BASIC RESEARCH STUDIES

Endothelial cell response to different mechanical forces

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Purpose: Endothelial cells (ECs) are subjected to the physical forces induced by blood flow. The aim of this study was to directly compare the EC signaling pathway in response to cyclic strain and shear stress in cultured bovine aortic ECs.
Materials and Methods: The ECs were seeded on flexible collagen I–coated silicone membranes to examine the effect of cyclic strain. The membranes were deformed with a 150–mm Hg vacuum at a rate of 60 cycle/min for up to 120 minutes. For a comparison of the effect of shear stress, ECs from the same batch as used in the strain experiments were seeded on collagen I–coated silicone sheets. The ECs were then subjected to 10 dyne/cm^2 shear with the use of a parallel flow chamber for up to 120 minutes. Activation of the mitogen-activated protein kinases was assessed by determining phosphorylation of extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 with immunoblotting.
Results: ERK, JNK, and p38 were activated by both cyclic strain and shear stress. Both cyclic strain and shear stress activated JNK with a similar temporal pattern and magnitude and a peak at 30 minutes. However, shear stress induced a more robust and rapid activation of ERK and p38, compared with cyclic strain.
Conclusions: Our results indicate that different mechanical forces induced differential activation of mitogen-activated protein kinases. This suggests that there may be different mechanoreceptors in ECs to detect the different forces or alternative coupling pathways from a single receptor. (J Vasc Surg 2000;32:789-94.)

Vascular endothelial cells (ECs) line the luminal surface of blood vessels and are exposed to the forces of the circulation, such as fluid shear stress, circumferential distention, and blood pressure. These mechanical forces are now well recognized as playing important roles in regulating EC function and vascular

remodeling.[1] For example, systemic and local abnormalities of mechanical forces have been implicated in a variety of pathologic events such as atherosclerosis and intimal hyperplasia.[2] The mechanical forces of the circulation are also thought to be involved in the arterialization and the development of anastomotic intimal hyperplasia of vein grafts.[3,4] Studies in which cultured cells and ex vivo perfusion devices are used have documented that physical forces are capable of inducing alterations in EC phenotype and EC expression and production of a variety of substances implicated in vessel growth and coagulation.[5-7] However, the mechanism by which vascular cells sense and respond to external force is not clearly known.

The mitogen-activated protein (MAP) kinase family is a class of signal transduction proteins that are capable of transmitting extracellular signals to cytoplasmic and nuclear pathways. Our laboratory has recently demonstrated that cyclic strain activated several members of the MAP kinase family including

extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 in cultured bovine aortic and pulmonary ECs.[8,9] Other laboratories have also reported that fluid shear stress activates ERK and JNK in cultured ECs.[10-12] However, a fundamental issue is whether ECs detect and respond to the different mechanical forces by the same mechanoreceptors and/or signaling pathways. Comparison of the responses by merely reviewing the reports in the literature is inadequate, because there is no uniformity in cell type, passage levels, culture conditions, or even analytic methods.

The aim of this study was to directly compare the signaling pathway in response to shear stress and cyclic strain by studying identical cells and using the same analytic methods. We show here that the magnitude and time course of MAP kinase activation induced by shear stress were different from those by cyclic strain, suggesting that the mechanoreceptors and/or transduction pathways involved with shear stress might not be the same as those involved with cyclic strain.

MATERIALS AND METHODS

Culture of bovine aortic ECs. Bovine aortic ECs were obtained by scraping the intimal surface of aorta obtained from freshly killed calves at a local slaughterhouse. Cells were maintained in Dulbecco modified Eagle medium F-12 (GIBCO BRL, Gaithersburg, Md), which was supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah), 5 µg/mL deoxycytidine/thymidine (Sigma Chemical, St Louis, Mo), and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 250 ng/mL, [GIBCO BRL]). The cells were grown to confluence at 37°C in a humidified incubator with 5% carbon dioxide. Bovine aortic ECs were identified by their typical cobblestone appearance and indirect immunofluorescence staining for factor VIII antigen. Cells used in this study were between passages 3 and 6. The ECs were synchronized before experimentation by incubating in media containing 0.1% fetal calf serum for 2 days and in serum-free media for 12 hours.

Experimental design. The ECs ($40,000/cm^2$) were seeded on flexible silicone membrane coated with collagen I (Flexcell International Corp, McKeesport, Pa) and synchronized as above. Cyclic strain was applied using a Flexercell Strain Unit (Flexcell FX-2000; Flexcell International Corp), which consists of a vacuum manifold with recessed ports controlled by computer program. Cyclic strain was applied by deforming the membrane with 150 mm Hg of vacuum, which produced an average strain of 10% at a frequency of 60 cycle/min for up to 120

minutes.[13] The ECs grown on stretch plates but that were not applied cyclic strain served as static control.

The ECs from the same batch as used in the stretch experiment were seeded on collagen I–coated silicone sheets at the same density ($40,000/cm^2$). The silicone sheeting (SF Medical, Hudson, Mass) was bonded on 75×38-mm glass slides (Fisher Scientific, Pittsburgh, Pa). After confluence was attained, ECs on the collagen I–coated silicone sheeting were synchronized as above. The ECs were then subjected to a 10 $dyne/cm^2$ shear using a parallel flow chamber (CytoDyne, San Diego, Calif) for up to 120 minutes.[14] The ECs grown on the glass slides but that were not subjected to shear stress served as static control. In a preliminary study, we compared the activities of MAP kinases of the static control at the beginning of experimentation (0 minutes) and static control at the end of experimentation (120 minutes). There were no significant differences among these static controls, which ensure that MAP kinases were not activated with time. At least three separate experiments were performed with ECs harvested from different calves.

Immunoblotting. Activation of the MAP kinases was assessed by determining phosphorylation of ERK, JNK, and p38 with immunoblotting using phospho-specific antibodies as previously described.[8] In brief, after exposure to the mechanical forces, cells were washed in ice-cold Hanks' Balanced Salt Solution (Sigma Chemical) twice and scraped in lysis buffer containing 50 mmol/L HEPES, pH 7.4, 150 mmol/L sodium chloride, 10% glycerol, 1% Triton X-100, 1.5 mmol/L sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Cell extracts were sonicated and centrifuged at 15,000g for 10 minutes, and supernatants were collected. Protein content was measured by BioRad protein assay system (BioRad, Hercules, Calif). Laemmeli sample buffer was added to equal amounts of each sample and boiled for 5 minutes. Samples were resolved on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham, Arlington Heights, Ill). The gels were routinely stained with Gelcode Blue to ensure equal loading of proteins. The membrane was probed with primary antibodies and horseradish peroxidase–conjugated secondary antibody (New England Biolabs, Beverly, Mass) with washing as suggested by the manufacturer. Immunodetection was carried out by chemiluminescence (Amersham) and quantitated with a phosphoimager densitometer (Molecular Dynamics, Sunnyvale, Calif). Antiactive MAP kinase antibody (Promega, Madison, Wis), phospho-specific p38 MAP kinase antibody (New England Biolabs), and
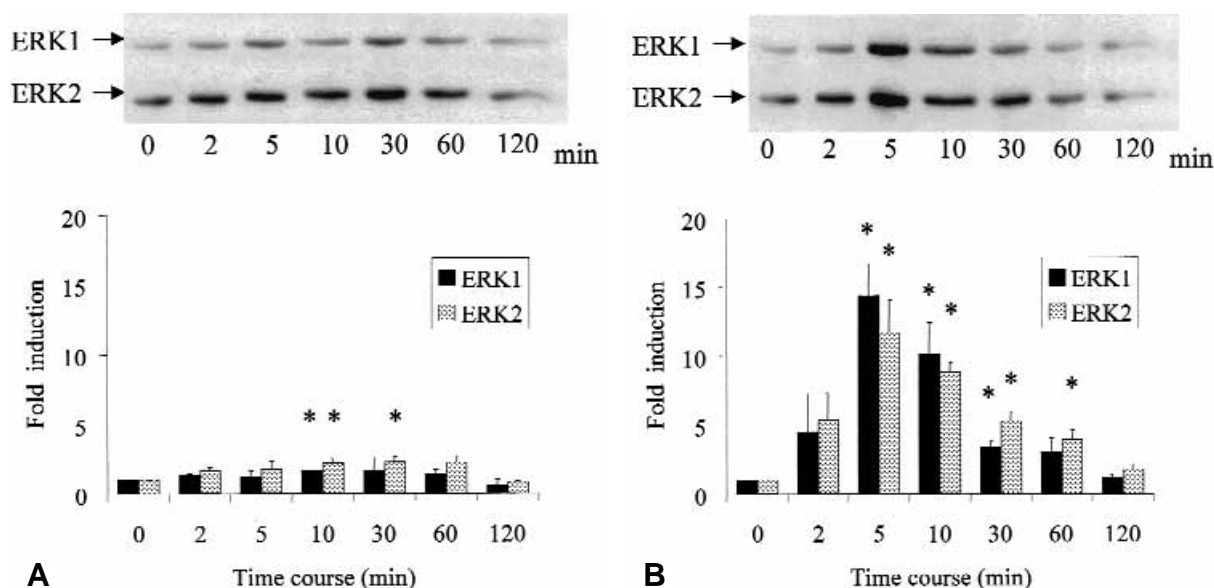
Fig 1. Time course of ERK 1/2 activation by cyclic strain and shear stress. Bovine aortic ECs were exposed to cyclic strain **(A)** or shear stress **(B)** for the indicated times (n = 3). ERK 1/2 activity was analyzed with immunoblot using antiactive MAP kinase antibody as described under "Materials and Methods." A representative immunoblot analysis showing the phosphorylation level of ERK 1 and ERK 2 is on the *top*, and the densitometric analysis of ERK 1/2 activity is at the *bottom*. *Asterisk* indicates statistical significance ($P < .05$) compared with static control (0 minutes). There was a significant difference between cyclic strain and shear stress activation of both ERK 1 and ERK 2 by two-way ANOVA.

phospho-specific JNK monoclonal antibody (New England Biolabs) were used as the primary antibodies for detecting phosphorylated ERK 1/2, p38, and JNK 1/2, respectively.

Statistical analysis. Data are presented as mean ± SE. The Student *t* test was used for analyzing densitometric data for each experiment compared with the respective static control (time, 0 minutes). Two-way analysis of variance (ANOVA) was used for comparing magnitude of activation between shear stress and cyclic strain experiments. A *P* value less than .05 was considered significant.

RESULTS

ERK 1/2 activation induced by mechanical forces. To determine the activation of ERK 1/2 induced by shear stress or cyclic strain, we measured the phosphorylation of ERK 1/2 with the use of an antiactive MAP kinase antibody. As shown in Fig 1, *A*, cyclic strain activated both ERK 1 and ERK 2 in the time-dependent manner. The time for peak activation ranged from 10 to 30 minutes, and the activation returned to static level at 120 minutes. Densitometric analysis showed that the peak activation of ERK 1 and ERK 2 was 1.6- and 2.3-fold greater than that of the static control, respectively.

On the other hand, activation of ERK 1/2 induced by shear stress was more robust and rapid compared with cyclic stretch ($P < .05$). Activation of both ERK 1 and ERK 2 occurred as early as 2 minutes, reached a peak at 5 minutes, then returned to basal levels at 60 minutes as shown in Fig 1, *A*. The peak activation of ERK 1 and ERK 2 was 14.4- and 11.7-fold greater than that of the static control, respectively (Fig 1, *B*). The static control in both the strain and shear stress groups, which were serum-starved, had minimal activation.

JNK activation. Both cyclic strain and shear stress activated JNK with similar magnitude and temporal pattern. As shown in Fig 2, immunoblotting demonstrated phosphorylation of both JNK 1 and JNK 2 in a time-dependent manner. The densitometric data indicated that cyclic strain induced 3.8-fold JNK 1 activation and 2.4-fold JNK 2 activation at 30 minutes. Shear stress also activated JNK 1/2 with a peak at 30 minutes, and the magnitude of the peak activation of JNK 1 and JNK 2 was 4.7- and 4.0-fold greater than that of static control, respectively. There was no statistical difference in JNK 1/2 activation by shear stress and cyclic strain.

p38 activation. Although cyclic strain induced small activation of p38 (1.7-fold compared with stat-
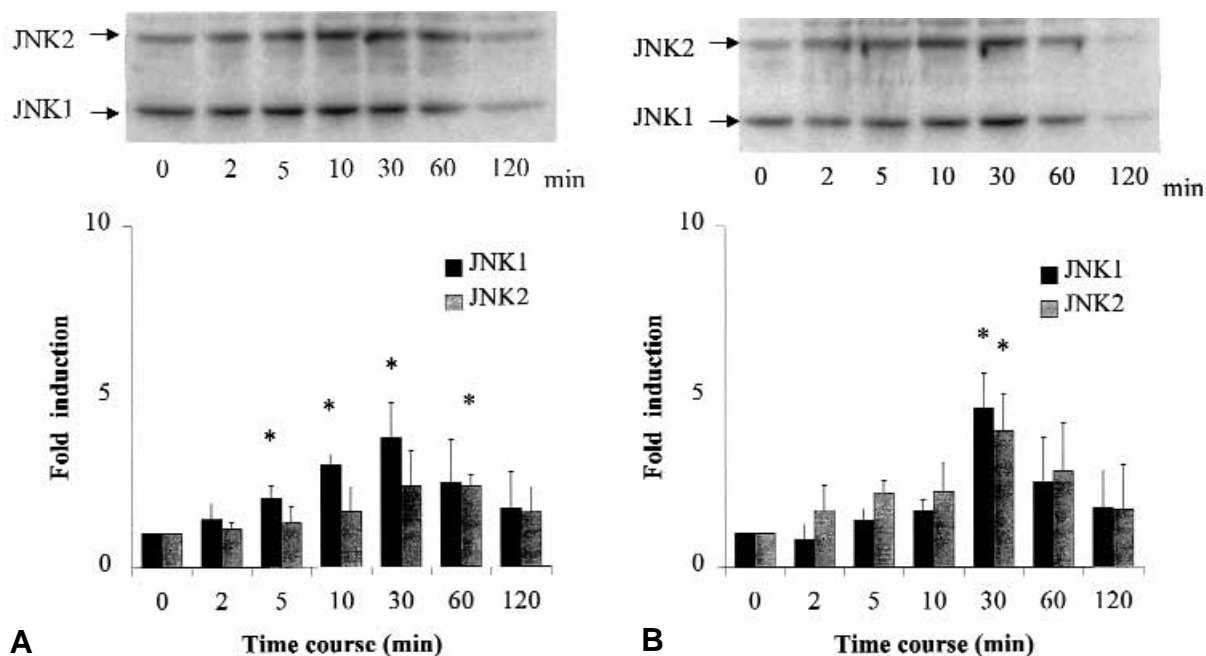
Fig 2. Time course of JNK 1/2 activation by cyclic strain and shear stress. Bovine aortic ECs were exposed to cyclic strain **(A)** or shear stress **(B)** for the indicated times (n = 4). JNK 1/2 activity was analyzed with immunoblot using phospho-specific JNK 1/2 antibody as described under "Materials and Methods." A representative immunoblot analysis showing the phosphorylation level of JNK 1 and JNK 2 is at the *top*, and the densitometric analysis of JNK 1/2 activity is at the *bottom*. *Asterisk* indicates statistical significance ($P < .05$) compared with static control (0 minutes). There was no significant difference between cyclic strain and shear stress activation of both JNK 1 and JNK 2 by two-way ANOVA.

ic control) with peak at 30 minutes as shown in Fig 3, in this series of experiments this was not statistically significant compared with the static level ($P = .09$). In contrast, shear stress induced a robust and rapid activation of p38. Activation of p38 occurred as early as 2 minutes after applying shear stress, reached peak values at 5 minutes, and was sustained for at least 2 hours. Densitometric data indicated that the activation at 5 minutes was 11.8-fold and by 2 hours was still 7.6-fold compared with that of static control. p38 activation induced by shear stress was significantly ($P < .05$) greater than that of cyclic strain.

DISCUSSION

The novel findings of this study are that activation of ERK and p38 induced by shear stress was much more rapid and robust compared with activation by cyclic strain, whereas JNK was activated by both shear stress and cyclic strain with a similar magnitude and temporal pattern. In addition, this is the first report that we are aware of that demonstrates p38 activation induced by shear stress.

Under normotensive conditions, the large conduit arteries, such as the aorta, stretch by 5% to 10%

between diastole and systole, and the mean shear stress has been calculated to be between 10 and 20 $dyne/cm^2$.[15,16] This is the rationale for the comparison of the effects of 10% cyclic strain and 10 $dyne/cm^2$ shear stress. Although it would be useful to assess activation of ECs under pulsatile shear stress, this force is extremely complicated and results in both laminar and nonlaminar flow patterns.[17,18] We chose the laminar steady shear stress system because it is simple, well established, and has been characterized extensively.

ERK 1 and ERK 2 were activated 1.6- and 2.0-fold at 30 minutes by cyclic strain, but activated 14.4- and 11.7-fold at 5 minutes by shear stress. These findings are consistent with previous independent data.[8,10] To explain these differences between cyclic strain and shear stress, we hypothesize that mechanoreceptors and/or transduction pathways involved with shear stress are different from those involved with cyclic strain. Deformation of the silicone membrane by cyclic strain may result in engagement of adhesion molecules on the basal membrane (abluminal side of cell) and extracellular matrix. In support of this hypothesis, our previous report sug-

gests that some integrins and focal adhesion kinase play an important role in transducing cyclic strain.[19,20] Furthermore, Ishida et al[21] activated β_1 integrin by applying the anti-integrin β_1 antibody, 8A2, and demonstrated that ERK 1 and ERK 2 were activated with a peak at 20 minutes. Although the temporal pattern of ERK 1/2 activation induced by 8A2 was much slower than that seen on exposure of ECs to shear stress in their paper, it has a pattern similar to cyclic strain as shown in our current study. Taken together, these reports suggest that integrins are likely candidates as mechanoreceptors.

On the other hand, it is likely that the forces induced by shear stress are acting not only on the luminal surface, but as a dragging force against the abluminal side of the cell. The latter force might be capable of activating other molecules, including integrins.[21-23] For example, Osawa et al's[24] laboratory has demonstrated that platelet-endothelial cell adhesion molecule–1 was tyrosine phosphorylated by shear stress as early as 30 seconds. Kuchan et al[25] and Gudi et al[26] have proposed that the cell membrane itself act as a mechanoreceptor. They hypothesize that shear stress alters the physiologic properties of the membrane, such as increasing permeability, fluidity, and perturbation, which in turn activates G protein on the cytosolic face of the membrane as early as the first second after applying flow. These accumulating data suggest the possibility that mechanoreceptors existing on the luminal surface of the cell membrane or the membrane itself are capable of being activated by shear stress. This activation might cause a much more rapid and robust induction of MAP kinase activity compared with integrin-related response. Many other potential mechanoreceptors such as cell-cell contact-complex, cytoskeleton, and ion channels have been proposed.[23] Further studies are obviously needed to definitively identify and characterize the mechanoreceptor(s).

In our studies, only JNK was activated with a similar magnitude and temporal pattern by both shear stress and cyclic strain (both with peak at 30 minutes). This suggests that it is possible that the mechanoreceptor and upstream pathway leading to JNK activation for shear stress may be similar to those for cyclic strain, but different from mechanoreceptors responsible for ERK or p38 activation in ECs exposed to shear stress. In this regard, Park et al[27] have shown that filipin, which disrupts caveolae, inhibited ERK activation induced by shear stress, but had no effect on JNK activation. Because the caveolins play an important role in the early phase of signal transduction,[12,28] this result is consistent with the hypothesis that the mechanoreceptors or upstream pathway resulting in JNK activation is different from those responsible for ERK activation.
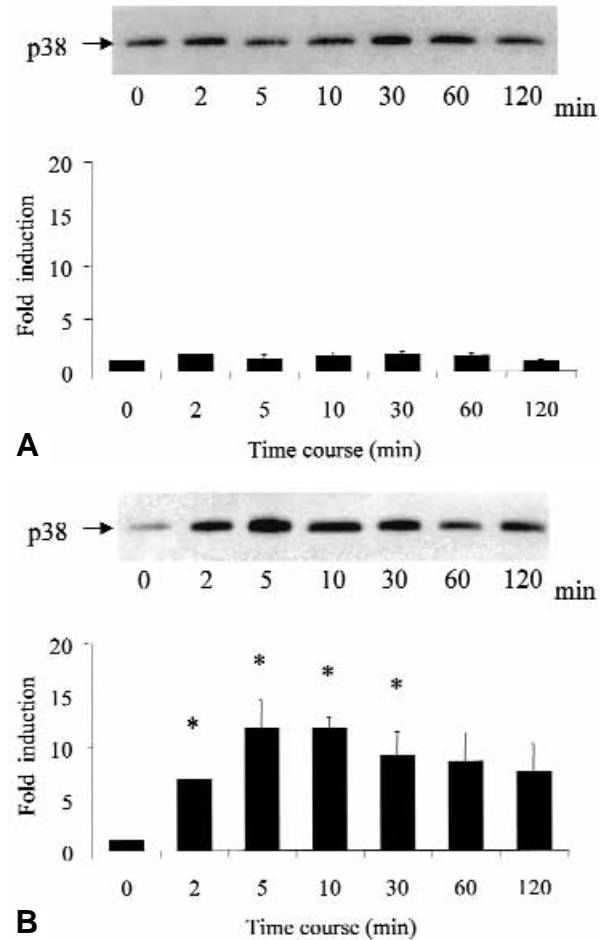
Fig 3. Time course of p38 activation by cyclic strain and shear stress. Bovine aortic ECs were exposed to cyclic strain **(A)** or shear stress **(B)** for the indicated times (n = 3). p38 activity was analyzed with immunoblot using phospho-specific p38 MAP kinase antibody as described under "Materials and Methods." A representative immunoblot analysis showing the phosphorylation level of p38 is at the *top,* and the densitometric analysis of p38 activity is at the *bottom. Asterisk* indicates statistical significance ($P < .05$) compared with static control (0 minutes). There was a significant difference between cyclic strain and shear stress activation of p38 by two-way ANOVA.

p38 was activated only mildly by cyclic strain, whereas shear stress robustly induced p38 activity. Activation of p38 was sustained for a relatively long term compared with the other MAP kinases induced by shear stress. The upstream pathway of p38 remains unknown. Rousseau et al[29] indicated that vascular endothelial growth factor (VEGF) activated p38, which causes stress fiber formation and cell migration. Shyy et al (oral communication, January 1999) have observed that shear stress causes tyrosine-phosphorylation of VEGF receptor and suggest that

VEGF receptor may be one of the mechanoreceptors to activate p38 in response to shear stress.

In conclusion, we show that ECs detect and respond to different mechanical forces in different manners in terms of MAP kinase activation. The ECs are constantly exposed to these different mechanical forces simultaneously, and the mechanical forces acting on the vascular wall are different between artery (high shear/high stretch) and vein (low shear/low stretch), trunks of vascular tree (high shear) and branched portion (low shear, turberlent flow), pathologic stenotic region (low shear/high stretch at prestenotic region, low shear/low stretch at post-stenotic region and turbulent flow) and normal vascular bed, and arterial graft (normal shear and stretch) and vein graft (low shear/high stretch) for coronary arterial bypass grafting. These major questions remain: how do ECs integrate these signals from different forces, and how do ECs influence physiology and pathology of vascular function and morphology in this varied hemodynamic environment. Further studies to clarify the molecular basis of signal coupling to physical force are needed.

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