Matrix-specific p21-activated kinase activation regulates vascular permeability in atherogenesis

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Elevated permeability of the endothelium is thought to be crucial in atherogenesis because it allows circulating lipoproteins to access subendothelial monocytes. Both local hemodynamics and cytokines may govern endothelial permeability in atherosclerotic plaque. We recently found that p21-activated kinase (PAK) regulates endothelial permeability. We now report that onset of fluid flow, atherogenic flow profiles, oxidized LDL, and proatherosclerotic cytokines all stimulate PAK phosphorylation and recruitment to cell–cell junctions. Activation of PAK is higher in cells plated on fibronectin (FN) compared to basement membrane proteins in all cases. In vivo, PAK is activated in atherosclerosis-prone regions of arteries and correlates with FN in the subendothelium. Inhibiting PAK in vivo reduces permeability in atherosclerosis-prone regions. Matrix-specific PAK activation therefore mediates elevated vascular permeability in atherogenesis.

Introduction

Atherosclerosis involves the progressive accumulation of lipids, immune cells, and ECM in the vessel wall, which can decrease blood flow or rupture to cause acute thrombosis. Endothelial cell dysfunction is the key initiating event in atherogenesis, resulting in decreased flow-induced dilation and inflammatory gene expression (Ross, 1999). Activated endothelium recruits monocytes, which differentiate into macrophages. Elevated permeability of the endothelium is believed to allow entry of lipoproteins into the vessel wall, which become oxidized and propagate endothelial dysfunction (Steinberg, 1997). Macrophages engulf low-density lipoprotein (LDL) and other lipoproteins and become foam cells, which can be visualized as fatty streaks in the vessel wall. In the continued presence of high LDL cholesterol and oxidant stress, fatty streaks progress to advanced atherosclerotic plaques (Ross, 1999).

Despite the systemic nature of most atherogenic stimuli, atherosclerosis is a focal disease affecting discrete regions of the vasculature, such as vessel curvatures and bifurcations. These regions are characterized by complex flow patterns, including flow reversal, flow gradients, secondary flows with rapid changes in flow direction, and, in some regions, turbulence (VanderLaan et al., 2004). We group all of these flow patterns under the rubric of disturbed flow. Endothelial cells sense the force of flowing blood, termed shear stress, and different blood flow patterns regulate endothelial behavior. Regions of blood vessels exposed to undisturbed, unidirectional laminar flow (henceforth termed laminar flow) are protected from atherosclerosis, and in vitro prolonged laminar flow stimulates expression of athero-protective genes (Traub and Berk, 1998; Brooks et al., 2004). By contrast, disturbed flow patterns stimulate proatherosclerotic events, including increased monolayer permeability; decreased antioxidant capacity; and enhanced expression of proinflammatory genes, such as ICAM-1, VCAM-1, and monocyte chemotactic protein-1 (MCP-1; Jo et al., 1991; De Keulenaer et al., 1998; Phelps and DePaola, 2000; Brooks et al., 2004). The correlation between flow patterns and endothelial monolayer permeability has recently been demonstrated in vivo, where vascular permeability is inversely proportional to time-average shear stress and correlated with increased flow oscillation and flow gradients (Himburg et al., 2004; LaMack et al., 2004). Interestingly, onset of laminar shear stimulates many of the same responses as disturbed shear; however, in laminar shear, these events are down-regulated as cells adapt, whereas in disturbed shear, they are sustained. Thus, failure to adapt is thought to be critical for responses to disturbed shear (Orr et al., 2006).

The molecular mechanisms involved in flow-induced endothelial permeability are unknown. Although vesicular transport and transcellular channels may contribute to endothelial permeability,
paracellular pore formation is most likely the major pathway for macromolecule transport across arterial endothelium (Ogunrinade et al., 2002). Paracellular permeability is limited by cell–cell interactions, especially those in tight junctions (TJs). Multiple molecular mechanisms implicated in regulation of endothelial paracellular permeability include changes in gene expression, phosphorylation of junctional components, myosin-dependent contractility, and stability of cortical actin (Ogunrinade et al., 2002). Many signaling pathways regulate permeability, most of which affect cortical actin or myosin (Yuan, 2002). Actin remodeling is regulated by the Rho family of small GTPases, including Rho, Rac, and Cdc42 (Jaffe and Hall, 2005). The p21-activated kinase (PAK) family of Ser/Thr kinases is important for Rac and Cdc42-induced cytoskeletal remodeling, affecting both actomyosin contractility and the stability of actin filaments (Bokoch, 2003). Recently, PAK was shown to stimulate paracellular pore formation and increased endothelial cell permeability in response to a wide range of cellular stimuli (Stockton et al., 2004). PAK-mediated permeability responses require the localization of active PAK to cell–cell junctions, where PAK stimulates the phosphorylation of myosin light chain to induce contractility (Stockton et al., 2004). In addition, PAK can also promote paracellular pore formation by phosphorylating VE-cadherin, which results in its arrestin-dependent internalization (Gavard and Gutkind, 2006). PAK contains multiple domains that bind scaffolding proteins, such as Nck and Grb2, capable of regulating PAK localization (Lu et al., 1997; Puto et al., 2003). Interestingly, both PAK localization to cell–cell junctions and PAK-mediated permeability were inhibited with a cell-permeable peptide corresponding to the Nck-binding sequence of PAK (Stockton et al., 2004).

Shear stress activates the integrin family of ECM receptors, and new integrin ligation mediates effects of flow on Rac, Cdc42, and Rho activity (Jalali et al., 2001; Tzima et al., 2001, 2002, 2003). Flow-induced GTPase regulation mediates cell alignment in the direction of flow and stimulates the transcription factor NF-κB, which is important for expression of inflammatory genes in the endothelium (Tzima et al., 2002). The idea that integrin ligation mediates these effects suggested that alterations in the subendothelial matrix composition would affect which integrins become ligated, resulting in differential signaling in response to flow. Indeed, shear stress activates NF-κB when endothelial cells are plated on a fibronectin (FN) or fibrinogen matrix, but not when cells are plated on collagen or laminin. Furthermore, FN and fibrinogen were deposited at sites of disturbed flow in vivo, which correlated with expression of NF-κB target genes (Orr et al., 2005). These results suggest that matrix remodeling plays a causal role in atherogenesis. In this work, we investigate the role of flow and ECM in endothelial permeability in atherogenesis.

Results

Flow stimulates matrix-specific PAK activation and localization to cell–cell junctions

The N terminus of PAK contains a Rac/Cdc42 binding domain that overlaps an autoinhibitory domain (AID) such that binding of active GTPases alleviates an inhibitory interaction between the AID and the C-terminal kinase domain (Bokoch, 2003). PAK activation results in autophosphorylation at multiple sites (Gatti et al., 1999; Chong et al., 2001), including Ser141 at the end of the AID. Phosphorylation of this residue prevents the interaction of the AID with the kinase domain to maintain the active conformation. Flow-induced integrin signaling activates both Rac and Cdc42 (Tzima et al., 2002, 2003), suggesting that PAK might be activated. Using PAK Ser141 phosphorylation as a marker, bovine aortic endothelial (BAE) cells were examined. Cells were plated for 4 h on coverslips coated with either FN or diluted matrigel (MG), which, under these conditions, adsorbs to the glass as a thin layer similar to FN. MG was used as a model for normal basement membrane proteins. We found that flow stimulated biphasic PAK activation on FN; however, no significant activation occurred in cells on MG (Fig. 1 A). Collagen also failed to support PAK activation under these conditions (unpublished data). Immunofluorescence staining showed that activated PAK localized to cell–cell borders (Fig. 1 B). No major changes in cell–cell junctions themselves were noted on this time scale (see Fig. 4). Consistent with previous results (Stockton et al., 2004), this localization was abrogated by the addition of a cell-permeant peptide that blocks the binding of PAK to Nck (unpublished data).

Responses to the onset of laminar shear are transient but otherwise resemble events triggered by disturbed flow (Orr et al., 2006). We therefore determined PAK activity in endothelial cells exposed to different flow patterns for longer times. BAE cells plated on FN were stimulated for 24 h with flow profiles derived from the athero-protective common carotid artery (CCA) or the athero-prone internal carotid sinus (ICS; Fig. 1 C; Gelfand et al., 2006). Matrix specificity was not determined in this assay because cell-derived matrices deposited over this extended time course could affect signaling responses. Consistent with the adaptation to flow hypothesis, cells stimulated with ICS flow showed elevated PAK phosphorylation compared with cells stimulated with CCA flow (Fig. 1 D).

Matrix-dependent PAK signaling regulates flow-induced endothelial permeability

Because PAK regulates permeability of endothelial monolayers (Stockton et al., 2004; Gavard and Gutkind, 2006), we tested whether matrix-specific PAK activation correlates with permeability. To assay flow-mediated endothelial cell permeability, we developed a novel transwell assay that used a modified cone and plate device adapted to 75-cm transwell chambers (Fig. 2 A). Using this system, we applied shear to endothelial cell monolayers and assessed the movement of a tracer across the filter. Membranes were then fixed and stained to ensure that no cell loss occurred during the assay. Consistent with previous results, we found that laminar flow transiently increased endothelial cell permeability, which returned to baseline by 4 h (Fig. 2 B).

To determine whether these effects are matrix specific, BAE cells were plated on either FN or diluted MG for 4 h. Endothelial cells formed a complete monolayer with both adherens junctions and TJs as assessed by β-catenin and ZO-1 staining but deposited very little endogenous matrix (Fig. S1, available online). To test whether matrix deposition per se is necessary for the flow-induced changes in permeability, we applied shear stress to cells plated on collagen or laminin matrices. Consistent with previous results (Orr et al., 2006), we found that laminar flow transiently increased endothelial cell permeability, which returned to baseline by 4 h (Fig. 2 B).
at http://www.jcb.org/cgi/content/full/jcb.200609008/DC1). Onset of flow triggered a greater increase in permeability in cells on FN compared with MG or collagen IV (Fig. 3 A). In addition, the low level of permeability in cells on MG was enhanced in a dose-dependent manner when overlaid with FN (Fig. 3 B). Matrix proteins alone without cells did not differentially affect permeability (Fig. S2).

To test whether PAK is involved in flow-induced permeability, cells were either transfected with a construct encoding the PAK AID or treated with a cell-permeant peptide that contains the Nck-binding sequence from PAK. This peptide was previously shown to mimic the dominant-negative effects of kinase-dead PAK, including inhibition of endothelial permeability (Kiosses et al., 2002; Stockton et al., 2004). The peptide blocked the flow-induced increase in permeability by ~80%, whereas an inactive control peptide containing mutations in key proline residues involved in Nck binding (Kiosses et al., 1999) had no effect (Fig. 3 C). Though transfection efficiency with the PAK AID was ~50%, the decrease in flow-induced permeability approached 50%, indicating that it is also highly effective (Fig. 3 D). In addition to HRP, Alexa 488–labeled BSA was also used to determine flow-induced permeability. Absolute permeability to both BSA and HRP were similar (Fig. 3, A and E), and both showed sensitivity to PAK inhibition (Fig. 3 E).

Disturbed flow is known to increase permeability compared with steady or arterial flow patterns (Phelps and DePaola, 2000). To confirm these results in our system, BAE cells on FN were exposed to CCA or ICS flow for 4 h, and permeability was assessed. ICS flow increased monolayer permeability nearly twofold compared with CCA flow (Fig. 3 F). Immunofluorescence revealed that active PAK was localized to cell–cell junctions after 4 h of ICS flow but not after CCA flow (unpublished data). The blocking peptide also inhibited permeability induced by ICS flow (Fig. 3 F) as well as junctional phospho-PAK staining (not depicted). Taken together, these data show that matrix-specific PAK activation triggered by onset of flow or prolonged disturbed flow mediates enhanced endothelial monolayer permeability.

Flow stimulates PAK-dependent paracellular pore formation

Multiple growth factors and other bioactive substances use a pathway in which PAK regulates phosphorylation of MLCK to increase cellular contractility, thereby inducing endothelial cell permeability through formation of paracellular pores (Stockton et al., 2004). To test whether flow induces PAK-dependent paracellular pores, BAE cells were treated with either the control or
PAK-Nck inhibitory peptide, sheared for 30 min, and assayed for the presence of paracellular pores by staining for the adherens junction protein β-catenin. Flow induced the formation of paracellular pores, which was strongly reduced by the pretreatment with the PAK-Nck inhibitory peptide (Fig. 4).

Effects of cytokines and oxidized LDL (oxLDL)

Although flow patterns regulate susceptibility to atherosclerosis, a number of soluble factors also promote atherosclerotic plaque development and likely contribute to endothelial permeability in atherosclerosis. OxLDL stimulates endothelial cell permeability through a Rho-dependent pathway (Essler et al., 1999; Siess et al., 1999). In early atherogenesis, activated endothelial cells and macrophages produce MCP-1, which also stimulates endothelial permeability (Stamatovic et al., 2003), as do the macrophage-derived cytokines TNFα and IL-1β (Martin et al., 1988; Brett et al., 1989). Furthermore, mice deficient in either MCP-1 or TNFα show reduced atherosclerosis (Gu et al., 1998; Ohta et al., 2005). We previously showed that TNFα-induced endothelial permeability was reduced by the PAK-Nck inhibitory peptide (Stockton et al., 2004). To analyze the matrix dependence of these factors, PAK phosphorylation was assessed in endothelial cells plated on FN or MG. Though the time courses were distinct, MCP-1, TNFα, and oxLDL stimulated PAK phosphorylation in cells on FN but not on MG (Fig. 5). In all cases, phosphorylated PAK localized to cell–cell junctions, and this localization was inhibited by the Pak-Nck peptide (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200609008/DC1).

Figure 4. Flow stimulates PAK-dependent formation of paracellular pores. BAE cells plated on FN were treated with either control or PAK-Nck inhibitory peptide (20 µg/ml for 1 h) and stimulated with flow for 30 min, and cell–cell junctions were visualized by staining for β-catenin. (A) Representative β-catenin stains are shown. Arrows indicate the presence of a paracellular pore. (B) The total number of paracellular pores per high power field was determined. 20 fields were counted per experiment (n = 3).

Figure 3. Flow-induced endothelial permeability is matrix and PAK dependent. (A) BAE cells plated on transwell membranes coated with collagen IV, MG, or FN were stimulated with laminar flow at 12 dynes/cm². HRP leak across the membrane was assessed during the first hour of flow; data are presented as absolute solute permeability as described in Materials and methods. Values are means ± SD (n = 3). (B) Endothelial cells were plated on transwells coated with MG and increasing concentrations of FN, stimulated with 12 dynes/cm² laminar flow for 1 h, and HRP leak across the membrane was assessed. Values are means ± SD (n = 3). (C) Endothelial cells plated on FN-coated transwell membranes were treated with either control peptide or PAK-Nck inhibitory peptide (20 µg/ml for 1 h), HRP leak across the membrane was assessed during the first hour of steady laminar flow at 12 dynes/cm². Values are means ± SD (n = 4). (D) Endothelial cells plated on FN-coated transwell membranes were transfected with HA-tagged PAK AID. At 24 h after transfection, monolayers were exposed to flow, and HRP leak across the membrane was assessed during the first hour. Values are means ± SD (n = 3). Transfection efficiency ranged from ~35 to ~50% as determined by immunocytochemistry. (E) Endothelial cells plated on FN-coated transwell membranes were treated with either control or PAK-Nck inhibitory peptide (20 µg/ml for 1 h). Alexa 488–conjugated BSA leak across the membrane was assessed during the first hour of steady laminar flow at 12 dynes/cm². Data (means ± SD; n = 3) are shown as absolute solute permeability. (F) Endothelial cells plated on FN-coated transwell membranes were stimulated for 4 h with CCA or ICS flow. HRP leak across the membrane was assessed during the last 1-h period. Some cells were pretreated with the PAK-Nck inhibitory peptide (20 µg/ml for 1 h) and stimulated with ICS flow in the continued presence of the peptide. Values are means ± SD (n = 3).
We next examined monolayer permeability. All of these factors triggered matrix-dependent increases in permeability (Fig. 6A) that were inhibited by the PAK-Nck blocking peptide (Fig. 6B) and by expression of the PAK AID (Fig. 6C). Thus, effects of a number of atherogenic soluble factors on PAK-dependent permeability are strongly modulated by the ECM.

**PAK is phosphorylated in vivo**

Areas of disturbed flow in vivo show elevated endothelial cell permeability (Himburg et al., 2004; LaMack et al., 2005). These regions also show deposition of FN in the subendothelial ECM and expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1; Orr et al., 2005). We therefore tested whether permeability, PAK activity, and FN correlate in vivo. The carotid arteries from young (20-wk-old) ApoE−/− mice fed either a chow or Western diet were isolated and processed for immunohistochemistry. In mice on a chow diet, the carotid sinus displayed some monocyte infiltration but no foam cell formation. PAK phosphorylation was observed specifically in the atherosclerosis-prone region of these vessels but not nearby athero-resistant regions (Fig. 7A). Nearby sections showed FN in the subendothelial matrix in the same regions of the artery (Fig. 7A). Furthermore, enhanced expression of VCAM-1 was detected in these regions, indicating endothelial activation. The opposite side of the carotid sinus can develop atherosclerosis in some cases but in this mouse shows no FN, VCAM-1, or phospho-PAK staining (Fig. 7B). PAK phosphorylation, VCAM-1, and FN were all enhanced by the Western diet within the carotid sinus (Fig. 7C) but not in athero-resistant regions of the CCA (Fig. 7D). Monocytes recruited to atherosclerosis-prone regions of arteries from these mice also stained positively for phospho-PAK. Thus, PAK activation correlates with subendothelial FN and inflammatory markers.

To determine whether active PAK localizes to cell–cell junctions in atherosclerosis in vivo, aortas from ApoE−/− mice on a chow diet were fixed, excised, and examined en face. Staining for platelet-endothelial cell adhesion molecule 1 (PECAM-1) confirmed the ability to visualize endothelial cell
Aortas from C57Bl/6 mice were used as a source for healthy, measuring leakage of Evans blue dye into the vascular wall. Vascular permeability within the aorta was then assessed by animals on a high-fat Western diet (Reddick et al., 1994). Sclerotic lesions, though plaque development is slower than under these conditions are reported to develop moderate atherosclerosis, 32-wk-old male ApoE−/− mice (chow diet) were given intraperitoneal injections of the PAK-Nck blocking peptide or a control peptide. Mice were killed, and the carotid arteries were removed and embedded in paraffin. Nearby sections were stained for PAK phospho-Ser141, FN, and VCAM-1, and shown at high magnification (40×) with lower magnification (10×) views of the entire vessels shown as insets. A and B show different areas of the same carotid sinus, and C and D show the carotid sinus and CCA, respectively, from the same animal. A 3D representation of the artery indicates the location of the sections. Bars: 50 μm; [insets] 200 μm. (E) Male ApoE−/− mice fed a chow diet for 10 wk were killed, the aortic arch was excised, and en face staining was performed for PAK phospho-Ser141. Representative images are shown at both 20× and 40× magnifications. Bars: (left) 200 μm; (right) 100 μm.

PAK inhibition reduces permeability in atherosclerosis in vivo

To determine whether PAK is responsible for the increased permeability during development of atherosclerosis, 32-wk-old ApoE−/− mice (chow diet) were given intraperitoneal injections of the PAK-Nck blocking peptide or a control peptide. Mice under these conditions are reported to develop moderate atherosclerotic lesions, though plaque development is slower than in animals on a high-fat Western diet (Reddick et al., 1994). Vascular permeability within the aorta was then assessed by measuring leakage of Evans blue dye into the vascular wall. Aortas from C57Bl/6 mice were used as a source for healthy, atherosclerosis-free vessels. Each mouse received 1 mg of peptide at 24 h and 1 h before Evans blue injection via the tail vein. After 30 min, leakage of dye into the aorta was assessed. Although little Evans blue accumulated in the aorta of C57Bl/6 mice, in ApoE−/− mice, dye was apparent at the lesser curvature of the arch and at branch points for major arteries in both the nontreated and control peptide–treated animals (Fig. 8), consistent with known athero-prone regions. The Pak-Nck peptide inhibited 67% of the increase in permeability, relative to healthy vessels. These data suggest that PAK makes an important contribution to permeability in atherogenesis.

Discussion

These data support the concept that remodeling of the subendothelial ECM plays a crucial role in atherogenesis. Previous work demonstrated a correlation between enhanced vascular permeability and atherosclerosis (Ogunrinade et al., 2002). In this work, we present evidence for ECM-specific activation of PAK by atherogenic stimuli, leading to increased permeability. PAK activation may be initiated by disturbed flow, though as atherosclerosis develops, soluble factors such as oxLDL and cytokines produced by immune cells and activated endothelium most likely make major contributions. Importantly, PAK activation at athero-prone sites in vivo correlates with areas of FN deposition. Finally, inhibiting PAK function in vivo reduced permeability in athero-prone regions.

The mechanisms regulating the matrix specificity of PAK activation are presently unclear. Flow-induced Rac activation is equivalent on all matrices (unpublished data), suggesting that there may be matrix-specific signals that inhibit PAK activation. Known mechanisms limiting PAK activation include binding of PAK to Nischarin or hPIP1 and dephosphorylation by the phosphatases PP2A and POPX1/2 (Xia et al., 2001; Koh et al., 2002; Kumar and Vadlamudi, 2002; Alahari et al., 2004). Phosphorylation of PAK by protein kinase A also inhibits PAK activation (Howe and Juliano, 2000). Further examination of matrix-specific PAK activation will be an interesting avenue for future work.

The current data suggest that reducing either PAK activation or localization to cell–cell junctions should reduce the permeability of the endothelial cell layer. Recently, the Ser/Thr kinases Akt and protein kinase G (PKG) were found to phosphorylate PAK at Ser21 within the Nck-binding sequence, inhibiting the interaction between PAK and Nck (Zhou et al., 2003; Fryer et al., 2006). Because blocking the PAK–Nck interaction inhibits localization of PAK to cell–cell borders and decreases endothelial permeability, these kinases might decrease permeability in a similar manner. Indeed, both Akt and cyclic GMP/PKG can decrease vascular permeability (Pearse et al., 2003; Chen et al., 2005; Moldobaeva et al., 2006). Whether PAK is the relevant target for these effects remains to be explored.

The mechanisms by which permeability is elevated in the plaque endothelium are not well understood. Dissolution of intercellular interactions during endothelial cell division and apoptosis, both of which are elevated at athero-prone sites in vivo (Weinbaum et al., 1985; Lin et al., 1988), has been suggested as...
a possible mechanism. However, the correlation between endothelial cell turnover and enhanced permeability in vivo is weak (Penn and Chisolm, 1991; Malinauskas et al., 1995). A more likely mechanism involves TJ's in athero-prone regions, which are discontinuous compared with athero-resistant regions (Okano and Yoshida, 1994). Changes in TJ protein expression, phosphorylation, and reorganization could all contribute to decreased barrier function (Ogunrinade et al., 2002). Both flow and cytokines induce permeability too rapidly for changes in gene expression to be an attractive mechanism. Shear stress stimulates occludin phosphorylation on Ser/Thr residues, which could alter occludin localization to TJ's or function (Sakakibara et al., 1997; DeMaio et al., 2001). VEGF stimulates PAK-dependent VE-cadherin phosphorylation, resulting in its arrestin-dependent internalization and the formation of paracellular pores (Gavard and Gutkind, 2006). Myosin light chain phosphorylation triggers cell contraction and the formation of paracellular pores (Stockton et al., 2004), and contractility appears to be a common pathway for endothelial cell permeability by multiple atherogenic stimuli (Takeya et al., 1993; Essler et al., 1999; Siess et al., 1999; Ogunrinade et al., 2002; Stamatic et al., 2003). PAK inhibition decreases myosin phosphorylation and contractility in endothelial cells (Kiosses et al., 1999; Stockton et al., 2004). Thus, effects of PAK on the cytoskeleton appear to be involved in regulation of permeability, though other events, such as VE-cadherin and occludin phosphorylation, are likely to contribute.

PAK regulates cytoskeletal organization, proliferation, and movement in many cell types, making PAK activity by itself an unlikely target for long-term therapy. For example, PAK inhibition in mice with a cell-permeable peptide was recently shown to mimic Alzheimer's disease (Zhao et al., 2006). However, specific interactions, such as Nck, may offer more attractive therapeutic targets. The ECM dependence of PAK activity may provide an especially attractive means for therapeutic intervention that would be less perturbing than global inhibition of kinase activity.

Materials and methods

Cell culture, transfection, and shear stress

BAE cells (a gift from H. Sage, Hope Heart Institute, Seattle, WA) were maintained in low-glucose DME containing 10% calf serum (CS), 10 U/ml penicillin, and 10 μg/ml streptomycin (Invitrogen). Cells were plated for 4–24 h on 38–× 75-mm2 glass slides (Corning) precoated with collagen IV (20 μg/ml in PBS; Sigma-Aldrich), MG (1:100 dilution in serum-free media; Calbiochem), or FN (10 μg/ml in PBS). After 4 h, cells were fully attached and spread and formed a confluent monolayer. Slides were then loaded onto a parallel plate flow chamber in 0.5% CS, and 12 dynes/cm2 shear stress was applied for varying times as previously described (Orr et al., 2005). To stimulate BAE cells with athero-prone (ICS) or athero-protective (CCA) shear stress profiles, BAE cells were plated as described except in a custom Petri dish and stimulated as previously described (Blackman et al., 2002). Human hemodynamic shear stress profiles were developed from MRI-generated near-wall velocity profiles of normal carotid arteries (Gelfand et al., 2006). Transient transfection of HA-tagged PAK AID was accomplished by Effectene (QIAGEN) using the manufacturer's protocols.

Immunoblotting and immunocytochemistry

Cell lysis and immunoblotting were performed as previously described (Orr et al., 2002). Antibodies used include rabbit anti–phospho-PAK (Ser141; 1:5,000; Biosource International) and rabbit anti-PAK (1:1,000; Cell Signaling Technologies). For immunocytochemistry, cells were fixed with PBS containing 2% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked for 1 h in PBS containing 1% BSA and 10% goat serum. Primary antibodies were incubated with cells in blocking buffer as follows: rabbit anti–phospho-PAK (Ser141; 1:1,500 overnight), rabbit anti–β-catenin (1:200 overnight; Santa Cruz Biotechnology, Inc.), and mouse anti–ZO-1 (1:500 overnight). Cells were then incubated in 1 μg/ml Alexa 488–conjugated goat anti–rabbit IgG or goat anti–mouse IgG (Invitrogen). Slides were mounted with Fluoromount G, and images were taken using the 60× oil-immersion objective on a microscope (Diaphot; Nkon) equipped with a video camera (CoolSnap; Photometrix) using the Inovision ISS software program.

Permeability assays

A novel transwell well-flow device was developed to assay macromolecule permeability across an intact endothelial monolayer using previously established methods (Stockton et al., 2004). In brief, a previously developed cone-and-plate flow device was adapted to accept a 7.5-mm chamber transwell insert (Blackman et al., 2002). Custom flanges mounted on the lid of the Petri dish hold inlet and outlet tubing for the top and lower chambers, respectively, to inject and remove HRP without interrupting flow. Transwell chambers (3.0-μm pore size; Costar) were coated with either MG or FN, and BAE cells were allowed to attach for 4–24 h. Some transwells were coated with a fixed concentration of MG followed by increasing concentrations of FN. For flow experiments, cells on 7.5-mm chambers were serum deprived for 4 h in phenol red-free DME containing 0.5% CS and 2% dextran (w/v) and loaded onto the flow device stage, and shear stress was applied using the modified cone-and-plate device. At desired times, the medium was replaced with fresh medium containing 60 μg/ml HRP (Sigma-Aldrich) or Alexa 488–conjugated BSA (Invitrogen). After 1 h, medium was removed from the lower chamber, and cells were fixed in 2% formaldehyde and stained with Coomassie blue to detect cell loss or examined by immunocytochemistry for Ser141 phosphorylated PAK. For cytokine and LDL-induced permeability assays, cells grown on 6.5-mm filters were serum deprived for 4 h in phenol red-free DME containing 0.5% CS and transferred to fresh medium containing soluble factors for 90 min. HRP was then added to the top well to give a final concentration of 60 μg/ml. After 30 min, medium from the bottom well was removed, incubated with 0.5 mM guaiacol, 50 mM Na2HPO4, and 0.6 mM H2O2, and formation of Ophenylene diamine was determined by measure of absorbance at 470 nm. Alexa 488–conjugated BSA was measured using a spectrophuorometer (Fluorolog; Jobin Yvon). Results are shown as a fold increase in HRP activity or in absolute solute permeability. Solute permeability coefficients for the endothelial monolayer were calculated as P = ΔC ΔtS/ΔCVA, where

Figure 8. PAK inhibition reduces permeability in vivo. C57Bl/6 and ApoE−/− mice fed a chow diet were treated with PAK-Nck inhibitory or control peptides, and leakage of Evans blue dye into the aortas was assessed as described in Materials and methods. Images were recorded by bright field microscopy. Results were quantified by extracting the dye and measuring absorbance at 620 nm. Values were normalized to the dry weight of the aorta (n = 3). * P < 0.05. Representative images are shown.
formaldehyde, and the aortic arch, left carotid sinus, and right carotid sinus were processed for paraffin embedding. For Evans blue assays, six male C57Bl/6 and nine male ApoE-deficient mice (The Jackson Laboratory) were maintained on chow diets for 8 or 32 wk, respectively.

Immunohistochemistry

5-μm paraffin sections were obtained for immunohistochemistry. Immunohistochemistry for adhesion molecules VCAM-1 (Santa Cruz Biotechnol- ogy, Inc.) was performed as previously described (McPherson et al., 2001). After microwave antigen retrieval with antigen unmasking solution (Vector Laboratories), rabbit anti-FN (1:400; Sigma-Aldrich) and rabbit anti-Ser141 phosphorylated PAK (1:250) were applied. Detection of antibodies was with Vectastain Elite kit (Vector Laboratories). Visualization was with diaminobenzidine (DakoCytomation). For en face staining, the aortic arch was cut into rings and stained for either PECAM-1 or Ser141 phosphorylated PAK using Alexa 488–conjugated goat anti–rabbit secondary antibodies to detect localization. Rings were then cut, opened, and mounted between two coverslips for en face viewing by fluorescence microscopy. Images were acquired using the 10× or 40× objective on a microscope (BX51; Olympus) equipped with a digital camera (DP70; Olympus) using ImagePro Plus software (Media Cybernetics).

Permeability to Evans blue in vivo

Mice were injected intraperitoneally with 0.1 ml of either control peptide or the PAK-Nck inhibitory peptide (10 mg/ml) at 24 h and 1 h before Evans blue injection. Evans blue (0.1 ml of 1% dye in PBS) was injected into the tail vein. After 30 min, mice were killed with ketamine/xylazine and perfused through the left ventricle with 10 ml of 4% formaldehyde in PBS, and the aorta was excised from the cusp to the renal artery branches. Bright field microscopy of excised aortas was performed using the 0.5 and 1.2× objectives on a microscope (SZX12; Olympus) equipped with a DP70 digital camera using ImagePro Plus. Aortas were dried and weighed. Evans blue was extracted by incubation in formamide for 24 h at 60°C, and absorbance at 620 nm was determined. Concentration curves for pure Evans blue were used to calculate the total amount of dye extracted, and this value was normalized to the weight of the isolated aorta.

Online supplemental material

Independent of matrix composition, the 4-h plating time is sufficient to allow both adherens and TJ formation, as assessed by staining cells for β-catenin andZO-1, respectively (Fig. S1). Matrix-specific effects on monolayer permeability are not due to differences in matrix permeability, which shows no difference between MG and FN (Fig. S2). Localization to cell–cell junctions is required for PAK-dependent permeability (Stockton et al., 2004), and TNFα, MCP-1, and oxidized LDL all stimulate active PAK localization to cell–cell junctions, which was abrogated by the addition of the PAK-Nck inhibitory peptide (Fig. S3). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200609008/DC1.

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