

The Subendothelial Extracellular Matrix Modulates JNK Activation by Flow

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Abstract—Atherosclerosis begins as local inflammation of artery walls at sites of disturbed flow. JNK (c-Jun NH₂-terminal kinase) is thought to be among the major regulators of flow-dependent inflammatory gene expression in endothelial cells in atherosclerosis. We now show that JNK activation by both onset of laminar flow and long-term oscillatory flow is matrix-specific, with enhanced activation on fibronectin compared to basement membrane protein or collagen. Flow-induced JNK activation on fibronectin requires new integrin ligation and requires both the mitogen-activated protein kinase kinase MKK4 and p21-activated kinase. In vivo, JNK activation at sites of early atherogenesis correlates with the deposition of fibronectin. Inhibiting p21-activated kinase reduces JNK activation in atheroprone regions of the vasculature in vivo. These results identify JNK as a matrix-specific, flow-activated inflammatory event. Together with other studies, these data elucidate a network of matrix-specific pathways that determine inflammatory events in response to fluid shear stress. (*Circ Res.* 2009;104:995-1003.)

Key Words: shear stress ■ atherosclerosis ■ JNK

Atherosclerosis is an inflammatory disease in which endothelial cell (EC) activation leads to leukocyte recruitment into artery walls, followed by formation of plaques containing lipid-laden macrophages and smooth muscle cells.¹ Plaques can occlude vessels and cause ischemia, or rupture to cause stroke or myocardial infarction. Whereas systemic risk factors such as hypertension, smoking, and obesity play important roles in atherogenesis, plaques show a predilection for vessel branch points and regions of high curvature, where flow is low and shows a variety of complex patterns that are grouped together under the term disturbed flow.² These areas show increased EC turnover, altered redox regulation, and upregulation of proinflammatory genes that contribute to atherosclerotic progression.^{3,4} By contrast, areas of high laminar shear show downregulation of proatherogenic genes and upregulation of atheroprotective genes and are resistant to atherosclerosis.⁵

In vitro, acute application of laminar flow to unstimulated cells transiently activates inflammatory events and is often used to investigate EC responses to flow. Interestingly, oscillatory flow activates most of the same events in a sustained manner, recapitulates features of atheroprone regions of arteries in vivo, and has been used to model complex flow profiles found in vivo. Together, these methods have been widely used to study flow signaling associated with atherosclerosis.

Previous work in our laboratory showed that integrins are converted to a high affinity state downstream of a cell–cell

junction mechanosensory complex consisting of platelet endothelial cell adhesion molecule-1, VE-cadherin, and vascular endothelial growth factor receptor 2.⁶ In response to shear stress, this complex stimulates phosphoinositide 3-kinase (PI3K), which then leads to integrin activation. Subsequent binding of newly activated integrins to extracellular matrix (ECM) initiates downstream signals. Consistent with this model, several studies have shown that the subendothelial ECM modulates a subset of endothelial responses to flow.^{7,8}

ECs in the vasculature normally adhere to a basement membrane composed mainly of laminin, collagen (Coll) IV, and entactin/nidogen. In vivo, areas of disturbed shear show expression of atherogenic genes, such as the adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule (VCAM)-1, and increased deposition of fibronectin (FN). These changes occur at early times in Apolipoprotein E (ApoE)^{-/-} mice and even in atherosclerotic wild-type mice.⁷ Fibrinogen (FG) is deposited at these sites at later stages of atherosclerosis. In general terms, adhesion to basement membranes or Coll is associated with a quiescent cell phenotype, while binding to FN or FG is associated with proliferation and migration in many cellular systems.⁹ Matrix remodeling may therefore promote an activated EC phenotype in these regions.

In vitro, integrin activation in response to flow has been linked to shear responses including activation of mitogen-activated protein kinases (MAPKs), Rho family GTPases and

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nuclear factor (NF)- κ B.^{10–12} Our work has shown that in ECs plated on FN, but not on basement membrane or Coll, shear activates the inflammatory mediators NF- κ B and p21-activated kinase (PAK), suggesting that early changes in the subendothelial matrix in atheroprone regions of the vasculature may contribute to atherogenesis through promotion of an activated EC phenotype.^{7,13}

The c-Jun NH₂-terminal kinases (JNKs) are MAPKs traditionally considered stress-activated protein kinases. This subfamily includes JNK1 and JNK2, which are ubiquitously expressed, and JNK3, which is expressed mainly in the heart, brain, and testis.¹⁴ JNKs can regulate cell proliferation, apoptosis, migration, cytoskeletal rearrangements, inflammation, metabolic disease, neurodegenerative disease, oncogenesis, and cancer progression.¹⁵ JNK is activated by inflammatory cytokines and environmental stresses, including UV irradiation, osmotic stress, redox stress, and mechanical stress.¹⁶ JNK stimulates activation of the activator protein-1 transcription factor, resulting in the expression of inflammatory genes such as monocyte chemoattractant protein-1, interleukin-8,^{17,18} and VCAM-1.¹⁹

JNK has been shown to be activated in response to onset of laminar shear, and its activation appears to involve G proteins, PI3K γ , small GTPases, Src, and the upstream kinase MEKK1.^{20–22} JNK has also been implicated in atherosclerosis because both feeding mice the JNK inhibitor SP600125 and genetic deletion of JNK2 decreased atherosclerotic plaque formation in ApoE^{-/-} mice.²³ These links between JNK, inflammation, and atherosclerosis prompted us to investigate the activation of JNK in the context of fluid shear stress, ECM remodeling, and atherosclerosis.

Materials and Methods

Bovine aortic ECs (BAECs) were cultured as described.¹³ Laminar or oscillatory flow was applied to cells on coverslips using a parallel plate flow chamber.¹³ For Western blots, cells were lysed in SDS sample buffer and run on SDS polyacrylamide gels, transferred to poly(vinylidene difluoride) membranes, and probed with antibodies according to standard protocols. For immunoprecipitations, cells were lysed in buffer with 1% Triton X-100 and 0.5% Nonidet P-40 plus protease and phosphatase inhibitors and incubated with primary antibody and protein G-Sepharose, and beads were washed and eluted with SDS sample buffer. Eluates were analyzed by Western blotting as above. Immunohistochemistry was performed essentially as described previously.⁷ For details, see the expanded Materials and Methods section in the online data supplement at <http://circres.ahajournals.org>.

Results

JNK Activation by Shear Stress Is Matrix-Specific

JNK is typically activated by phosphorylation by the MAPK kinases (MAPKKs) MKK4 and MKK7, which phosphorylate T183 and Y185 on JNK within the activation motif Thr-Pro-Tyr.²⁴ In BAECs, both total and phospho-specific JNK antibodies recognized a major p54 and minor p46 band, which comigrate with JNK2 and JNK1 (Figure 1A). The p54 JNK2 band was 5.2-fold more intense than the p46 JNK1 band (Figure 1B); thus, subsequent studies focused on the p54 band. However, the minor p46 JNK1 band behaved similarly in all of the assays.

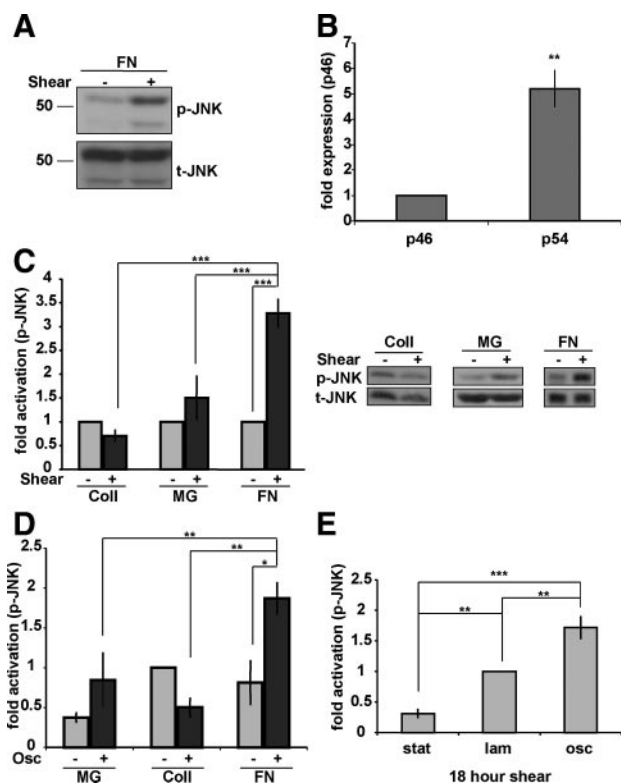


Figure 1. Shear stress–induced JNK activation is matrix specific. **A**, Representative Western blot of BAECs plated on glass slides coated with FN and exposed to 45 minutes of shear stress, followed by immunoblotting total cell lysate for phosphorylation site–specific T183/Y185 JNK antibody and total JNK antibody. Both antibodies recognized a major p54 band and minor p46 band. Numbers to the left of blots indicate molecular mass standards in kilodaltons. **B**, BAEC total cell lysates were quantified for relative expression of p54 and p46 JNK bands using total JNK antibody. Values are means \pm SEM following normalization to p46 band ($n=4$). **C**, BAECs plated on Coll I, diluted MG, or FN for 4 hours were exposed to acute onset laminar shear for 45 minutes, and activation of JNK was assessed by immunoblotting total cell lysates. Values are means \pm SEM following normalization to total JNK ($n=3$ to 4). **D**, BAECs plated on MG, Coll I, or FN for 4 hours were exposed to 18 hours of oscillatory shear. Phosphorylation of JNK was assessed by immunoblotting total cell lysates for activated and total JNK. Values are means \pm SEM following normalization for total JNK ($n=3$). **E**, BAECs plated on FN for 4 hours were held static or exposed to 18 hours of laminar or 18 hours of oscillatory shear. Values are means \pm SEM following normalization for total JNK ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Previous studies demonstrated that onset of shear stress–stimulated JNK activity maximally at 30 to 60 minutes (elsewhere^{21,25} and unpublished data, 2007). To determine whether the activation of JNK by shear stress depends on the subendothelial matrix, BAECs were plated on glass slides coated with either Coll I, MG (a solubilized basement membrane preparation), or FN for 4 hours, which is sufficient time to spread and form a confluent monolayer. Cells were then subjected to laminar shear stress (12 dyn/cm²) in a parallel plate flow chamber for 45 minutes. Cells on FN showed a marked increase in JNK phosphorylation in response to flow, whereas cells on MG or Coll showed little or no activation compared to static controls (Figure 1C).

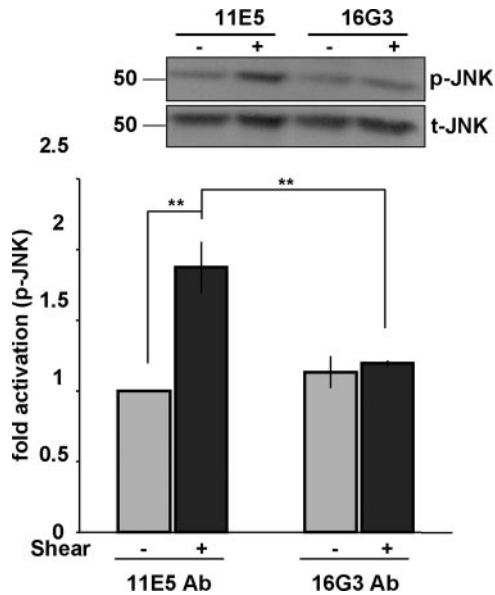


Figure 2. Requirement for new integrin ligation. BAECs were plated on FN for 4 hours, followed by incubation with 50 $\mu\text{g}/\text{mL}$ 11E5 control antibody or 16G3 anti-FN antibody for 1 hour. Cells were then exposed to onset of shear for 45 minutes. Phosphorylation of JNK was assessed by immunoblotting total cell lysates for activated and total JNK. Values are means \pm SEM normalized for total JNK ($n=4$). $**P<0.01$. A representative blot is shown.

Atheroprone regions of arteries in vivo experience complex flow patterns that are often modeled in vitro by oscillatory flow. To determine whether the effect of ECM also applies to longer term stimulation by oscillatory flow, BAECs plated on different matrices were exposed to oscillatory shear for 18 hours. Activation of JNK was again highly matrix-dependent, with a significant increase in JNK in response to flow seen only in cells on FN (Figure 1D). Additionally, cells on FN exposed to 18 hours of laminar shear showed low JNK phosphorylation as compared to cells exposed to oscillatory flow (Figure 1E).

Previous studies showed that a number of flow-dependent signals are initiated downstream of de novo integrin binding to the ECM beneath the endothelium, which occurs after their conversion to a high-affinity state.^{6,10,26} Given the matrix specificity of JNK activation by shear stress, we next asked whether new integrin ligation following flow mediates this response. To test this idea, BAECs that were stably adherent to FN were preincubated for 1 hour with either the anti-FN antibody 16G3, which blocks integrin binding sites on FN, or the control antibody 11E5 before being exposed to the acute onset of laminar shear stress. Treatment with anti-FN antibody under these conditions does not cause cell detachment or disruption of cytoskeletal organization but prevents formation of new integrin binding following integrin activation by shear.^{10,26,27} Blocking FN abrogated JNK phosphorylation in response to shear stress, indicating that new integrin ligation is required for activation of JNK by flow (Figure 2).

JNK Activation in Atheroprone Areas In Vivo

We next tested whether these findings were relevant to activation of JNK in ECs at atheroprone sites in vivo.

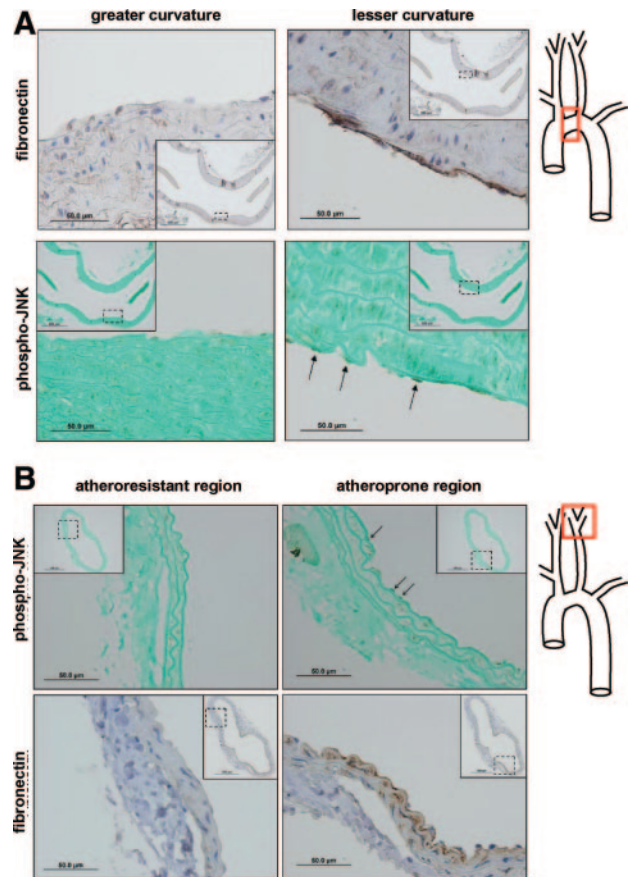


Figure 3. JNK activation and FN in vivo. A, Serial sections from aortas of C57Bl/6 mice were stained for phospho-T183/Y185 JNK (bottom) or FN (top). Left images show greater curvature; right, lesser curvature. Insets show lower magnification views of the same vessels with boxes indicating enlarged areas. Images are representative of results from 3 mice. B, Serial sections from the carotid sinus of ApoE^{-/-} mice fed a Western diet for 10 weeks, stained for phospho-JNK and FN as in A. Left images show atheroresistant region; right, atheroprone region. Insets are lower magnification view of the entire vessel with boxes indicating the enlarged areas. Images are representative of results from 4 mice. Illustrations indicate regions from which sections were taken.

Previous work from our laboratory showed increased deposition of FN in atheroprone segments of arteries even in wild-type mice that do not develop atherosclerosis; furthermore, FN localization correlates with inflammatory gene expression.⁷ We therefore performed immunohistochemical analysis of phospho-JNK and FN in aortas from wild-type C57BL/6 mice. The greater curvature of the aortic arch is exposed to high laminar shear and is resistant to atherosclerosis, whereas the lesser curvature of the aortic arch is exposed to disturbed flow and is susceptible to atherosclerosis. Staining revealed a continuous FN stain beneath the endothelial layer of the atheroprone lesser curvature of the aortic arch, with phospho-JNK staining localized to nuclei of the ECs in the same region (Figure 3A). By contrast, the greater curvature showed very little FN or phospho-JNK staining. To quantify the correlation between FN and phospho-JNK, the aortic arches from 3 different mice were

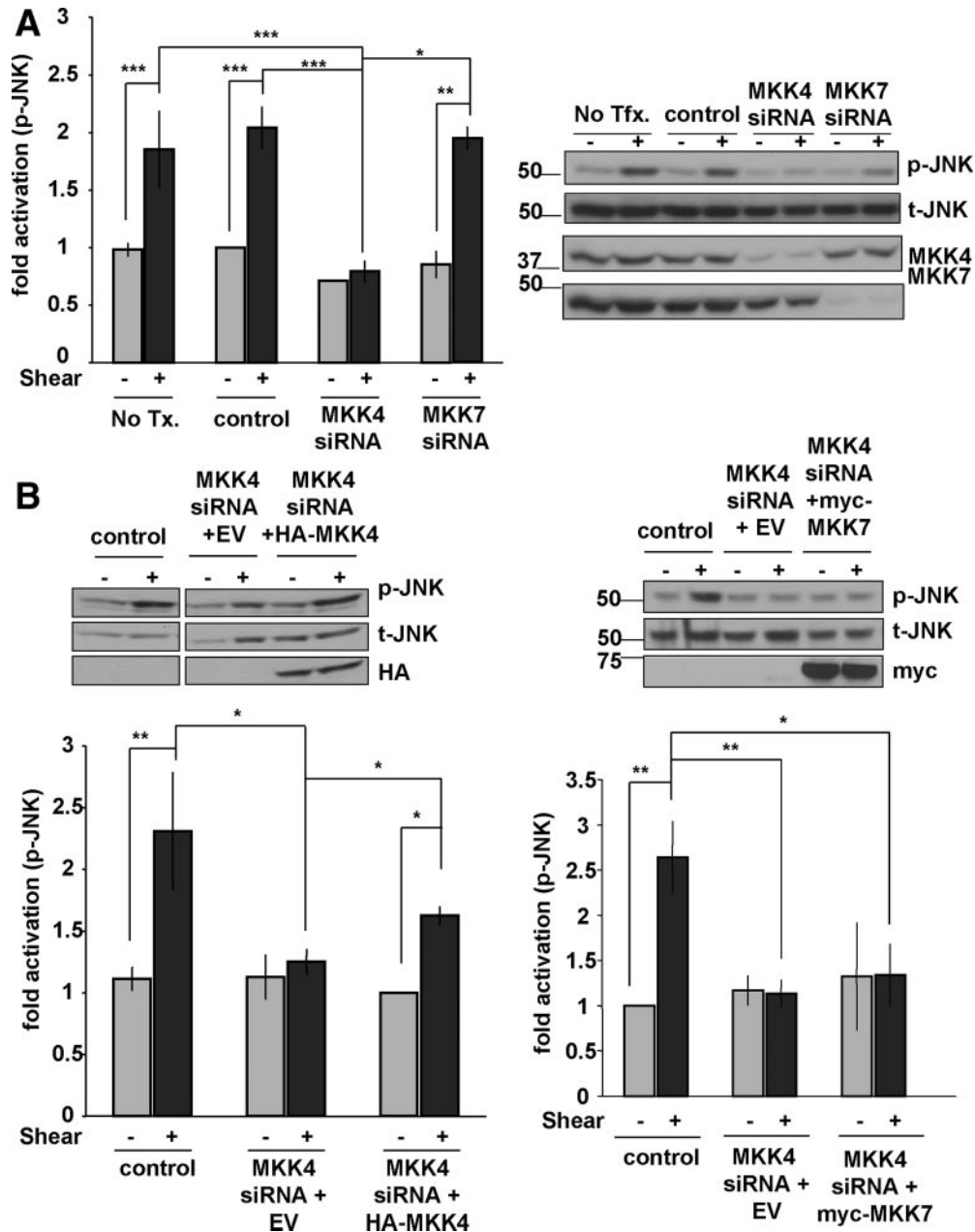


Figure 4. MKK4 is required for JNK activation by shear stress. A, BAECs were plated on FN-coated slides overnight, then either left untreated or transfected with control, MKK4, or MKK7 siRNA. After 48 hours, cells were either untreated or exposed to laminar shear for 45 minutes. Phosphorylation of JNK was assessed by immunoblotting total cell lysates for activated and total JNK. Knockdown of MKK4 and MKK7 was assessed by immunoblotting with anti-MKK4 and anti-MKK7 antibodies. Values are means±SEM normalized for total JNK (n=4). A representative blot is shown. B, For MKK4 rescue, BAECs on FN-coated glass slides were transfected with control siRNA or cotransfected with MKK4 siRNA and empty vector, MKK4 siRNA, and HA-MKK4 or MKK4 siRNA and myc-MKK7. After 48 hours, cells were stimulated and JNK activity as assessed as in A. Values are means±SEM (n=3 to 4). Representative blots are shown. **P*<0.05, ***P*<0.01, ****P*<0.001.

split into equal sized sections and scored for staining of FN and phospho-JNK in each section. FN colocalized with phospho-JNK with a frequency of 0.88, whereas for the random distribution (null hypothesis), colocalization occurred with a frequency of 0.54, *P*=0.003.

To further investigate whether phospho-JNK is present in atherosclerotic lesions in vivo, hypercholesterolemic ApoE^{-/-} mice that develop atherosclerotic plaque at regions of disturbed flow were examined.²⁸ These mice were fed a high-fat, Western diet for 10 weeks to accelerate plaque

formation. Mice were then euthanized and the carotid arteries stained for FN and phospho-JNK (Figure 3B). The carotid sinus is a classic site of atherosclerosis and previous work showed staining for the ECM proteins FN and the inflammatory markers intercellular adhesion molecule-1 and VCAM-1 in this region.⁷ We observed pronounced staining of FN in the carotid sinus of these mice which colocalized with phospho-JNK staining, whereas nearby regions of the vessel wall that did not experience disturbed flow showed little staining for any of these proteins.

MKK4 in Shear-Induced JNK Activation

We next investigated the upstream MKK in this pathway. To date, only MKK4 and MKK7 have been found to mediate JNK activation. MKK4, MKK7, or control small interfering (si)RNAs were used to test which of these MAPKKs mediate shear-induced JNK activation. Transient transfection of BAECs with MKK4 and MKK7 siRNAs decreased the level of those proteins by approximately 80% to 90% (Figure 4A). Whereas depletion of MKK4 inhibited shear-induced JNK phosphorylation, neither MKK7 nor control siRNAs had significant effects (Figure 4A). To confirm this result, a rescue experiment was performed in which BAECs were cotransfected with MKK4 siRNA together with either empty vector, hemagglutinin (HA)-tagged MKK4, or myc-tagged MKK7. Only the HA-MKK4 significantly rescued shear-induced JNK phosphorylation (Figure 4B).

We also assessed the role of these MAPKKs by measuring their activation in response to flow. BAECs were plated on either Coll or FN and exposed to short-term laminar shear. Activation was assessed by Western blotting with antibodies that recognize phosphorylated MKK4 or phosphorylated MKK7. Consistent with the matrix specificity of JNK activation, MKK4 phosphorylation increased in cells plated on FN but decreased to slightly below baseline levels in cell on Coll (Figure 5A). By contrast, MKK7 phosphorylation was unaffected by shear on either matrix (Figure 5B). Finally, we examined cells in oscillatory shear. MKK4 phosphorylation was significantly elevated after 18 hours of oscillatory shear (Figure 5C), whereas MKK7 showed no change (C.H., unpublished data, 2008). Taken together, these data confirm the matrix dependence for this pathway and identify MKK4 as the MAPKK responsible for ECM-dependent JNK activation in response to shear.

PAK in Shear-Induced JNK Activation

The p21-activated kinase (PAK) Ser/Thr kinases have also been implicated in activation of JNK in some systems.²⁹ PAK is an interesting candidate because, like JNK, it is activated by flow in a matrix-specific manner, and is activated in atheroprone areas of arteries.⁸ In those experiments, PAK was found to mediate local breakdown of cell-cell junctions and increased vascular permeability. Inactive PAK is a homodimer in which its N-terminal autoinhibitory domain (PAK AID) binds and inhibits the C-terminal kinase domain *in trans*. Binding of active Rac or Cdc42 to PAK relieves this autoinhibition, allowing autophosphorylation of the protein on Ser141 and Ser423, which lead to sustained activation.³⁰ Active PAK is recruited to target sites on the cell membrane via interaction of an N-terminal proline-rich sequence with Nck.

To investigate the involvement of PAK in JNK activation by flow, BAECs on FN were transfected with a vector encoding HA-tagged PAK AID or control vectors and exposed to onset of laminar shear. Compared to empty vector and myc-tagged PAK2 controls, PAK AID completely blocked JNK activation in response to shear (Figure 6A). To confirm these results, BAECs were treated with 20 $\mu\text{g}/\text{mL}$ of either a control peptide or a peptide that contains the Nck-binding sequence of PAK fused to the transduction

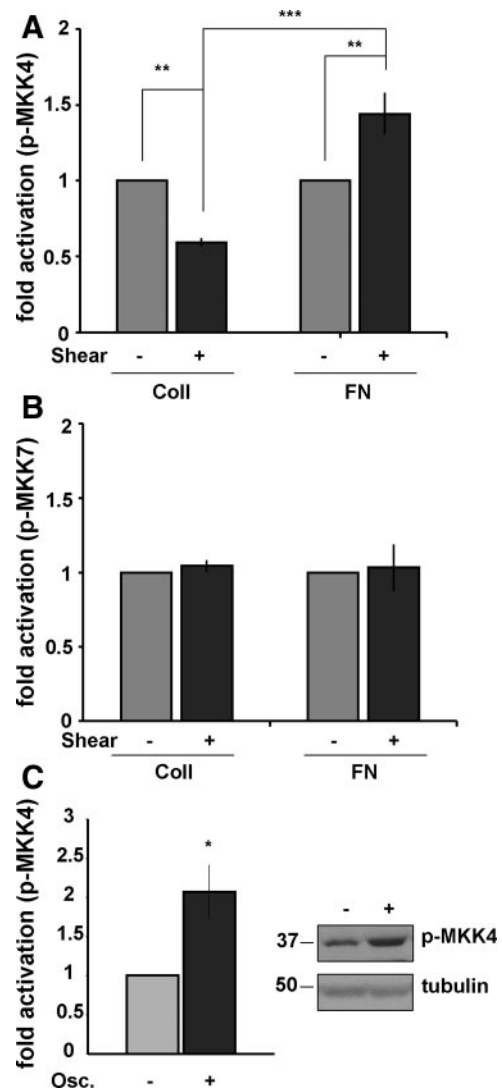


Figure 5. MKK4 activation by onset of shear and long-term oscillatory shear. A, BAECs plated on Coll I or FN for 4 hours were exposed to laminar shear for 45 minutes. Phosphorylation of MKK4 was assessed by immunoblotting total cell lysates for phospho-S257/T261 MKK4 and for total MKK4. Values are means \pm SEM normalized for total MKK4 ($n=3$). B, BAECs were transfected with myc-MKK7. After 48 hours, cells were plated on Coll I or FN for 4 hours, followed by laminar shear for 45 minutes. MKK7 was immunoprecipitated with anti-myc antibody and analyzed by Western blotting for phospho-S271/T275 MKK7 and total MKK7. Values are means \pm SEM normalized for total MKK7 ($n=3$). C, BAECs plated on FN-coated glass slides for 4 hours were exposed to 18 hours of oscillatory shear. Activation of MKK4 was assessed by Western blotting as in A ($n=3$). A representative blot is shown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

sequence from the HIV TAT protein; this peptide enters cells and blocks PAK function in several systems, both *in vitro* and *in vivo*.^{8,31–33} The blocking peptide efficiently inhibited the activation of JNK in response to onset of laminar shear. By contrast, a control peptide in which 2 critical prolines are mutated had no effect (Figure 6B). Thus, PAK appears to be required for activation of JNK by flow in cells on FN.

Conversely, we tested the effect of activating PAK in cells on Coll where JNK activity is suppressed. Cells were transfected with either wild-type PAK as a control or a constitu-

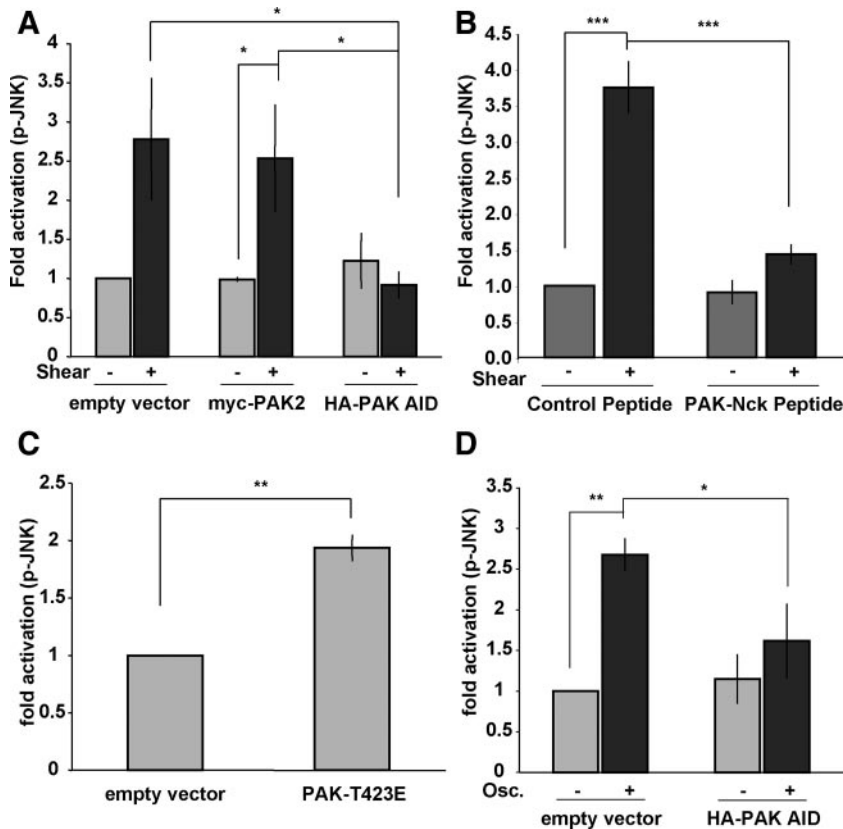


Figure 6. PAK is required for shear stress induced JNK phosphorylation. A, BAECs plated on FN-coated glass slides were transfected with empty vector, myc-PAK2, or HA-PAK AID constructs. After 48 hours, cells were exposed to laminar shear for 45 minutes. JNK activation was assessed as in Figure 1 (n=3). B, BAECs were plated on FN for 4 hours, followed by incubation with 20 μ g/mL either control peptide or PAK-Nck blocking peptide for 1 hour. Cells were then exposed to laminar shear for 30 minutes. JNK activation was assessed as in Figure 1 (n=3). C, Cells were transfected with either empty vector or myc-PAK T423E. After 48 hours, cells were plated on Coll I. JNK activation was assessed as in Figure 1 (n=3). D, BAECs plated on FN were transfected with empty vector or HA-PAK AID constructs. After 48 hours, cells were exposed to 18 hours of oscillatory shear. JNK activation was assessed as in Figure 1 (n=3). * P <0.05, ** P <0.01, *** P <0.001.

tively active PAK T423E mutant. We found that expression of even low levels of PAK-T423E increased JNK activity in the absence of flow (Figure 6C). Applying flow to these cells had no further effect (data not shown). This result shows that active PAK is sufficient to activate JNK in the absence of other stimuli.

PAK is activated in a sustained manner in response to oscillatory shear.¹³ To address whether PAK is required for activation of JNK under this condition, BAECs on FN were transfected with the HA-PAK AID construct and exposed to oscillatory shear for 18 hours. Inhibiting PAK strongly attenuated JNK activation compared to the empty vector control (Figure 6D). Taken together, the data show that PAK is the critical determinant of matrix-specific JNK activation by both onset of laminar shear and by oscillatory shear.

PAK Mediates JNK Activation in Areas of Disturbed Flow In Vivo

Given these data and the activation of PAK and JNK at similar sites in arteries, we wondered whether PAK is a critical determinant of JNK activation at regions of early atherogenesis in vivo. To address this question, C57BL/6 mice were injected with either control or PAK-Nck blocking peptide once a day for 3 days, and then carotid arteries were harvested and analyzed by immunohistochemistry for phospho-JNK. Mice treated with the control peptide had the expected phospho-JNK staining in the nuclei of the ECs in the carotid sinus, a well-described atheroprone region where flow is disturbed. By contrast, mice treated with the PAK-Nck blocking peptide showed an approximately 50% de-

crease in the number of cells that scored positive for JNK per length of vessel perimeter (Figure 7A and 7B; P <0.05). These results confirm the importance of PAK in JNK activation in a region of disturbed flow in vivo.

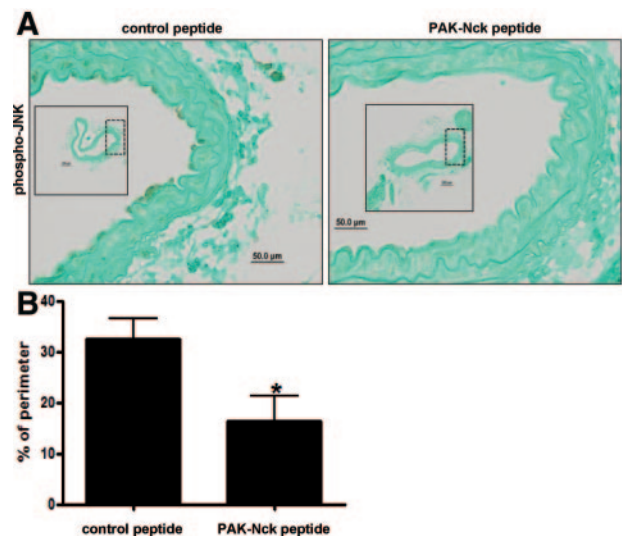


Figure 7. Blocking PAK reduces JNK activation in atheroprone sites in vivo. A, Representative images of 36-week-old C57BL/6 mice injected intraperitoneally with 0.1 mL of either control peptide or PAK-Nck blocking peptide (10 mg/mL) daily for 3 days. Carotid sinuses were isolated and stained with anti-JNK phospho-T183/Y185. B, Images were analyzed for percentage of perimeter phospho-JNK staining normalized to total vessel perimeter. Values are means \pm SEM (n=3). * P <0.05.

Discussion

These results show that JNK is activated in a matrix-specific manner in response to fluid shear stress. JNK is activated at early times by acute onset of laminar shear and at later times by oscillatory shear in cells on a FN matrix, but little or no activation occurred in cells on basement membrane protein or type I Coll. JNK activation is also seen in vivo at atheroprone regions of arteries coincident with FN in the subendothelial matrix. Matrix-specific activation of JNK by shear stress requires new integrin ligation, because blocking integrin binding to FN abrogated its activation. JNK activation by shear also requires MKK4 and PAK. Both kinases are activated by acute onset of laminar flow or by oscillatory shear stress in a matrix-specific manner, and inhibiting either kinase blocked JNK activation. Furthermore, a small peptide inhibitor of PAK decreased JNK activation in atheroprone areas of the vasculature in vivo. Taken together, these results suggest that JNK is activated by disturbed flow in ECs adherent to FN in vivo.

JNK is known to play a key role in expression of cytokines and adhesion molecules that mediate leukocyte recruitment and activation.^{18–21,34} Inhibiting or deleting JNK also reduced atherosclerosis in ApoE^{-/-} mice.¹⁴ JNK can mediate apoptosis under stressful conditions¹⁶ and thus could contribute to the elevated rates of apoptosis at atheroprone regions in vivo. Taken together, the data therefore suggest that ECM-dependent JNK activity is likely to play a significant role in atherogenesis.

ECs are normally adhered to a basement membrane in which the major components are laminin, which binds integrins $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_2\beta_1$, and Coll IV, which binds integrins $\alpha_2\beta_1$ and $\alpha_1\beta_1$.^{35,36} Injury, inflammation, and angiogenesis that promote vascular remodeling lead to deposition of a provisional matrix containing proteins including FN and FG, which bind primarily $\alpha_5\beta_1$ and α_v integrins.³⁷ Binding of these integrins to matrix is associated with an activated cell phenotype, with increased cell migration and proliferation,^{9,38} consistent with the EC phenotype in atheroprone regions.^{39,40} This matrix remodeling most likely contributes to resolving inflammation or injury in response to acute insults but under chronic stresses may have deleterious aspects.

Previous work in our laboratory showed that the altered subendothelial matrix at atheroprone sites influences activation of the transcription factor NF- κ B, a key regulator of inflammatory gene expression during atherosclerotic progression.⁷ We also recently reported that matrix-specific NF- κ B activation by flow is mediated by matrix-specific activation of PAK.¹³ That JNK, a third key inflammatory mediator, is activated in a matrix- and PAK-dependent manner therefore indicates, first, that matrix remodeling is a major determinant of endothelial activation in this system. It also reveals that the ECM regulates EC phenotype through a network of pathways in which PAK appears to be the central determinant, accounting for both JNK and NF- κ B, as well as mediating effects on junctional integrity and permeability more directly.

The result that shear-induced JNK activation is among the events that depend on new integrin ligation also provides additional support for the junctional mechanotransducer model. In this model, integrin activation and ECM binding

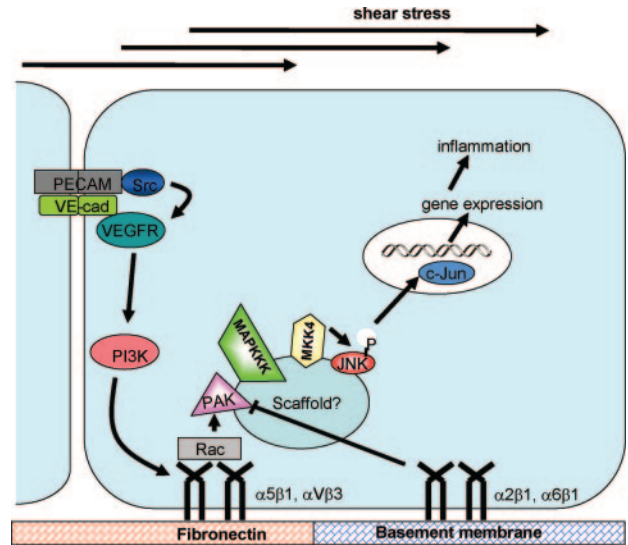


Figure 8. Model for shear-induced JNK activation. Shear stress stimulates a junctional mechanosensory complex consisting of platelet endothelial cell adhesion molecule 1 (PECAM-1), VE-cadherin, and vascular endothelial growth factor receptor 2 (VEGFR2), leading to activation of PI3K. PI3K lipids trigger activation of a pool of low-affinity integrins, which then bind to the ECM and trigger a variety of signals. In cells on FN, Rac and PAK are activated, leading to activation of JNK, which induces inflammatory gene expression. In cells on Coll or basement membrane proteins, PAK is not activated, which prevents activation of JNK.

occur downstream of a junctional complex that stimulates integrin activation through PI3K (Figure 8). These data fit well with previous results describing a role for PI3K γ in JNK activation by shear stress.²² Thus, a series of events beginning with rapid activation of signaling proteins in cell–cell junctions, followed by integrin activation and binding, leading to JNK activation and leukocyte recruitment appear to be components of a single pathway. Our results also show that MKK4 and PAK are upstream of matrix-specific JNK activation. Despite being identified as a potential activator of JNK some years ago,⁴¹ how PAK affects this pathway remains unknown. Upstream kinases such as MEKK1 or scaffold proteins such as JNK-interacting proteins are possible targets. Further work will be required to address this question.

Distinct from our results, it has been reported that laminar flow inhibits JNK activation in ECs by inflammatory cytokines such as tumor necrosis factor.^{42–44} This effect was proposed to be one of the means by which long term laminar shear is atheroprotective. Under these conditions, inhibition of JNK was mediated through MEK5 and ERK5/BMK1, which inhibited the MAPKKK ASK1 by inducing thioredoxin interacting protein.^{42,43,45} These results may appear to conflict with ours and those of other labs that report activation of JNK by flow.^{20–22,46,47} However, studies demonstrating suppression of JNK by laminar shear used cells plated on denatured Coll (gelatin). Thus, the matrix specificity of JNK activation may resolve the discrepancy.

In conclusion, our data provide evidence for JNK activation in atheroprone regions of the vasculature through a pathway that involves both disturbed flow and ECM remodel-

eling. Together with previously published results, these data suggest that JNK contributes to atherogenesis in vivo. These results raise many questions for future work. Which factors determine local PAK activation ECM remodeling in areas of disturbed flow in vivo are major unsolved questions. Although roles for JNK in expression of inflammatory genes such as monocyte chemoattractant protein-1, interleukin-8, VCAM-1, and prostaglandin D synthase,^{18,20,48} and in EC apoptosis and cell migration¹⁵ have been identified, elucidation of the specific roles for JNK during atherogenesis will also be an important area for future research. Understanding the inflammatory pathways controlled by flow may provide novel therapeutic strategies for modulating atherosclerotic progression.

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Disclosures

None.

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Materials and Methods

Cell culture and application of shear stress

BAECs were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS; Atlanta Biologicals), 10 U/ml penicillin and 10 µg/mL streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were maintained in DMEM:F12 medium containing 10% FBS, 1% bovine brain extract, 60 µg/mL heparin (Sigma), 10 U/ml penicillin, and 10 µg/ml streptomycin. Endothelial cells were plated on glass slides coated with the indicated matrix proteins (20 µg/mL FN, 40 µg/mL Coll I (Sigma) or 1:100 dilution in serum-free media of MG (Calbiochem). After 4h they formed a confluent monolayer, at which time they were either kept under static (no flow) conditions or loaded onto parallel plate flow chambers. Cells were exposed either to laminar flow at 12 dynes/cm² in media containing 0.2% FBS for the indicated times as described¹. Flow experiments lasting 18h used medium containing 1% FBS in order to maintain viability of cells for these longer times. Oscillatory flow was generated using an infusion-withdrawal pump (New Era NE-1050) combined with a peristaltic pump (Cole-Parmer) to superimpose an average forward flow component of 0.6 dynes/cm² (with peak to peak variation of 1.92 dynes/cm² at 4 Hz) to allow for nutrient delivery.

Transfections

Transient transfection of control luciferase siRNA (5'-CGUACGCGGAAUACUUCGATT-3'), MKK4 siRNA (5'-GAAGGUGCUGAUCCUAAGATT-3'), MKK7 siRNA (5'-

GAACUGCAAGACGGACUUUTT-3'), pcDNA 3.1 empty vector, HA-MKK4, myc-MKK7, myc-PAK2, HA-PAK AID, myc-PAK T423E was accomplished using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For control peptide and PAK-Nck blocking peptide (EZ Biolabs), cells were pretreated with 20 µg/mL for 1 hr. before shear application.

Cell lysis, immunoprecipitation and immunoblotting

For whole cell lysates, cells were lysed in Laemmli buffer (0.1M Tris pH6.8, 10% β-mercaptoethanol, 20% glycerol, 4% SDS). For immunoprecipitation, cells were collected in lysis buffer pH7.4 (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% NP-40) containing phosphatase and protease inhibitor cocktails (Sigma) and clarified by centrifugation at 14,000 rpm for 15 min at 4°C.

Lysates were incubated with anti-myc antibody (Santa Cruz) for 2 h at 4°C, followed by Gammabind G Sepharose (GE Healthcare) at 4°C for 2h. Beads were washed with lysis buffer three times and eluted with 2X Laemmli buffer. Samples were run on a 10% SDS-PAGE gel, transferred to PVDF membrane (BioRad) and blocked with 5% milk in TBS with 0.01% Tween 20. Blots were incubated with 1:1000 primary antibody phospho-JNK, total-JNK, phospho-MKK4, MKK4, MKK7, total-ERK (Cell Signaling Technology), phospho-MKK7 (BioSource), JNK1 (Affinity BioReagents) or 1:10000 tubulin overnight at 4°C. Blots were washed, incubated with 1:5000 secondary goat anti-rabbit HRP antibody (Jackson Laboratories) or 1:2500 secondary goat anti-mouse (Jackson Laboratories) for 1 h at room temperature and developed using ECL reagents (Pierce) and film (Kodak).

Immunohistochemistry(IHC)

5 µm paraffin sections were used for IHC. Following microwave antigen retrieval (AR) with antigen unmasking solution (Vector Labs), rabbit anti-phospho-JNK 1:100 (BioSource) and FN antibodies 1:400 (Sigma-Aldrich) were applied overnight at 4°C. Goat anti-FG antibodies (1 µg/mL, Accurate Chemical) was applied as for other antibodies, but required no antigen retrieval. Slides were visualized with Vectastain Elite Kit (Vector Lab), and visualized with diaminobenzidine (DAB; Deko Corp). Sections were counterstained with either Hematoxylin I or Fast Green. Images were acquired using the 10X or 40X objective on an Olympus BX51 microscope equipped with an Olympus DP70 digital camera using the ImagePro plus software program in the Academic Computing Health Sciences Center at the University of Virginia.

Quantification and statistical analysis

Immunoblots was quantified using Image J software, and p values were obtained by one-way ANOVA with the Newman-Keuls multiple comparisons post-test, two-way ANOVA with the Bonferroni post-test, and two-tailed Student's t-test using GraphPad Prism software or the Microsoft Excel program. To quantify the correlation between FN and phospho-JNK in Fig 3A, the aortic arches from 3 different mice were divided into 12 equal sized sections, which were then scored for FN and phospho-JNK staining. FN co-localized with phospho-JNK 88% of the time. To determine the statistical significance of these data, we calculated the expected overlap for a random distribution (the null hypothesis). 50,000 paired vectors were created with the same density of FN and

phospho-JNK staining as that observed experimentally but with locations randomized.

Overlap was observed in 54% of these instances. Overlap \geq 88% (as observed experimentally) occurred in 272 of the 50,000 runs ($p < 0.003$).

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