Differential Effect of Shear Stress on Extracellular Signal-regulated Kinase and N-terminal Jun Kinase in Endothelial Cells

G12- AND Gβγ-DEPENDENT SIGNALING PATHWAYS

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Shear stress differentially regulates production of many vasoactive factors at the level of gene expression in endothelial cells that may be mediated by mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK) and N-terminal Jun kinase (JNK). Here we show, using bovine aortic endothelial cells (BAEC), that shear stress differentially regulates ERK and JNK by mechanisms involving Gβγ and pertussis toxin (PTx)-insensitive G-protein-dependent pathways, respectively. Shear activated ERK with a rapid, biphasic time course (maximum by 5 min and basal by 30-min shear exposure) and force dependence (minimum and maximum at 1 and 10 dyn/cm² shear stress, respectively). PTx treatment prevented shear-dependent activation of ERK1/2, consistent with a Gβγ-dependent mechanism. In contrast, JNK activity was maximally turned on by a threshold level of shear force (0.5 dyn/cm² or higher) with a much slower and prolonged time course (requiring at least 30 min to 4 h) than that of ERK. Also, PTx had no effect on shear-dependent activation of JNK. To further define the shear-sensitive ERK and JNK pathways, vectors expressing hemagglutinin epitope-tagged ERK (HA-ERK) or HA-JNK were cotransfected with other vectors by using adenovirus-polylysine in BAEC. Expression of the mutant αG52(G203), antisense Gα2 and a dominant negative Ras (N17Ras) prevented shear-dependent activation of HA-ERK, while that of αG52(G204) and antisense αG2 did not. Expression of a Gβγ-carboxyl terminator of β-adrenergic receptor kinase (βARK-et), and N17Ras inhibited shear-dependent activation of HA-JNK. Treatment of BAEC with genistein prevented shear-dependent activation of ERK and JNK, indicating the essential role of tyrosine kinase(s) in both ERK and JNK pathways. These results provide evidence that 1) Gβγ-protein, Ras, and tyrosine kinase(s) are upstream regulators of shear-dependent activation of ERK and 2) that shear-dependent activation of JNK is regulated by mechanisms involving Gβγ, Ras, and tyrosine kinase(s).

Endothelial cells lining the inner vessel wall are in direct contact with flowing blood, which generates a frictional force, hemodynamic shear stress, acting on the surface of the endothelium. Hemodynamic shear stress controls vascular tone, vessel wall remodeling, interaction of blood cells with endothelium, coagulation, and fibrinolysis (1). The focal pattern of atherosclerotic lesions in areas of low and/or unstable shear stress further highlights the importance of shear stress in the atherogenic process (2, 3). Endothelial cells play a key role in shear-dependent vascular changes, sensing shear stress by an unidentified mechanoceptor(s) followed by production of autocrine and paracrine factors (1). For example, hemodynamic shear stress selectively and differentially regulates production of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, platelet-derived growth factor-B, basic fibroblast growth factor, transforming growth factor β-1, tissue plasminogen activator, endothelial nitric oxide synthase, and endothelin at the level of gene expression (4–13). Resnick et al. (6) identified a conserved cis-acting shear stress response element with the 6-base pair core binding sequence within the 5’ promoter region of the platelet-derived growth factor-B chain. Interestingly, this shear stress response element has been identified in other shear-sensitive genes including intercellular adhesion molecule-1, tissue plasminogen activator, and transforming growth factor β-1, suggesting its role in shear-dependent regulation of these genes. Furthermore, NF-κB has been shown to bind to the shear stress response element (15). Shear stress induces biphasic expression of c-Fos, c-Jun, and c-Myc as well as activation of DNA binding activities of NF-κB and transcription factor activator protein 1 (AP-1) (14, 16). These immediate early response genes and transcription factors are likely to be involved in the regulation of shear-dependent gene expression.

Mitogen-activated protein kinase family members including ERK and JNK (also known as stress-activated protein kinase), have been proposed to be important signaling components linking extracellular stimuli to cellular responses including cellular growth, differentiation, and metabolic regulation (reviewed in Refs. 17 and 18). Mitogen-activated protein kinases can be activated by various external stimuli such as growth factors (nerve and epidermal growth factors), ligands acting on G-protein-coupled receptors (α2-adrenergic agonists and lysophosphatidic acid) and physical stresses (ultraviolet radiation and hyperosmolarity) (17, 18). Recently, shear stress also has

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The abbreviations used are: AP-1, transcription factor activator protein 1; βARK-et, carboxyl terminus of the β-adrenergic receptor kinase; BAEC, bovine aortic endothelial cells; ERK, extracellular signal-regulated kinase; pERK, phospho-ERK; GST-c-Jun, c-Jun (amino acids 5–89) fused to glutathione S-transferase; JNK, N-terminal Jun kinase; MBP, myelin basic protein; GDPβS, guany1-5’-yl thio phosphate; DME M, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; HA, hemagglutinin; AdpL, adenovirus-polylysine; PAGE, polyacrylamide gel electrophoresis; PTx, pertussis toxin; CTx, cholera toxin.

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been shown to stimulate ERK in endothelial cells (19, 20). Tseng et al. (19) showed that GDP/S treatment and inhibition of protein kinase C blocked shear-dependent ERK activation in fetal BAEC.

In this study we examined the effect of shear stress on JNK and ERK in adult BAEC. Shear stress activated ERK and JNK with markedly different time courses and force dependence. Contrary to the previous report (19), shear-stimulated activation of ERK is regulated by mechanisms involving a PTX-sensitive α2g, Ras, and a tyrosine kinase(s). On the other hand, shear-dependent activation of JNK is not PTX-sensitive but is regulated by Gαγ, Ras, and pathways dependent on tyrosine kinase(s).

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** BAEC harvested from descending thoracic aortas were maintained (37 °C, 5% CO2) in a growth medium (DMEM (1 g/liter glucose; Life Technologies, Inc.) containing 20% fetal calf serum (FCS; Atlanta Biologicals) without antibiotics) (21). Cells used in this study were between passages 5 and 10. For shear experiments, 1 million cells/glass slide (75 × 38 mm; Fisher) were seeded in growth medium. The next day, the medium was changed to a starvation medium (phenol red-free DMEM containing 0.5% FCS with 25 mM HEPES) and incubated for 1–2 days. Where indicated, PTx (0.1 μg/ml; List Biologicals) was added 18 h prior to shear exposure.

**Plasminogen, Adenovirus, and Transfection—** Antisense α2g, and α2b vectors were prepared by using the EcoRI fragments of α2g, and α2b (kind gift of Dr. R. Reed (Johns Hopkins University)) and EcoRI-cut pcDNA3 (Invitrogen) as described (22). Two G2a mutants containing point mutations at Gly383→Thr (α2g; G203) and Gly386→Ala (α2b; G204) inserted into pCWO were kind gift of Dr. G. L. Johnson (University of Colorado) (23). Vectors expressing N17Ras and βARK-ct fused to CD8 in pcDNA3 (24) and HA-ERK2 and HA-JNK1 in pSRa (25) were kindly provided by Dr. S. Gutkind (National Institute of Dental Research) and Dr. A. Lin (University of Alabama at Birmingham), respectively. Endotoxin-free DNAs used in all transfection experiments were prepared by using a maxiprep kit following the manufacturer's instructions (Quiggen).

For transfection studies, BAEC (2.5 × 105 cells/glass plate) were grown overnight in the growth medium and washed in Hanks' buffered salt solution just prior to transfection using the method of adenovirus conjugated to polylysine (AdpL) as described (26). Briefly, a replication-defective adenovirus d11014 (kind gift of Dr. R. Reed (Johns Hopkins University)) was cross-linked to polylysine (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl carbodiimide-HCl), and small aliquots (1 × 105 particles/ml) were stored at −80 °C (26). AdpL was conjugated to DNA on the day of transfection by incubating 100 μl of HEPES-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.3), 50 μl of AdpL, and 2 μg of plasmid DNA per glass plate for 30 min at room temperature followed by an additional 30-min incubation at room temperature with 2 μg of polylysine in 100 μl of HEPES-buffered saline. AdpL-DNA conjugate mixed with 2 ml of DMEM containing 0.5% FCS was added to the cells. One hour later, 10 ml of DMEM containing 10% FCS was added to the cells and incubated overnight. The next day, medium was changed to fresh DMEM containing 10% FCS, incubated for 24 h, and serum-starved overnight in the starvation medium. Under identical conditions, the transfection efficiency of β-galactosidase DNA in pcMV (ATCC) ranged from 25 to 40% (data not shown) as determined by a 5-bromo-4-chloro-3-indoyl β-D-galactoside staining method in 4% paraformaldehyde fixed monolayers (27).

**Shear Stress Studies—** The glass slide containing a BAEC monolayer was assembled into a parallel plate shear chamber forming a flow channel (220-μm height × 2.5-cm width × 6.2-cm length) between the monolayer and fabricated polycarbonate plate as described (28). Nonpulsatile, laminar shear stress was controlled by changing the flow rate of the starvation medium delivered to the cells using the constant head flow loop or a syringe pump (KD Scientific) as described (21, 28).

**Preparation of Cytosol and Soluble Lysates—** Following shear exposure, cells were washed in ice-cold phosphate-buffered saline and resuspended in a 0.5-ml extraction buffer (50 mM Hepes, pH 7.53, 1.5 mM EDTA, 0.1 mM vanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) and immediately frozen at −80 °C until cell fractionation. Cell extracts were sonicated and centrifuged for 1,000 × g for 10 min, and the supernatants were further centrifuged at 20,000 × g for 30 min to separate crude cytosolic fractions (cytosol). For preparation of detergent-soluble lysates, shear-exposed cells were scraped in the extraction buffer containing 1% Triton X-100, solubilized for 1 h, and centrifuged at 20,000 × g for 30 min. The entire fractionation and solubilization procedures were performed at 4 °C. Protein content of each sample was measured by using a Bio-Rad DC assay kit.

**In Vitro Kinase Assays—** Soluble lysates were resolved on 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore) and probed with antibodies specific to phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY), ERK1/2 (Upstate Biotechnology), or phosphorylated forms of ERK1/2 (Ref. 29; New England Biolabs). Goat anti-mouse (or rabbit) IgG conjugated to alkaline phosphatase was used as secondary antibody and developed either colorimetrically using a Bio-Rad kit or by a chemiluminescent detection method as suggested by the manufacturer (New England Biolabs).

**Mitogen-activated Protein Kinase Assays—** Cytosol or soluble lysates (5 μg each) were used to phosphorylate myelin basic protein (MBP) (Sigma) and e-Jun (amino acids 5–89) fused to glutathione S-transferase (GST-e-Jun) (30). Briefly, each substrate was phosphorylated in buffer A (50 mM β-glycerophosphate, pH 7.33, 2.5 mM EGTA, 0.1 mM vanadate, 20 mM MgCl2, 1 mM dithiothreitol, 70 μM ATP, and 2,000 cpm/pmol [γ-32P]ATP) for 5 min at 30 °C. MBP phosphorylation was stopped either by spotting aliquots to P-81 filter papers followed by washing and counting (filter assay) as described (31) or by boiling in Laemmli sample buffer followed by 12.5% SDS-PAGE, Coomassie staining, and autoradiography. Phosphorylated bands were cut and counted in a scintillation counter. For “in-gel kinase” assays, cytosols (20 μg) were resolved in a 12.5% SDS-PAGE minigel containing immobilized MBP (0.4 mg/ml) followed by phosphorylation of renatured kinases and autoradiography as described (31). For immunocomplex assays, antibodies specific for ERK1/2 (Upstate Biotechnology, catalog number 06-182), JNK1 (Pharmingen, clone G203–333), and HA (Boehringer Mannheim) were incubated with the soluble lysates (50–100 μg) for 1 h at 4 °C, followed by addition of Protein A-agarose (for ERK and HA antibodies) or Protein G-agarose (for JNK antibody) beads. The immunocomplex was washed four times in the extraction buffer containing 1% Triton X-100 and twice in buffer C (20 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophosphatase, 0.1 mM vanadate, and 2 mM dithiothreitol). The washed immunocomplexes were incubated in 20 μl of buffer C containing either MBP or GST-e-Jun (5 μg each) and 50 μCi of [γ-32P]ATP for 20 min at 30 °C followed by SDS-PAGE, autoradiography, and quantitation of radioactivity incorporated into each band in a scintillation counter.

**RESULTS**

**Shear Stress Stimulates ERK Activity—** Immunoblot studies of BAEC lysates with a phosphotyrosine antibody showed that exposure of cells to shear stress (0, 5, 10, and 20 dyn/cm2 for 5 min each) increased tyrosine phosphorylation of 42–44-kDa bands (p44/p42) in a shear intensity-dependent manner (Fig. 1A). Additional immunoblot studies showed that an ERK antibody (Upstate Biotechnology) recognized ERK1 (p44) and ERK2 (p42) in both control and shear-stimulated BAEC (data not shown).

To test directly whether shear stress stimulated ERK activity, cytosols obtained from BAEC exposed to shear stress were used in an *in vitro* phosphorylation assay using MBP as a substrate (filter assay; Fig. 1B). Exposure of BAEC to shear stress (5–20 dyn/cm2) for 5 min stimulated ERK activity by 2–3-fold (Fig. 1B). The minimum shear intensity required to stimulate ERK was 1 dyn/cm2. At 10 dyn/cm2 of shear intensity, MBP phosphorylation activity reached a maximal level and did not change further when shear was increased to 20 dyn/cm2. To determine the molecular weight and specificity of the shear-stimulated MBP kinase activity (Fig. 1B), an in-gel kinase assay using immobilized MBP was performed. As shown in Fig. 1C, the electrophoretic mobilities of the shear-stress-dependent MBP kinase activities were 44/42 kDa, which are consistent with the molecular masses of ERK1 and ERK2, respectively. The stimulation of p44/p42 phosphorylation activity
Fig. 1. Shear stress stimulates ERK activity. Cytosol or cell lysates prepared from BAEC exposed to shear stress were used to determine the activation of ERK. A, phosphotyrosine blot. Lysates from BAEC exposed to shear stress (0, 0.5, 1, 2, 5, 10, and 20 dyn/cm²) for 5 min were probed with a phosphotyrosine antibody. Shear-sensitive phosphotyrosyl proteins of p44/42 are marked. Shown is a representative result of four independent studies. B, filter assay. Cytosols obtained from cells exposed to shear for 5 min were used for phosphorylation of MBP that was quantitated by a filter assay. Results are expressed as percentage of control (no shear group). Each phosphorylation assay was performed in triplicate, and mean values obtained from two to four independent shear studies are shown. Where shown, the error bars indicate S.E. C, in-gel kinase assay. Cytosols obtained from shear-exposed (5 dyn/cm² for 0, 5, or 10 min) cells were separated on a gel containing immobilized MBP, and an in-gel kinase assay to phosphorylate the immobilized MBP was performed. Shear-sensitive p44/42 and shear-insensitive p66 are marked in an autoradiogram representative of four independent studies. The autoradiogram was quantitated by densitometry (OD × area), which was 9 (0 min), 69 (5 min), and 56 (10 min), respectively, D, immunocomplex assay. ERK1/2 was immunoprecipitated from the lysates obtained from shear-exposed (10 dyn/cm² for 0 or 10 min) BAEC by an ERK antibody. Immunoprecipitates were used to phosphorylate MBP, electrophoresed, and autoradiographed. MBP is marked in a representative autoradiogram of four independent studies.

Fig. 2. Shear stress stimulates ERK and JNK with different time courses. Cytosols obtained from BAEC exposed to shear stress (5 dyn/cm² for 0, 0.5, 1, 2, 5, 10, 30, and 60 min) were used to phosphorylate MBP (for the ERK assay) and GST-c-Jun (for the JNK assay). Phosphorylated proteins in both assays were separated by 12.5% SDS-PAGE followed by autoradiography. Representative autoradiograms are shown in the inset. The radioactivity of phosphorylated bands was quantitated by cutting and counting each band. The line graphs show mean ± S.E. of results obtained from at least three independent studies.

Shear Stress Stimulates JNK1 in a Time- and Threshold Force-dependent Manner—Shear force- and time-dependent activation of JNK was further characterized (Fig. 3). When exposed for 30 min to increasing intensities of shear stress (0–20 dyn/cm²), maximum stimulation of JNK activity occurred at the lowest shear force tested (0.5 dyn/cm²) in BAEC (Fig. 3, A and B). In contrast to the force-dependent activation of ERK (Fig. 1B), this result suggests that JNK is regulated by an “on or off” mechanism in response to the sustained increase in shear force. Shear-dependent activation of JNK was detectable after 30 min, reached a maximum by 4 h, and returned to basal level after 17 h of shear onset using 5 dyn/cm² (Fig. 3, C and D).

PTX Inhibits Shear-dependent Activation of ERK but Not JNK—Previous studies have shown that heterotrimeric G proteins regulate both ERK and JNK pathways in other cell types. Furthermore, G-proteins have been shown to mediate many shear-dependent signaling pathways in endothelial cells by PTX-sensitive and -insensitive pathways (8, 28, 32, 33). Therefore, we tested whether G-proteins regulate shear-stimulated ERK and/or JNK activation using PTX.

Treatment with PTX completely prevented shear-dependent phosphorylation of ERK1/2 stimulated by 2-, 5-, and 10-min shear exposure (Fig. 4, B and D, PTX groups). Essentially the same inhibitory effect of PTX on shear-dependent activation of ERK was also shown by in vitro MBP phosphorylation assay of cytosols (25 ± 7% of control, n = 6, p < 0.0001, 5 or 10 dyn/cm² of shear stress for 5 min, data not shown). On the other hand, cholera toxin (CTx), an activator of α₁G, had no effect on shear-dependent activation of ERK (Fig. 4, B and D, CTx groups). PTX treatment of BAEC using the conditions employed here resulted in maximal ADP-ribosylation of G₁proteins as evidenced by complete inhibition of in vitro PTX-catalyzed [³²P]ADP-ribosylation (Fig. 4C). The rapid and transient changes in the phosphorylation status of ERK1/2 were not due to changes in the amount of these enzymes as shown in Fig. 4A.

Unlike ERK, PTX had no significant effect (p > 0.18, n = 3 or 4) on shear-stimulated JNK1 activation at any time points examined in this study (Fig. 5). CTX treatment also had no significant effect on JNK1 activation by 30 and 60 min of shear (Fig. 5). However, at 5 min of shear. Ctx stimulated JNK activation of from 89.8 ± 17% of control (n = 4, no toxin group) to 197.7 ± 50% of control (n = 3, cholera toxin group, p = 0.02) (Fig. 5, A and B).

α₁G and Ras Regulate Shear-dependent ERK Activation—To determine the identity of the PTX substrate and the role of Ras
regulating shear-sensitive ERK, BAEC were co-transfected with HA-ERK and \( \alpha_i \) constructs or N17Ras with the AdpL method. Transfection of BAEC by this method did not have any significant effect on shear-dependent activation of endogenous ERK and JNK (data not shown). Shear stress was able to stimulate the activity of HA-ERK by 3–4-fold in the co-transfected BAEC (see pcDNA3 groups, Fig. 6, A and B). Expression of \( \alpha_{3i}(G203) \) and antisense \( \alpha_{3i} \) genes did not inhibit shear-stimulated phosphorylation of ERK (\( \alpha_{3i} \) construct, Fig. 6 B) suggesting the involvement of Ras as an upstream regulator.

**Gβ/γ and Ras Regulate Shear-dependent JNK Activation**—Previous studies have shown that JNK can be regulated by heterotrimeric G-proteins, \( \alpha_{12}, \alpha_{13}, \alpha_i \), or Gβ/γ, in a Ras- and Rac-dependent pathway in other cell types (24, 34, 35). Since dominant negative mutants of \( \alpha_{12}, \alpha_{13}, \alpha_i \) and Ras Regulate Shear-dependent JNK Activation have not been available, we decided to block Gβ/γ to test the role of heterotrimeric G-proteins and to use N17Ras in co-transfection studies with HA-JNK. Expression of \( \beta\)ARK-ct inhibits shear-dependent activation of JNK by 60% compared with the HA-JNK/pcDNA3 control (Fig. 7) suggesting the involvement of heterotrimeric G-proteins as one of its upstream regulators. Furthermore, expression of N17Ras completely prevented shear-dependent activation of JNK, showing its Ras dependence (Fig. 7).

**Tyrosine Kinase(s) Regulate Shear-dependent Activation of ERK and JNK**—Tyrosine kinases have been shown to regulate shear-dependent activation of both ERK and JNK (36, 37). Therefore, we pretreated BAEC with genistein to inhibit tyrosine kinases. Genistein treatment completely blocked shear-dependent activation of both ERK and JNK, indicating tyrosine kinase involvement in both pathways.

**DISCUSSION**

Shear stress regulates vessel wall function and structure by the mechanisms that include expression of multiple genes in endothelial cells (4–13). Focal development patterns of atherosclerotic lesions in the areas of low and/or disturbed shear
stress support the importance of shear stress in the pathogenesis of atherosclerosis (2, 3).

In this study we demonstrated the differential mechanisms regulating the activities of ERK and JNK in endothelial cells in response to shear stress. ERK activation was rapid and biphasic (peaks at 5 min and returns to basal by 30 min of shear) and required higher shear force (insensitive to 0.5 dyn/cm² and maximum by 10 dyn/cm²) compared with JNK activation (see Figs. 1B and 3A). In comparison, JNK activation required longer shear exposure (30 min) and showed a slower time course (peaks at 4 h and returns to basal at 17 h) (Fig. 3C) than ERK activation. Moreover, JNK activity was activated to the maximal level by shear force as low as 0.5 dyn/cm². This study also showed that PTx prevents shear-activated ERK, but not JNK. Further studies identified αi2, Ras, and a tyrosine kinase(s) as upstream regulators of shear-dependent ERK activity. We also showed evidence supporting the role of Gβ/γ, Ras, and a tyrosine kinase(s) in shear-dependent activation of JNK. These results indicate that arterial endothelial cells, which are in direct contact with shear stress, respond to this physiologic mechanical stimulus through differential G-protein-dependent mechanisms leading to activation of ERK and JNK.

Activation of ERK and JNK in endothelial cells by shear stress may result in the selective phosphorylation and activation of transcription factors leading to selective gene regulation events. ERK activation has been reported to result in phosphorylation of ternary complex factor/Etk, which in turn triggers induction of c-Fos and subsequent stimulation of AP-1 activity (18, 38, 39). Activated JNK has been shown to phosphorylate c-Jun, which in turn induces its own gene expression and subsequent increase in AP-1 activity (18, 31, 25, 40–43). In endothelial cells, shear stress has been shown to induce biphasic increases in mRNA levels of c-Fos and c-Jun (14), which could have resulted in increased DNA binding activities of AP-1 (16). Interestingly, shear-dependent activation of ERK (5 min) and JNK (30 min to 4 h) precedes the first (20 min) and second phase (60–90 min of shear) of the AP-1 activation, respectively (Figs. 2 and 3 and Ref. 16). Although not directly proven, this temporal relationship suggests possible roles of ERK and JNK as upstream regulators of AP-1 activity.

Our study strongly indicates that shear-dependent activation of ERK and JNK is regulated by signaling pathways involving two different heterotrimeric G-proteins (most likely to be Gi-protein for ERK and αi or αi12/αi13 for JNK). Several lines of evidence support our conclusion. First, the effect of PTx on shear-dependent ERK activation (Fig. 4) points to αi2 and/or αi3 as its regulator, since BAEC are known to express these two αi-proteins but not αi1 and αi4 (44). Second, co-transfection studies with αi mutants and antisense αi constructs further demonstrate the specific effect of αi3 as an activator of ERK (Fig. 6). Mutations of Gly residues within the invariant sequence (Asp-Val-Gly203-Gly204-Gln) of all Go-subunits have been shown to produce dominant negative or null phenotypes, when expressed in cells, depending upon cell types and effector systems studied (45, 46, 28). In our study, only αi2 (G203) inhibited shear-dependent ERK activation while αi3 (G204) was without any effect. This specific effect of αi2 (G203) is not likely due to a simple scavenging of Gβ/γ, since both αi2 (G203) and αi3 (G204) mutants retain the ability to bind Gβ/γ, although they lose guanine nucleotide binding capabilities (45). Winitz et al. (23) showed that the expression of αi2 (G203), but not αi3 (G204), inhibits cPLA2 and speculated that Gly203 mutation may interfere with the conformational change required for its interaction with a specific effector. Similar mutations of αi12 corresponding to Gly203 and Gly204 also resulted in a dominant negative and a null phenotype, respectively, in vivo (46). The specific effect of antisense αi2 but not antisense αi3 provides further support for the essential role of αi2 in shear-dependent activation of ERK. Third, the inhibitory effect of βARK-ct, a Gβ/γ scavenger (47), on shear-dependent JNK activation suggests the role of heterotrimeric G-proteins in its pathway. Many examples of G-protein-dependent activation of ERK and JNK have been documented in other cell types. For example, ERK can be activated by Gi-coupled receptor agonists, presumably by stimulating the release of Gβγ-subunits of Gi-proteins and subsequent activation of Ras-dependent or -independent cascades (47–52). Gβ/γ and constitutively active mutants of αi2, αi12, and αi13 have been shown to activate JNK through Ras and Ras-c1-dependent pathways (24, 34). Since PTx and CTx had no significant effect on shear-dependent activation of JNK (Fig. 5), αi2/αi13 and αi3 classes of G-proteins are the possible candidates. Last, if ERK and JNK were stimulated by the same
**G-protein, PTx should have blocked shear stimulation of both ERK and JNK, and we found this was not the case (Fig. 4 and 5).**

Tyrosine kinases, such as Pyk2 and Src-related kinases, have been shown to be essential intermediates providing links between G-proteins and signaling pathways of ERK and JNK (36, 37). In our system, genistein blocked shear stimulation of ERK and JNK (Fig. 8), although the identity of the tyrosine kinase(s) remains to be determined. A recent study by Ishida et al. (53) provides further evidence that shear stress increases tyrosine phosphorylation of many as yet unidentified molecules. Identifying the tyrosine kinase(s) will greatly enhance the understanding of molecular events occurring in response to shear. One of the potential downstream effector molecules that can be activated by tyrosine kinases includes Shc leading to the recruitment and activation of the Grb2, mSOS, and Ras pathway (37). This pathway may be important in shear stress signaling, since we showed that Ras plays an essential role in shear-dependent activation of ERK and JNK (Figs. 6 and 7). Although we have not addressed the exact sequence of events, shear-dependent activations of ERK and JNK do involve similar signaling components including heterotrimeric G-proteins and tyrosine kinase(s) acting on Ras-dependent pathways as described by previous investigators in other G-protein-dependent ERK and JNK pathways (36, 37, 47–52).

Endothelial cells are believed to possess a flow-sensing system(s), mechanoreceptor(s) (1). Based on the evidence provided in the present study, one possibility is that there is one mechanoreceptor that can activate two different classes of G-proteins leading to ERK and JNK pathways. Another alternative is that shear stress may be mediated by two different mechanoreceptors, one coupled to Gq-proteins and the other coupled to non-Gq-proteins directly or indirectly.

Recently, shear-dependent activation of ERK has been reported (19, 20). Tseng et al. (19) also reported that GDPβS completely inhibits shear stress stimulation of ERK activity in BAEC. While their results of shear-dependent activation of ERK are in general agreement with ours, these authors concluded that shear-dependent ERK activation was not inhibited by PTX treatment of fetal BAEC (12 dyn/cm²) for 5 min). This discrepancy of PTX sensitivity may be due to the different cell types used (fetal versus adult BAEC). Other possibilities include subtle differences in cell culture and starvation conditions, and use of DMEM containing 0.5% FCS for 1–2 days in our studies versus Hanks' buffered salt solution for an unspecified length of time (19). A similar difference has been reported previously that shear-dependent production of NO/cGMP was PTX-sensitive in BAEC but not in human umbilical endothelial cells (28, 32).

**What is the physiological and pathological importance of the shear-dependent activation of ERK and JNK?** The remarkably different shear time- and force-dependent responses of ERK and JNK suggest two possibilities that could occur in vivo. First, a relatively large change of shear stress (amplitude) even for a few minutes may be required to activate ERK. Second, even a very small change in shear stress level could activate JNK, however, if the change is sustained for longer than 30 min (duration). ERK regulates cellular growth and metabolic responses in response to many growth factors and other stimuli acting on G-protein-coupled receptors (17, 18). However, the role of JNK is less well characterized. JNK can be activated by growth factors, cellular stresses, and G-protein-coupled receptors, which can induce growth, as well as apoptotic responses, probably depending upon cell environment and types (25, 54, 55). It remains to be determined whether ERK and JNK pathways are directly responsible for the apparent beneficial effect of shear stress on vessel wall, focal patterns of atherosclerotic lesion development, and vascular remodeling (1). Unlike arterial endothelial cells, which are constantly exposed to shear stress, endothelial cells used in our studies were grown in static conditions and exposed to shear stress only during shear experiments ("static-to-shear" in vitro model). Therefore, extrapolation of our results to in vivo situations needs to be confirmed in vivo. This kind of "static-to-shear" in vitro system has been extremely valuable to demonstrate specific phenomena and signaling pathways induced by shear stress that are potentially proatherogenic as well as proatherogenic responses (1). It is likely that coordinated responses in ERK and JNK may lead to differential expression of multiple shear-sensitive genes. Dysregulation or loss of "check and balance" between ERK and JNK may contribute to the pathogenesis of atherosclerosis.

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