previously that BMK1 activation by H₂O₂ in smooth muscle cells and fibroblasts was regulated by herbimycin A, a broad specificity tyrosine kinase inhibitor (17). In a similar manner, BMK1 activation by flow was blocked by herbimycin A in a concentration-dependent manner (Fig. 4A), suggesting involvement of an herbimycin A-sensitive tyrosine kinase(s) in flow-induced BMK1 activation.
c-Src has been shown to be activated rapidly by flow in BAEC and HUVEC (14, 22). The flow-induced c-Src activation was blocked by herbimycin A, detected using both antibody clone 28 (a gift from Dr. J. Yano) (23), which recognizes the activated form of Src (Fig. 4B), and an in vitro kinase assay using enolase as a substrate (data not shown). In addition, we demonstrated previously that activation of BMK1 by H$_2$O$_2$ in fibroblasts is dependent on c-Src because BMK1 activation by H$_2$O$_2$ was diminished in Src$^{-/-}$ fibroblasts (17). Based on these observations, we anticipated that c-Src would be required for BMK1 activation by flow (17). PP1 is a Src family tyrosine kinase inhibitor (24) and has been shown to block H$_2$O$_2$-induced BMK1 activation, which is dependent on Src in smooth muscle cells and fibroblasts (17). To test the effect of PP1 on flow-induced BMK1 activation in EC, we pretreated EC with 10 or 50 $\mu$M PP1 for 15 min. PP1 failed to block BMK1 activation by flow, suggesting that flow-induced BMK1 activation is independent of c-Src in BAEC (Fig. 4C). As a control for PP1 uptake and Src inhibition, we showed that these concentrations of PP1 blocked flow-induced ERK1/2 activation (Fig. 4C) and p130$^{cas}$ tyrosine phosphorylation (data not shown), which are both dependent on c-Src (22). The effects of PP1 on in vivo Src activity stimulated by flow could not be evaluated by any available methods because PP1 is a competitive inhibitor of ATP for Src; thus, PP1 would not be present in sufficient concentrations to inhibit the enzyme after c-Src immunoprecipitation.4

To examine further the involvement of c-Src in BMK1 activation by flow, we overexpressed kinase-inactive, dominant-negative chicken Src in BAEC using a recombinant adenovirus vector (Ad.KI-Src). A recombinant adenovirus vector encoding $\beta$-galactosidase (Ad.LacZ) was used as a negative control. The transfection efficiency increased with increasing virus concentration; at a multiplicity of infection of 500 for Ad.LacZ, transfection efficiency was $>$80% measured 48 h after transfection by $\beta$-galactosidase staining. Infection with Ad.KI-Src increased total Src expression in a concentration-dependent manner from a multiplicity of infection of 250 to 500 as measured by Western blotting (Fig. 5). Inhibiting Src had no effect on BMK1 activity stimulated by flow (Fig. 5), consistent with the conclusion that c-Src is not involved in flow-stimulated BMK1 activation. As a positive control, we showed that Ad.KI-Src at a multiplicity of infection of 500 completely inhibited flow-stimulated p130$^{cas}$ tyrosine phosphorylation, which we have shown to be Src-dependent.3 These results indicate that a tyrosine kinase other than c-Src mediates BMK1 activation by flow.

**Effects of Ca$^{2+}$ on Shear Stress-induced BMK1 Activation—** Flow stimulates a rapid increase in intracellular Ca$^{2+}$ (25). To determine whether the flow-mediated increase in intracellular Ca$^{2+}$ is necessary for BMK1 activation, we used BAPTA/AM to chelate intracellular Ca$^{2+}$. BMK1 activation by flow was completely blocked by 30 $\mu$M BAPTA/AM and by 30 $\mu$M BAPTA/AM plus 2 mM EGTA (Fig. 6), suggesting that flow activates BMK1 through Ca$^{2+}$-dependent pathways in BAEC. Previous data suggest that flow stimulates both Ca$^{2+}$ influx into the cells and Ca$^{2+}$ release from internal stores (25). To determine which Ca$^{2+}$ source mediates BMK1 activation, we

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3 J. H. Hanke, personal communication.
Fig. 3. **Shear stress, H_2O_2, and osmotic stress stimulate BMK1.** Growth-arrested BAEC were exposed to flow (shear stress = 12 dynes/cm^2) for 20 min or were stimulated with various concentrations of H_2O_2 or sorbitol for 20 min. BMK1 kinase activity and protein were analyzed as described for Fig. 1. **Top panel,** representative autoradiogram showing BMK1 kinase activity; **bottom panel,** Western blot analysis showing BMK1 protein levels.

**Fig. 4.** **Effects of herbimycin A and PP1 on BMK1 activation by shear stress.** Growth-arrested BAEC were pretreated with 0.1% Me_2SO for 16 h (control) or the indicated concentrations of herbimycin A (HA) for 16 h (A and B) or PP1 for 15 min (C), followed by exposure to flow (shear stress = 12 dynes/cm^2) for 20 min. BMK1 kinase activity and protein were analyzed as described for Fig. 1. **Top panel** of A and C, representative autoradiograms showing BMK1 kinase activity; **bottom panel** of A and middle panel of C, Western blot analysis showing BMK1 protein levels; **bottom panel** of C, Western blot analysis showing ERK1/2 kinase activity with phospho-ERK1/2 antibody (New England BioLabs); **top panel** of B, Western blot analysis showing c-Src activities with antibody clone 28, which recognizes the active form of c-Src (23); **bottom panel** of B, Western blot analysis showing total c-Src protein levels.

Chelated extracellular Ca^{2+} by 2 mM EGTA or depleted internal Ca^{2+} stores by 10 μM thapsigargin, an inhibitor of endoplasmic Ca^{2+} pumps. Flow-induced BMK1 activation was completely blocked by thapsigargin, but not by EGTA alone (Fig. 6), suggesting that BMK1 activation is dependent on the mobilization of Ca^{2+} from internal stores. To determine whether an increase in intracellular Ca^{2+} is sufficient for BMK1 activation, we treated cells with the Ca^{2+} ionophore A23187. At A23187 concentrations known to increase intracellular Ca^{2+} to >1000 nM (1 and 10 μM), BMK1 activity was not stimulated (data not shown). These results suggest that increases in intracellular Ca^{2+} are necessary, but not sufficient, for BMK1 activation by flow.

**Effects of ROS and NO on Shear Stress-induced BMK1 Activation.—** Flow increases intracellular ROS, as evidenced by increases in oxidized proteins (21) and inhibition of aconitase activity.\(^5\) ROS production has been suggested to mediate mechanotransduction leading to MAP kinase activation in EC stimulated by flow (21). BMK1 is activated by flow and is also known to be activated by H_2O_2 (Fig. 3), suggesting that intracellular ROS generated by flow may mediate BMK1 activation in BAEC. To test this hypothesis, we utilized BSO (a selective inhibitor of γ-glutamylcysteine synthetase) and NAC (a well known precursor of glutathione synthesis) to change the levels of GSH, the intracellular reduced form of glutathione, which is the most important cellular non-protein-reducing thiol antioxidant (20). Treatment of cells with varying concentrations of NAC (3–30 mM) did not attenuate flow-induced BMK1 activation (Fig. 7A). In fact, a slight increase was observed (Fig. 7A). Treatment of cells with BSO (0.1–1 mM) also did not significantly modulate flow-induced BMK1 activation (Fig. 7B). However, as expected, exposure to BSO or NAC resulted in a dose-dependent depletion or accumulation of endothelial GSH, respectively (Fig. 7C), indicating that BSO and NAC were biologically active. The efficacy of treatment with BSO and NAC was shown by the findings that H_2O_2-induced BMK1 activation was altered by BSO and NAC in the anticipated manner, with a small enhancement by BSO and dramatic inhibition by NAC (Fig. 7D). We also showed that NAC treatment inhibited phenylmethylsulfonate-stimulated superoxide production in EC (data not shown), indicating that NAC treatment increased antioxidant defenses toward both superoxide and H_2O_2. Finally, we introduced superoxide dismutase into BAEC using liposome-encapsulated superoxide dismutase as described previously (26). However, there was no significant effect on BMK1 activation by flow (Table I). Taken together, these results indicate that flow-induced BMK1 activation is not mediated by ROS.

NO production is rapidly increased in response to increased shear stress (2, 27). To determine the role of NO in flow-induced BMK1 activation, two structurally independent nitric-oxide synthase inhibitors, N^G^-nitro-l-arginine and N^G^-monomethyl-l-arginine, were used to block NO production. Neither nitric-oxide synthase inhibitor had a significant effect on either basal or flow-stimulated BMK1 activity (Fig. 8, A and B). In addition, exogenous NO generated by four different NO donors

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5. A. Wu, C. Yan, and B. C. Berk, unpublished observations.
(diethylenetriamine-NO, S-nitroso-N-acetylpenicillamine, sodium nitroprusside, and 3-morpholinosydnonimine) had no significant effect on either basal or flow-stimulated BMK1 activity (Table I). These results suggest that NO production by EC is not required for flow-induced BMK1 activation.

**Effects of PKA, PKC, PKG, CaM Kinase, PI 3-Kinase, and Arachidonic Acid Metabolism on BMK1 Activation by Shear Stress**—To evaluate the role of other cell signaling molecules shown to be stimulated by flow in BMK1 activation, we studied PKA, PKC, PKG, CaM kinase, PI 3-kinase, and arachidonic acid metabolism using specific inhibitors or activators (Table I). When BAEC were pretreated with 1 μM phorbol 12,13-dibutyrate for 24 h to down-regulate PKC or with 1 μM Go6850 for 15 min to inhibit both Ca2+-dependent and Ca2+-independent PKC isozymes, there was no change in flow-induced BMK1 activation (Table I). We previously showed that PKC inhibition by these treatments significantly inhibited ERK1/2 activation (7, 11). Stimulation of PKA or PKG activity with 8-bromo-cAMP or 8-bromo-cGMP (100–1000 μM) for 30 min also did not significantly alter flow-induced BMK1 activation (Table I). Inhibition of CaM kinase activity by KN-62 (10 μM for 2 h) did not block flow-induced BMK1 activation (Table I). Pretreatment of BAEC with two different PI 3-kinase inhibitors, wortmannin (10–100 nM) and LY294002 (1–10 μM), for 30 min did not inhibit BMK1 activation by flow. In addition, there was no effect of inhibiting arachidonic acid metabolism by blocking cyclooxygenase with 10 μM indomethacin or lipoxygenase with 10 μM nordihydroguaiaretic acid for 30 min (Table I). These results suggest that flow activation of BMK1 is not mediated by

**Fig. 5.** Effects of overexpression of Ad.KI-Src on BMK1 activation by shear stress. BAEC at 70–80% confluence were infected with the indicated concentrations of Ad.KI-Src or Ad.LacZ. Two days after infection, cells were kept in a static state or exposed to flow (shear stress = 12 dynes/cm²) for 20 min. BMK1 kinase activity and protein were analyzed as described for Fig. 1. To confirm overexpression of Ad.KI-Src, cell lysates from the same samples were used for Western blot analysis with anti-c-Src antibody. Top panel, representative autoradiogram showing BMK1 kinase activity; middle panel, Western blot analysis showing total cellular c-Src protein levels; bottom panel, Western blot analysis showing total cellular c-Src protein levels. Values are from the densitometric quantification of relative c-Src expression (control + flow normalized to 1.0). MOI, multiplicity of infection.

**Fig. 6.** Effects of intracellular and extracellular Ca²⁺ chelation on BMK1 activation by shear stress. Extracellular calcium was chelated by adding EGTA (2 mM) in Ca²⁺-free flow buffer. To deplete intracellular calcium, growth-arrested BAEC were treated with BAPTA/AM (30 μM) for 30 min, BAPTA/AM (30 μM) plus EGTA (2 mM) for 30 min, or thapsigargin (1 μM) for 15 min prior to exposure to flow (shear stress = 12 dynes/cm²) for 20 min. BMK1 kinase activity and protein were analyzed as described for Fig. 1. Top panel, representative autoradiogram showing BMK1 kinase activity; bottom panel, Western blot analysis showing BMK1 protein levels.

**Fig. 7.** Effects of cellular redox state on BMK1 activation by shear stress. Growth-arrested BAEC were pretreated with the indicated concentrations of NAC for 45 min (A) or BSO for 16 h (B) and exposed to flow (shear stress = 12 dynes/cm²) for 20 min in the continual presence of NAC or BSO. BMK1 kinase activity and protein were analyzed as described for Fig. 1. Top panels, representative autoradiograms showing BMK1 kinase activity; bottom panels, Western blot analysis showing BMK1 protein levels. GSH levels were measured as described under “Experimental Procedures” (C). Quantification of BMK1 activity stimulated by 1 mM H₂O₂ for 20 min after pretreatment with the indicated concentrations of NAC for 1 h or BSO for 16 h was performed by densitometric analysis (D).
BMK1 Activation by Shear Stress

**TABLE I**

Summary of effects of various inhibitors and agonists on BMK1 activation by shear stress

| Drugs and agents | Effects on target molecules | BMK1 activity \(^a\) |
|------------------|-----------------------------|----------------------|
| Herbimycin A     | Inhibition of tyrosine kinase(s) | Decrease |
| PPI (10–50 μM)   | Inhibition of Src family kinases | No Δ |
| Ad-κI-Src        | Inhibition of c-Src | No Δ |
| Thapsigargin (1 μM) | Depletion of internal Ca\(^{2+}\) stores | Decrease |
| BAPTA/AM (30 μM) | Decrease in intracellular Ca\(^{2+}\) level | Decrease |
| EGTA (2 mM)      | Chelation of extracellular Ca\(^{2+}\) | No Δ |
| NAC (3–30 mM)    | Glutathione precursor | Slight increase |
| BSO (0.1–1 mM)   | Inhibition of γ-glutamylcysteine synthetase | No Δ |
| Liposome SOD\(^d\) | Increase in superoxide dismutase | No Δ |
| SIN-1 (100 μM)   | Generation of NO and O\(^{2-}\) | No Δ |
| SNAP (100 μM)    | Generation of NO | No Δ |
| SNP (100 μM)     | Generation of NO | No Δ |
| LNMMA (100–300 μM) | Inhibition of NOS | No Δ |
| LNA (10–300 μM)  | Inhibition of NOS | No Δ |
| PDBu (2 μM)      | Down-regulation of PKC | No Δ |
| Go69830 (2 μM)   | Inhibition of PKC | No Δ |
| 8-Br-cAMP (100 μM to 1 mM) | Activation of PKA | No Δ |
| 8-Br-cGMP (100 μM to 1 mM) | Activation of PKG | No Δ |
| KN-62 (10 μM)    | CaM kinases | No Δ |
| Wortmannin (10–100 μM) | Inhibition of PI 3-kinase | No Δ |
| LY294002 (1–10 μM) | Inhibition of PI 3-kinase | No Δ |
| Indomethacin (10 μM) | Inhibition of cyclooxygenase | No Δ |
| BSO (0.1–1 mM)   | Inhibition of lipooxygenase | No Δ |
| **TABLE I**

\(^a\) Shown are presumed mechanisms of action for the agents used.

\(^b\) Shown are the effects of drugs on flow-induced BMK1 activity.

\(^c\) No significant change observed.

\(^d\) SOD, superoxide dismutase; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; LNMMA, N\(^\text{\textsuperscript{\(O\)}}\)-monomethyl-L-arginine; LNA, N\(^\text{\textsuperscript{\(\text{\textpi}\)}}\)-nitro-L-arginine; PDBu, phorbol 12,13-dibutyrate; Br-, bromo-; NDGA, nordihydroguaiaretic acid.

**DISCUSSION**

The major finding of this study is that laminar shear stress stimulates BMK1 activity in endothelial cells. We found significant differences in the pathways responsible for activation of BMK1 compared with the closely related ERK1/2. First, although flow stimulated both BMK1 and ERK1/2, the time courses were markedly different. Flow-induced ERK1/2 activation was transient, reaching a maximum by 5–10 min and returning to base-line levels by 30 min (7, 10). In contrast, flow-stimulated BMK1 activation was delayed (onset at 10 min) and sustained (peak at 60 min). Second, the signal transduction events required for ERK1/2 and BMK1 activation by flow are different. It has been shown that c-Src is upstream of ERK1/2 activation in response to flow (22). In contrast, our results indicate that c-Src is not involved in flow-dependent activation of BMK1. Third, it has been proposed that ROS mediate ERK1/2 activation by flow (21), whereas no role for H\(_2\)O\(_2\) or superoxide in BMK1 activation was found in our study. Fourth, flow-stimulated BMK1 activation was dependent on intracellular Ca\(^{2+}\), which is not required for ERK1/2 activation (11). Finally, BMK1 activation by flow was not blocked by PKC down-regulation with phorbol 12,13-dibutyrate, whereas, ERK1/2 activation was inhibited by PKC down-regulation (11). Thus, BMK1 activation by flow defines a new signal transduction pathway in BAEC.

Activation of different MAP kinases by flow is likely to be important for selective regulation of gene expression in EC. The consensus DNA-binding site for the transcription factor activator protein 1 (AP-1) has been identified in negative and positive regulatory regions of many genes (28). As discussed below, ERK1/2 and BMK1 are likely candidates to mediate the increase in AP-1 activity observed in EC exposed to flow (29). ERK1/2 activation stimulates phosphorylation of the ternary complex factor Elk, which in turn increases c-Fos expression (28, 30). BMK1 has recently been reported to phosphorylate MEF2C, which in turn stimulates c-Jun expression (5), c-Fos and c-Jun, either as a Jun-Jun homodimer or as a Jun-Fos heterodimer, belong to the AP-1 family (28). Induction of c-Jun and c-Fos stimulates AP-1 activity (28, 30, 31). Flow increases c-Fos and c-Jun mRNA levels (32), which then stimulate AP-1 DNA binding activity (29). Activation of AP-1 activity is biphasic, rising 4-fold within 20 min and declining to near basal levels by 30 min before gradually increasing again (up to 30-fold) by 2 h (29). Flow-induced ERK1/2 activation (5–10 min) and BMK1 activation (20–60 min) precede the first phase (20 min) and the second phase (60–120 min) of AP-1 activation by flow. This temporal relationship suggests that ERK1/2 and BMK1 may be potential upstream regulators of AP-1 activity stimulated by flow.
Activation of a herbimycin A-sensitive tyrosine kinase other than c-Src was required for flow stimulation of BMK1. Recent studies from our laboratory (14) and others (21, 22) provide evidence that flow stimulates tyrosine phosphorylation of multiple proteins. It has been found that c-Src, a tyrosine kinase, was stimulated by flow within minutes (14, 22). In addition, c-Src appears to be upstream of the Ras-ERK1/2 pathway stimulated by flow in BAEC (22). Our previous studies showed that BMK1 activation by H$_2$O$_2$ was inhibited in fibroblasts prepared from transgenic mice lacking endogenous c-Src and that H$_2$O$_2$-induced BMK1 activation was blocked by the Src inhibitor PP1 (17), suggesting that c-Src is involved in BMK1 activation by H$_2$O$_2$ in fibroblasts. However, the present study indicates that BMK1 activation by flow is independent of c-Src. In fact, BMK1 activation by H$_2$O$_2$ in endothelial cells was also independent of c-Src because overexpression of kinase-inactive Src did not block H$_2$O$_2$-induced BMK1 activity (data not shown). This difference in c-Src involvement may be due to the presence of cell-specific signal transduction pathways (fibroblasts versus BAEC). A similar difference has been reported previously in that p130Cas activation by flow was c-Src-dependent in HUVEC, but not in fibroblasts. The nature of the non-Src herbimycin A-sensitive tyrosine kinase remains to be defined.

Another tyrosine kinase, focal adhesion kinase (FAK), has recently been shown to be activated by flow in BAEC (33). In addition, FAK activation was found to be necessary for the flow-induced activation of both ERK1/2 and JNK. Activation of FAK, ERK1/2, and JNK by flow was dependent upon actin microfilament integrity since pretreatment of cells with cytochalasin B attenuated the activation. However, we found that BMK1 activation by flow was not attenuated by disruption with cytochalasin D, suggesting that FAK is unlikely to be involved in flow-induced BMK1 activation. Thus, BMK1 activation in response to flow is via a signaling pathway separate from actin microfilament integrity.

We anticipated that BMK1 activation by flow would be mediated by ROS in EC because H$_2$O$_2$ stimulated BMK1 (Fig. 3) and flow increases ROS production in EC (21). In earlier studies, it has been shown that EC can generate significant amounts of superoxide (34, 35) and hydrogen peroxide (36) in response to various stimuli that activate PKC or raise Ca$^{2+}$ (15). However, exogenously added H$_2$O$_2$ changed intracellular Ca$^{2+}$ concentration in cultured EC (21, 38, 39). Laurindo et al. (15) demonstrated that increases in blood flow trigger free radical release both in vivo and in isolated perfused rabbit aortas. Potential sources of ROS production in EC include the enzymes of the mitochondrial electron transport chain, xanthine oxidase, cytochrome P-450, cyclooxygenase, lipooxygenase, and NAPDH oxidases. It is important to note that these enzymes are localized in different subcellular organelles and compartments. EC contain three major ROS detoxification systems, catalase, superoxide dismutase, and a glutathione redox cycle, which are also restricted to specific organelles. For example, catalase in EC is localized in peroxisomes (15). Therefore, local ROS production is dependent on both the ROS-generating enzymes and the status of antioxidant defense systems localized in a particular subcellular region. ROS generated by different stimuli therefore could be specifically compartmentalized and have different effects. This concept is supported by the observations that inhibition of the mitochondrial respiratory chain did not change intracellular H$_2$O$_2$ production near peroxisomes in normal EC or in EC in which glutathione reductase was inactivated (15). However, exogenously added H$_2$O$_2$ changed intracellular H$_2$O$_2$ production near peroxisomes only when the glutathione redox cycle was inactivated (15). These observations suggest that physiological effects produced by exogenously loaded ROS versus intracellularly generated ROS may be different. Our results indicate that exogenous H$_2$O$_2$ can activate BMK1, but that intracellular ROS generated by flow do not modulate BMK1 activity. These different results are best explained by the possibility that these two different sources of ROS target separate subcellular compartments.

In summary, our study demonstrates that shear stress potentially activates BMK1 activity in EC via a unique signaling pathway that is dependent on a tyrosine kinase other than c-Src and an increase in intracellular Ca$^{2+}$ concentration. Future studies to define the upstream and downstream molecular events involved in stress-stimulated BMK1 activation should enhance our understanding of the mechanisms by which shear stress regulates endothelial function.

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