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Matrix-specific protein kinase A signaling regulates p21 activated kinase activation by flow in endothelial cells

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Abstract

Rationale—Atherosclerosis is initiated by blood flow patterns that activate inflammatory pathways in endothelial cells. Activation of inflammatory signaling by fluid shear stress is highly dependent on the composition of the subendothelial extracellular matrix. The basement membrane proteins laminin and collagen found in normal vessels suppress flow-induced p21 activated kinase (PAK) and NF-κB activation. By contrast, the provisional matrix proteins fibronectin and fibrinogen found in wounded or inflamed vessels support flow-induced PAK and NF-κB activation. PAK mediates both flow-induced permeability and matrix-specific activation of NF-κB.

Objective—To elucidate the mechanisms regulating matrix-specific PAK activation.

Methods and Results—We now show that matrix composition does not affect the upstream pathway by which flow activates PAK (integrin activation, Rac). Instead basement membrane proteins enhance flow-induced protein kinase A (PKA) activation, which suppresses PAK. Inhibiting PKA restored flow-induced PAK and NF-κB activation in cells on basement membrane proteins, whereas stimulating PKA inhibited flow-induced activation of inflammatory signaling in cells on fibronectin. PKA suppressed inflammatory signaling through PAK inhibition. Activating PKA by injection of the PGI_2 analog iloprost reduced PAK activation and inflammatory gene expression at sites of disturbed flow *in vivo*, whereas inhibiting PKA by PKI injection enhanced PAK activation and inflammatory gene expression. Inhibiting PAK prevented the enhancement of inflammatory gene expression by PKI.

Conclusions—Basement membrane proteins inhibit inflammatory signaling in endothelial cells via PKA-dependent inhibition of PAK.

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Disclosures
None

Keywords

Shear stress; extracellular matrix; protein kinase A; p21 activated kinase; NF-κB

Introduction

Atherosclerosis, a chronic inflammatory disease of artery walls, originates as regions of local endothelial cell dysfunction characterized by enhanced permeability, inflammatory gene expression, and turnover[1]. While classic risk factors for atherosclerosis, including hypercholesterolemia, hyperglycemia, and smoking, occur throughout the vasculature, atherosclerotic plaques preferentially form at vessel curvatures, branch points, and bifurcations where blood flow is of lower magnitude and exhibits complex features including turbulence, oscillations, separation and reattachment, which we term disturbed flow[2]. Thus, flow patterns critically regulate the local susceptibility to atherosclerosis.

In vitro, laminar flow inhibits endothelial activation and turnover, whereas disturbed flow induces inflammatory signaling, enhanced turnover and other features of atherosclerosis-susceptible regions of arteries[3]. Interestingly, laminar and disturbed flow both initially activate NF-κB[4] and JNK[5], intercellular adhesion molecule-1 (ICAM-1) expression[6], and enhanced permeability[7]. However, in laminar flow, these events decrease at later times as the cells align in the direction of flow, whereas in disturbed flow, these events remain elevated[3]. Thus, the inability of cells to adapt to disturbed flow may mediate the differential cellular responses to these two flow patterns.

Integrins mediate an important subset of the endothelial response to flow. Shear stress stimulates integrin activation[8, 9], conversion from a low affinity to a high affinity state, which triggers new integrin-matrix binding; integrins thereby regulate both flow-induced endothelial cell alignment and proinflammatory gene expression[10]. Individual integrin heterodimers differ in both their ligand preferences and signaling responses, allowing cells to differentiate between matrix substrates. Endothelial cells are normally on a basement membrane comprised mainly of laminins, collagen and other glycoproteins. In contrast, during inflammation, angiogenesis or wound healing, provisional matrix proteins such as fibronectin and fibrinogen are deposited beneath the endothelium[11]. In *in vitro* flow models, flow-induced proinflammatory signaling (NF-κB)[11], proinflammatory gene expression[11], and endothelial permeability[7] are highly activated in endothelial cells on fibronectin or fibrinogen but not in cells on basement membrane proteins. Indeed, these pathways are actively suppressed in cells on collagen. In vivo, fibronectin is deposited beneath the endothelium at atherosclerosis-prone regions of arteries. Thus, matrix remodeling may play a critical role in atherogenesis.

The p21-activated kinase (PAK) family of Ser/Thr kinases regulate cell growth, migration, cytoskeletal organization and gene expression[12]. Shear stress stimulates PAK activation in cells on fibronectin but not in cells on basement membrane proteins or collagen[7], and PAK mediates matrix-specific activation of the proinflammatory transcription factor NF-κB[13, 14], inflammatory gene expression[13], and increased endothelial permeability[7] by flow. PAK is activated in athero-prone regions *in vivo*, which correlates with areas of fibronectin deposition and inflammatory gene expression[7]. However, the mechanisms mediating matrix-specific PAK activation remain unknown.

PAK is activated by the small GTPases Rac and Cdc42, [12] and suppressed by PAK inhibitory proteins (ex. nischarin, hPIP1)[15, 16], by dephosphorylation by phosphatases (PP2A, POPX1/2) [17,18], and by phosphorylation by protein kinase A (PKA)[19]. We therefore set out to elucidate the mechanism of differential PAK activation on different matrix proteins. Our results identify

PKA as the critical mediator of matrix-specific PAK activation and hence proinflammatory signaling through NF-κB.

Materials and Methods

Briefly, bovine aortic endothelial cells (BAECs) and human aortic endothelial cells (HAECs) on glass slides coated with specific matrix proteins were exposed to laminar or oscillatory flow using parallel plate flow chambers. Rac activation was assayed by affinity pulldown using GST-p21 binding domain (GST-PBD). Rac and Cdc42 were inhibited with dominant negative N17-Rac and N17-Cdc42 transfected using Lipofectamine 2000. PKA activation was determined by cAMP quantification, an *in vitro* kinase assay, and an affinity pulldown approach utilizing GST-PKI[20]. PAK activation was measured by Western blotting using an antibody to phospho-Ser141. NF-κB activation was measured by phosphorylation of the p65 subunit and by p65 nuclear translocation. ICAM-1 expression was measured by Western blotting and quantitative real time PCR. *In vivo* studies were carried out using either 10 week old or 36 to 38 week old male C57Bl/6J mice in which the effects of PKA activation or inhibition on PAK phosphorylation and inflammatory gene expression was determined. Detailed materials and methods are provided in the online supplement.

Results

Regulation of PAK activators

To determine the mechanism of matrix-specific PAK activation, we first investigated the upstream pathway by which shear stress activates PAK in endothelial cells (ECs) on fibronectin. Shear stress stimulates integrins, leading to activation of Rac and Cdc42, both of which can activate PAK[10, 21]. To determine if flow-induced activation of PAK requires new integrin ligation, we pretreated ECs on fibronectin with either the fibronectin blocking (16G3) or a control nonblocking anti-fibronectin antibody (11E5) for 30 minutes. This short term treatment does not significantly disrupt preexisting adhesions or induce cell rounding[10, 21]. Pretreatment with 16G3 but not 11E5 significantly inhibited flow-induced PAK activation (Figure 1A), indicating that shear stress stimulates PAK through integrin ligation.

To address the requirement for Rac and Cdc42,. ECs on fibronectin were transfected with dominant negative N17-Rac or N17-Cdc42. Transfection efficiencies were~70 to 80% (Online Figure I), which allowed biochemical analysis of the entire cell population. Only N17-Rac prevented flow-induced PAK activation (Figure 1B), indicating that Rac is the critical small GTPase in this pathway. To determine whether shear stress-induced Rac activation is matrix-specific, we plated ECs on collagen I, fibronectin, or diluted basement membrane protein (matrigel), which, under these conditions, coats the slides similarly to fibronectin without forming a gel[5, 7]. After 4h, cells formed a confluent monolayer, at which time they were sheared for 15 or 30 minutes. Surprisingly, flow activated Rac equally well on basement membrane protein and fibronectin (Figure 1C). Thus, the pathway upstream of PAK is matrix-independent, consistent with previous data that flow-induced reactive oxygen species production[13], which is thought to be regulated by Rac[22], also occurs independently of matrix composition.

Flow-induced PKA activation is matrix dependent

We next investigated possible PAK inhibitory pathways. PKA can directly phosphorylate and suppress PAK[19] and has known anti-inflammatory effects[23, 24]. To investigate this pathway, cells on matrigel or fibronectin were sheared and cAMP was assessed. Flow stimulated a transient increase in cAMP in cells on matrigel but not on fibronectin (Figure 2A). We next assayed PKA using an *in vitro* kinase assay (verified using the PKA activator Br-cAMP and

the PKA inhibitory peptide PKI; Online Figure II). PKA activity increased >2.5-fold in cells on matrigel but showed no change on fibronectin (Figure 2B). An affinity pull-down assay with the PKA pseudosubstrate PKI also showed an increase in ECs on matrigel but not on fibronectin (Figure 2C). Thus, flow stimulates cAMP and PKA on basement membrane protein but not on fibronectin.

Matrigel contains several matrix proteins as well as growth factors. We therefore assayed PKA activity in ECs on purified basement membrane proteins (collagen I, collagen IV, laminin). We also examined the provisional matrix protein fibrinogen, which is deposited into the subendothelial matrix in wounds and at early stages of atherosclerosis[11]. In ECs on collagen I or collagen IV, PKA activation increased ~2-fold after flow (Figure 2D), whereas cells on laminin showed no change and in cells on fibrinogen, PKA signaling decreased. These results suggest that collagens are the main component of matrigel that promote PKA activation in response to flow.

PKA regulates PAK activation

We next tested if basement membrane-associated PKA signaling prevents flow-induced PAK activation. ECs on matrigel were treated with membrane-permeable PKA inhibitory peptide (myristoylated $PKI_{14–22}$) for 15 minutes, and PAK activation was determined. PKI rescued flow-induced PAK activation on matrigel (Figure 3A) to near levels in cells on fibronectin (Figure 1A). siRNA against the PKA Cα catalytic subunit (~70% knockdown; Online Figure III) similarly restored flow-induced PAK activation in cells on matrigel (Figure 3B). These results show that PKA activation suppresses PAK on basement membranes.

Effects on inflammatory signaling

Low PAK activity mediates the inhibition of flow-induced NF-κB activation and inflammatory gene expression in ECs on basement membrane proteins[11, 13]. We therefore investigated whether PKA suppresses flow-induced NF-κB activation through PAK inhibition. When ECs on matrigel were treated with PKI flow-induced NF-κB activation was restored as indicated by nuclear translocation (Figure 3C; Online Figure IV) and phosphorylation of p65 on Ser536 (Figure 3D). Rescue of NF-κB in PKI-treated cells was blocked by a cell-permeant PAK inhibitory peptide (PNP)[13, 25] (Figure 3C/D),indicating PAK was involved. Additionally, expression of the NF-kB-dependent gene ICAM-1 in HAECs on matrigel showed similar dependence on PAK and PKA (Figure 3E). Taken together, these results indicate that PKA activation in cells on matrigel suppresses NF-κB by inhibiting PAK.

To further test this idea, ECs on fibronectin were treated with forskolin, a direct activator of adenylate cyclase[26], or iloprost, a prostacyclin analog that stimulates the Gαs-dependent activation of adenylate cyclase[27]. Both agents blocked flow-induced PAK activation on fibronectin (Figure 4A). These agents may also stimulate signaling through the Epac/Rap1 pathway, however, the Epac activating compound 8-pCPT-2′-O-Me-cAMP did not inhibit PAK activation on fibronectin (Online Figure V), implicating PKA as the primary regulator. Furthermore, pre-treatment with iloprost also inhibited flow-induced NF-κB phosphorylation (Figure 4B) and nuclear translocation (Figure 4C; Online Figure VI).

PKAin oscillatory flow

To investigate whether the matrix-specific events observed with onset of laminar shear apply to disturbed flow, ECs were exposed to oscillatory flow (±5 dynes/cm^2) for 18h. Assays for PAK, NF-κB, and PKA activation showed enhanced PAK and NF-κB activity on fibronectin compared to matrigel (Figure 5A/B/C). In contrast, oscillatory flow increased PKA activity ~2-fold in cells on matrigel compared to fibronectin (Figure 5D).

We next asked whether matrigel-associated PKA activation also mediates suppression of PAK activation and NF-κB-dependent gene expression. ECs were transfected with mCherry-tagged PKI, then plated on matrigel and exposed to oscillatory flow for 18 hours. Despite 40–50% transfection efficiencies, PKI significantly increased PAK activation in oscillatory flow compared to control vector (Figure 6A). Additionally, HAECs on fibronectin were sheared in the presence of the phosphodiesterase (PDE) inhibitor IBMX to stimulate cAMP accumulation and PKA activation. Forskolin and iloprost were not used in these experiments due to the amounts required to reach the target concentration in the large volume of flow medium. IMBX (100 μM) completely inhibited PAK activation (Figure 6B), NF-κB phosphorylation (Figure 6C) and ICAM-1 expression (Figure 6D) by oscillatory flow. Constitutively active PAK (T423E) restored NF-κB activation (Figure 6E) and ICAM-1 expression (Figure 6F) by oscillatory flow in the presence of IBMX. Taken together, these data show that, similar to onset of laminar shear, basement membrane protein suppresses inflammatory activation of ECs by oscillatory flow via PKA activation and PKA-dependent PAK inhibition.

Activating PKA limits PAK activation in vivo

PGI_2 analogs, such as iloprost, reduce inflammatory gene expression and vascular permeability both *in vitro* and *in vivo*[28, 29]. We therefore assessed whether iloprost mediates some of its protective effects through PAK inhibition using aged C57Bl/6J mice that show a modest level of chronic inflammation in regions of disturbed flow (e.g., the carotid sinus). Male C57Bl/6J mice (chow diet; 36–38 weeks old) received intraperitoneal injections of iloprost (20 μg; ~1 mg/kg). At 2.5 or 24 hrs post-injection, control mice showed PAK phosphorylation in the EC layer at the atheroprone carotid sinus, concomitant with ICAM-1 and VCAM-1 expression (Figure 7A). In contrast, mice treated with iloprost for 2.5 hrs show a significant reduction in PAK activation (Figure 7B) as well as ICAM-1 (Figure 7C) and VCAM-1 (Figure 7D) expression. PAK activity and ICAM-1 expression remained lower at 24 hours post-treatment; however this effect did not reach statistical significance, mostly likely because iloprost has a short half life *in vivo*[30]. Thus, activation of PKA can suppress PAK activation in atheroprone regions of arteries *in vivo*.

Since the effects of iloprost may result from secondary hemodynamic alterations, we also tested whether PKA inhibition could stimulate PAK activation and inflammation in otherwise healthy mice. Male C57Bl/6J mice (chow diet; 10 weeks old) were given retroorbital injections of PKI (24 μg, ~1.2 mg/kg) with or without the PAK inhibitory peptide (PNP; 50 μg, ~2.5 mg/kg). After 2.5 hrs, PKI treatment significantly increased PAK activation within the carotid sinus compared to control mice along with ICAM-1 and VCAM-1 staining (Figure 8). PNP did not significantly affect PAK activation, consistent with previous reports that it inhibits downstream PAK signaling but not PAK activation directly, but it significantly reversed the effects of PKI on ICAM-1 (Figure 8C) and VCAM-1 (Figure 8D). Taken together, these results suggest that PKA signaling limits inflammation at least in part through PAK inhibition.

Discussion

Previous work showed that PAK is activated by flow in cells on fibronectin but not collagen or basement membrane protein, and that it subsequently regulates EC inflammatory gene expression and endothelial monolayer permeability[5, 7, 13, 31, 32]. We now show, first, that PAK activation by flow is matrix-specific despite equivalent activation of its direct upstream element Rac, suggesting that basement membranes actively suppress PAK. PKA was identified as the critical suppressor of PAK in this system, based on the matrix-specific cAMP production and PKA activation, the restoration of PAK activation in cells on basement membranes by inhibitors of PKA, and the suppression of PAK in cells on fibronectin by activators of PKA. Collagen appears to be the active component of the basement membrane. Furthermore, we

show that PKA controls matrix-specific activation of NF-κB and downstream genes through PAK. Lastly, we show that pharmacological activation of PKA inhibits PAK activation at sites of disturbed flow *in vivo*. Taken together, these data demonstrate that basement membrane proteins inhibit PAK and downstream inflammatory signaling through PKA. These results further suggest that PAK inhibition may mediate a subset of the anti-inflammatory and barrier protective properties ascribed to PKA activators[24, 28, 29].

Although PKA has long been associated with the EC responses to flow[33], the molecular mechanism of activation is unknown. PKA is classically activated through Gαs-dependent activation of adenylate cyclase. While flow was reported to activate EC G protein-coupled receptors[34] and G proteins[35], this work implicated Gαi and Gαq subclasses, not Gαs. Furthermore, these G protein-dependent signals affected the early calcium-dependent nitric oxide (NO) production[36], but not the late calcium-independent NO production mediated by PKA. Ligation of the collagen-binding integrin α2β1 by the perlecan fragment endorepellin stimulates PKA[37], and direct application of mechanical force to β1 integrins can stimulate Gαs-dependent cAMP production and PKA activation[38]. However, there is currently no information about how integrins affect Gαs signaling.

Recently, PKA signaling was implicated in flow-induced EC alignment in cells plated on the connecting segment-1 (CS-1) fragment of fibronectin, a ligand for integrin α4β1[39]. Flow-inducedα4β1 ligation induced polarized PKA activation, which was required for flow-induced EC alignment [39]. However, ECs express high levels of the integrin α5β1, which is the endothelial fibronectin receptor in cells from multiple vascular beds[40, 41], whereas expression of α4β1 is less abundant and restricted to a small subset of ECs *in vivo*[42]. One potential implication of α4β1-dependent PKA activation is that α4β1 may serve as a protective signaling pathway limiting EC dysfunction induced by the α5β1-fibronectin interaction. In support of this idea, fibronectin stimulated the α5β1-dependent expression of NF-κB target gene MMP-9 in fibroblasts, which was diminished by α4β1 expression[43].

The molecular mechanism by which PKA inhibits PAK activation is currently unknown. Previous work (Howe and Juliano, 2000) suggested that PKA phosphorylates PAK directly[19] though at an unknown site. Alternatively, PKA could mediate PAK suppression indirectly through activation of eNOS and protein kinase G, which similarly phosphorylates PAK directly[44]. PKA-dependent PAK suppression could also result from regulation of protein phosphatases. PP2A dephosphorylates PAK limiting its activation[18], and PKA can directly stimulate PP2A phosphatase activity[45]. Future work will be required to determine the mechanism by which PKA inhibits PAK.

Multiple stimuli, including PGI_2 and adiponectin, reduce endothelial permeability through PKA signaling[24, 28, 46]. Proposed mechanisms for PKA-dependent barrier protection include inhibition of Rho[47] and activation of Rac[28]. Active PAK destabilizes EC cell-cell junctions by enhancing cortical actin contractility[32] and stimulating VE-cadherin internalization [31], and PAK inhibitors reduce endothelial permeability *in vitro*[7, 32] and *in vivo*[7 48]. Thus, PKA-dependent PAK inhibition may mediate, at least in part, PKA's barrier protective effects.

PAK signaling is classically associated with cell proliferation and migration[12]. As such, previous work has established PAK as a key regulator of vascular remodeling during development[49], angiogenesis[25], and restenosis[50]. Our work was the first to demonstrate a role for PAK signaling in EC inflammatory activation in atherogenesis. However, the therapeutic potential of long-term PAK inhibition is uncertain. Global PAK inhibitors are likely to induce deleterious side effects; a peptide inhibitor of PAK caused Alzheimer's-like symptoms in mice presumably due to inhibition of PAK3 in neuronal tissue[51]. As an alternative approach, identifying endogenous inhibitory pathways, such as PKA, may provide a more selective

reduction of PAK activation under defined circumstances. The PDE inhibitor IBMX blocks PAK activation and inflammatory gene expression by oscillatory flow, and PDE4 inhibitors are currently in clinical trials as immunosuppressive agents used to treat asthma and chronic obstructive pulmonary disease[52]. However, global PKA activation may also produce deleterious side effects, and identifying endothelial cell-specific molecular targets (ex. adenylate cyclase isoforms, PDEs, A kinase anchoring proteins) will likely be required for efficient therapeutic intervention. PKA has been found to inhibit NF-κB in other systems through unknown mechanisms; the current work therefore suggests that inhibition of PAK could play a wider role in this effect.

Novelty and Significance

What Is Known

- Vascular regions exposed to disturbed blood flow profiles are more susceptible to atherosclerotic plaque formation.
- Basement membrane proteins suppress flow-induced endothelial cell activation while wound-associated matrices such as fibronectin and fibrinogen support flow-induced endothelial cell activation.
- Enhanced p21 activated kinase (PAK) signaling in endothelial cells on fibronectin promotes flow-induced endothelial cell activation in both in *in vitro* and *in vivo* model systems; however the mechanisms mediating matrix-specific PAK signaling were unknown.

What New Information Does This Article Contribute

- Basement membrane proteins enhance flow-induced protein kinase A (PKA) signaling to suppress PAK activation.
- Inhibiting PKA reduces the protective effects of the basement membrane on flow-induced PAK activation and inflammation *in vitro* and at sites of disturbed flow *in vivo*, whereas activating PKA blocks PAK activation and inflammatory gene expression in both *in vitro* and *in vivo* model systems.

Regions of disturbed blood flow do not demonstrate significant endothelial cell activation until fibronectin is deposited into the endothelial cell matrix. In endothelial cells in culture, fibronectin promotes flow-induced endothelial permeability and inflammatory gene expression through a PAK-dependent pathway. PAK activation occurs concomitant with fibronectin deposition *in vivo*, and PAK inhibitors reduce vascular permeability and inflammation in mouse models of atherosclerosis. However, the mechanisms controlling matrix-specific PAK activation were unknown. By analyzing how flow activates PAK, we now show that matrix composition does not affect the signaling mediators upstream of PAK. Instead, the endothelial cell basement membrane limits PAK activation through enhanced PKA signaling. PKA possesses both barrier protective and anti-inflammatory properties, and PKA can phosphorylate and inhibit PAK. Basement membrane proteins significantly enhance flow-induced PKA signaling. PKA inhibitors promote flow-induced PAK activation and inflammation in cells on basement membrane proteins *in vitro* and at sites of disturbed flow in young C57Bl/6J mice. Stimulating PKA prevents PAK activation and inflammation in both *in vitro* flow systems and aged C57Bl/6J mice. These results identify a novel molecular mechanism regulating PAK signaling during endothelial cell activation and provide an alternative mechanism by which PKA exerts its protective effects on vascular physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations and acronyms

BAEC	Bovine aortic endothelial cell
CS-1	connecting segment-1
EC	endothelial cell
HAEC	human aortic endothelial cell
ICAM-1	intercellular adhesion molecule-1
IBMX	isobutylmethylxanthine
PAK	p21 activated kinase
PBD	p21 binding domain
PDE	phosphodiesterase
PKA	protein kinase A

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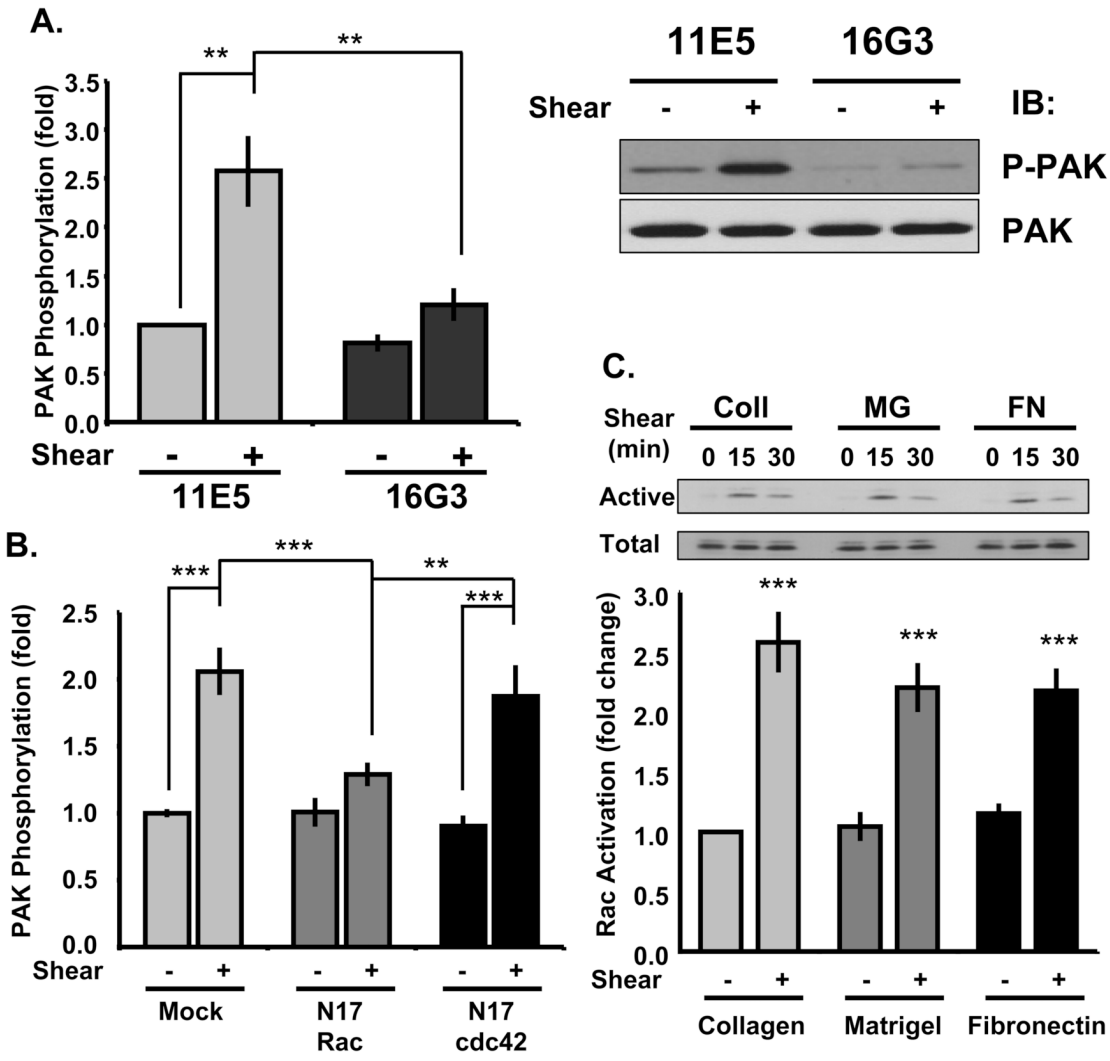

Figure 1. Analysis of the PAK upstream pathway

(A) BAECs on fibronectin were treated with fibronectin-blocking (16G3, 50 μg/ml) or nonblocking (11E5, 50 μg/ml) antibodies for 30 minutes. Cells were sheared for 15 minutes and PAK phosphorylation was determined by Western blotting for phospho-Ser141. Results are normalized to total protein. n = 3. (B) ECs expressing N17-Rac or N17-Cdc42 plated on a fibronectin matrix were sheared for 15 minutes. PAK activation was determined as described. n = 3–5. (C) ECs plated on collagen (Coll), matrigel (MG), or fibronectin (FN) were sheared for the indicated times and Rac activity was determined by affinity pulldown assays. Rac levels in the pulldown at the 0 and 15 minute time points were normalized to total Rac levels in whole cell lysates. n = 4–6. ** $p < 0.01$; *** $p < 0.001$ by multiple comparisons ANOVA.

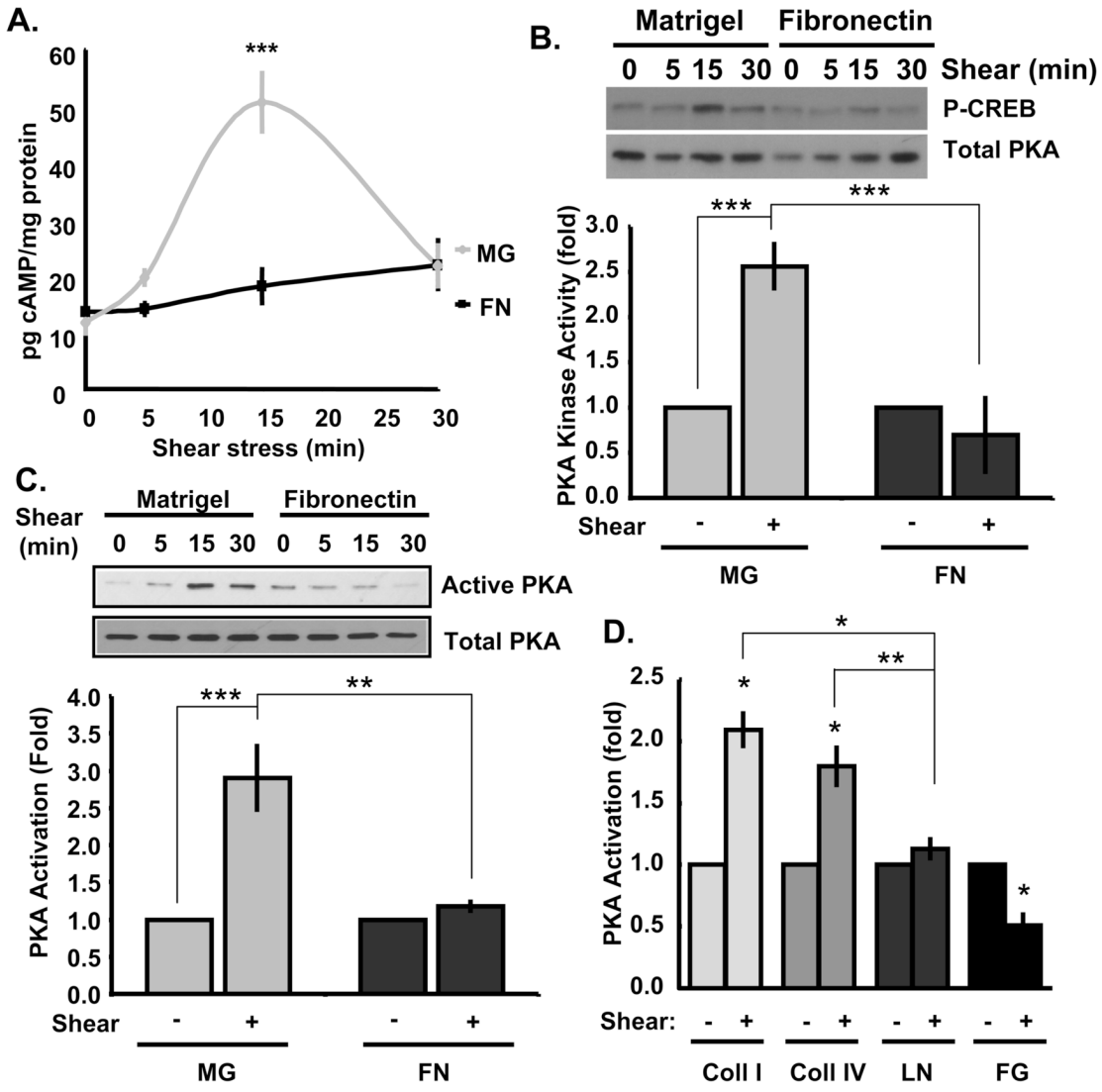

Figure 2. Matrix-specific PKA activation

BAECs plated on matrigel (MG) or fibronectin (FN)-coated slides were exposed to onset of laminar flow for the indicated times. (A) cAMP concentration was determined by competitive ELISA assay and normalized to total protein. n = 4–6. (B) Kinase activity in PKA immunoprecipitates was determined in a kinase assay using GST-CREB as substrate and antibodies specific for phosphorylated CREB. Results at the 0 and 15 minute time points were normalized to PKA levels in the immunoprecipitates and for total CREB. n = 3. (C) PKA activity was determined by affinity pulldown using GST-PKI. Active PKA in the pulldowns was normalized to total PKA in cell lysates using the 0 and 15 min. time points. n = 3. (D) ECs plated on slides coated with collagen I (Coll I), collagen IV (Coll IV), laminin (LN), or fibrinogen (FG) were sheared for 15 minutes, and PKA activity determined by GST-PKI pulldown. n = 3–5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by multiple comparisons ANOVA.

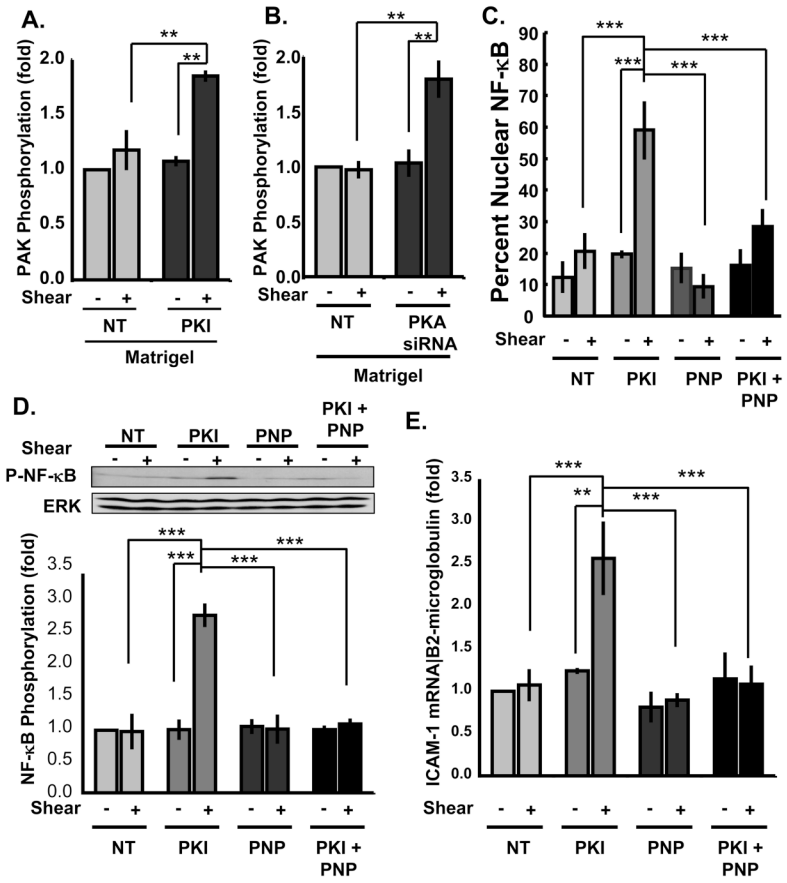

Figure 3. PKA inhibitors rescue inflammatory signaling on MG

(A) BAECs plated on matrigel were treated with the cell permeant PKA inhibitory peptide PKI (20 μM for 15 min.). Cells were then sheared for 15 minutes and PAK assayed as in Fig 1. n = 3. (B) ECs transfected with siRNA against the PKC Cα catalytic subunit (~70% knockdown) were plated on matrigel and sheared for 15 min. PAK was assayed as above. n = 3. (C,D) ECs plated on matrigel were treated with PKI (20 μM for 15 min.), the PAK inhibitory peptide (PNP; 20 μg/ml for 60 min.) or both. Cells were then sheared for 30 minutes and NF-κB nuclear translocation (C) and phosphorylation on Ser536 (D) were determined. Western blots were normalized to total protein, and representative blots are shown. n = 3. For nuclear translocation, more than 100 cells were counted for each condition and scored as positive or negative for nuclear NF-κB. NF-κB nuclear translocation was averaged from three independent experiments. (E) HAECs were plated on matrigel were treated with PKI (20 μM for 15 min.), the PAK inhibitory peptide (PNP; 20 μg/ml for 60 min.) or both and then sheared for 3 hours. ICAM-1 mRNA expression was determined using quantitative real time PCR and normalized toβ2-microglobulin mRNA. ** $p < 0.01$, *** $p < 0.001$ by multiple comparisons ANOVA.

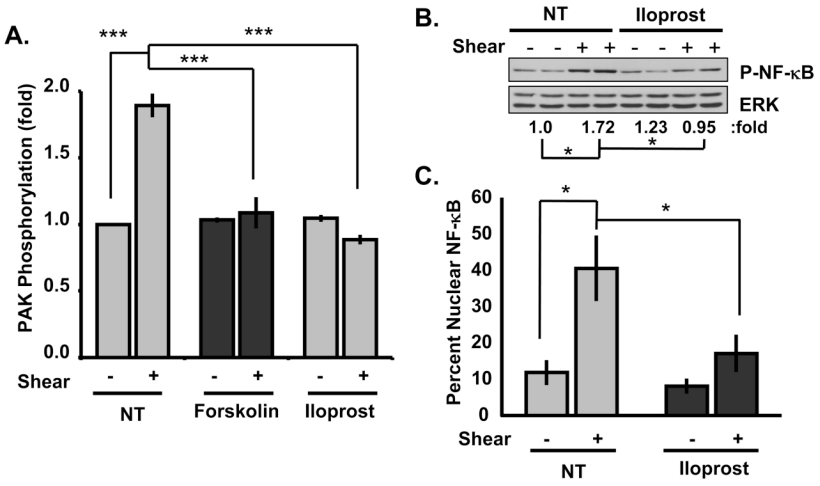

Figure 4. PKA activators inhibit inflammatory signaling on fibronectin

(A) BAECs plated on fibronectin were treated with forskolin (10 μM) or iloprost (1 μM) for 30 minutes. Cells were sheared for 15 minutes and PAK was assayed as in Figure 1. n = 3–6. (B,C) ECs plated on fibronectin were treated with iloprost (1 μM for 30 min.), sheared for 30 min., and (B) NF-κB phosphorylation on Ser536 and (C) nuclear translocation were determined as in Fig 3. n = 3. * $p < 0.05$, *** $p < 0.001$ by multiple comparisons ANOVA.

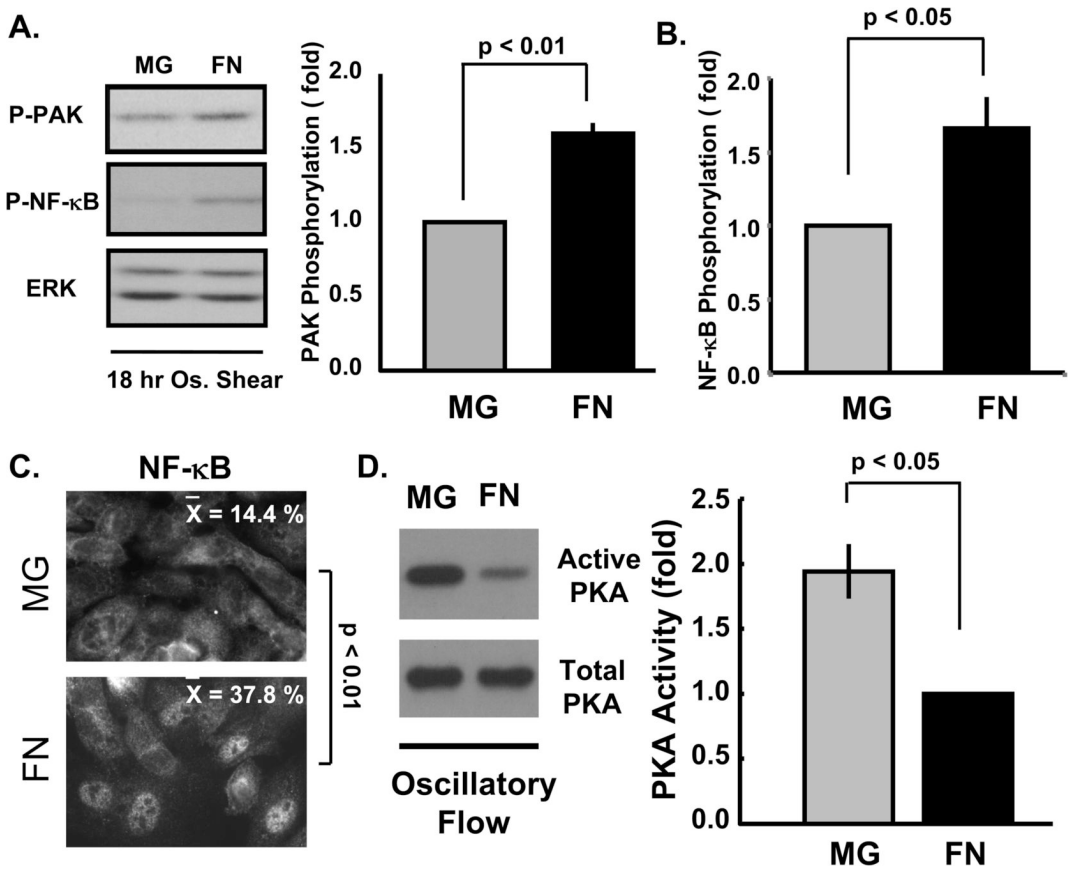

Figure 5. Matrix-specific signaling in oscillatory flow

BAECs plated on matrigel or fibronectin were exposed to oscillatory flow for 18 hours. Activation of (A) PAK and (A,B) NF-κB were determined by Western blotting with phospho-specific antibodies as previously described. n = 3–4. (C) Cells were fixed and NF-κB nuclear translocation was determined as in previous figures. Representative images are shown. n = 3 (D) PKA activity was determined using the GST-PKI affinity beads. Active PKA in the pulldown was normalized to total PKA in the lysate. n = 3. Statistical method was student's T-test.

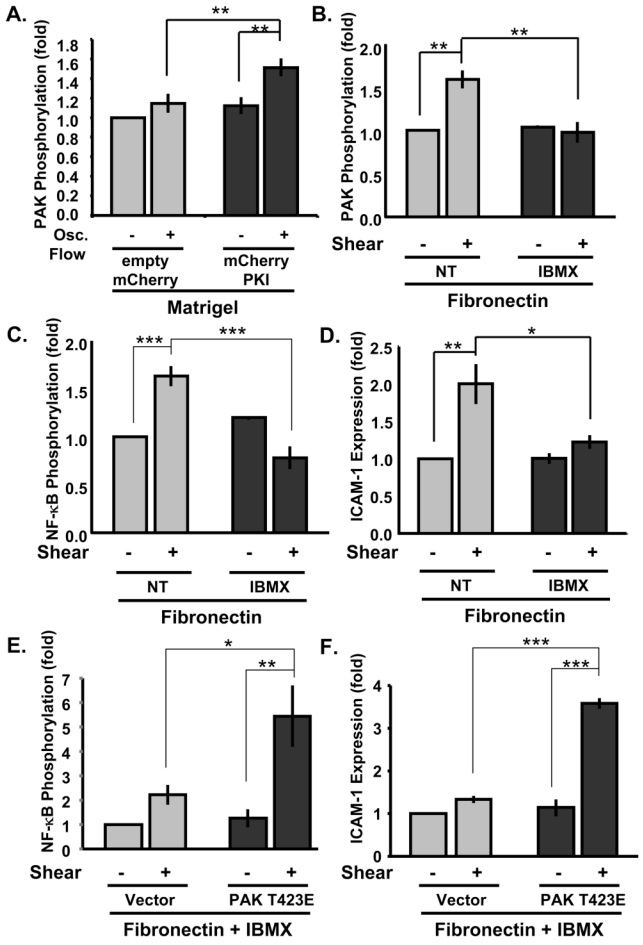

Figure 6. PAK mediates anti-inflammatory PKA signaling in oscillatory flow

(A) BAECs transfected (50% efficiency) with control, empty mCherry vector or mCherry-PKI were plated on matrigel and exposed to oscillatory flow for 18 hours. PAK activation was determined as in Fig 1. n = 4. (B) HAECs plated on fibronectin were exposed to oscillatory flow for 18 hours in the presence of the PDE inhibitor IBMX (100 μM) or vehicle control. (B) PAK phosphorylation and (C) NF-κB phosphorylation were determined. (D) ICAM-1 expression was determined by Western blotting and normalized to total protein. n = 3. (E,F) HAECs transfected with Myc-tagged T423E-PAK or control vector were plated on fibronectin and exposed to oscillatory flow in the presence of IBMX (100 μM) for 18 hours. (E) NF-κB phosphorylation and (F) ICAM-1 expression were determined by Western blotting as previously described. n = 3. * $p < 0.05$, ** $p < 0.01$ by multiple comparisons ANOVA.

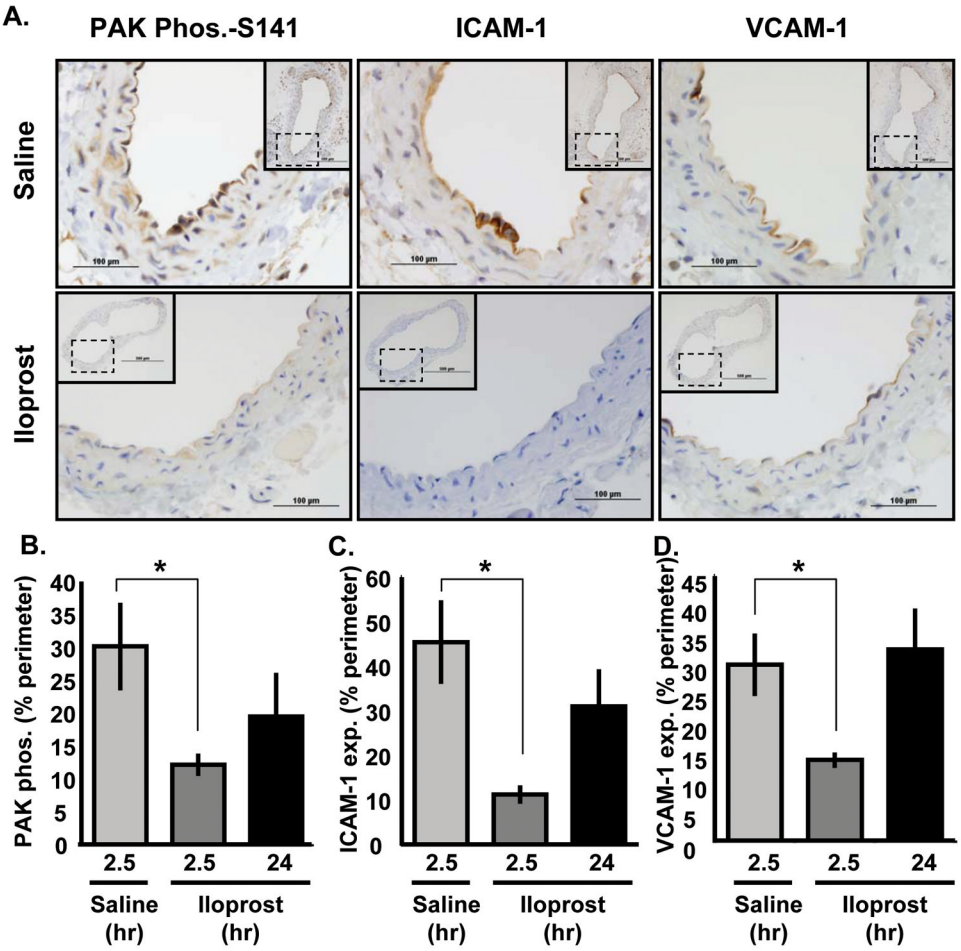

Figure 7. Iloprost reduces PAK activation in areas of disturbed flow in vivo

(A) C57Bl/6J mice at 36–38 weeks received intraperitoneal injection of iloprost (20 μg; ~1 mg/kg) or saline control. After 2.5 or 24 hours, carotid arteries were collected and analyzed for PAK phosphorylation (Ser141), ICAM-1 expression, and VCAM-1 expression by immunohistochemistry. 40X images show the atheroprone region at the outer wall of the carotid sinus. 10X images show the entire artery (insets). (B,C,D) The percent of the vessel lumen staining positive for (B) phosphorylated PAK, (C) ICAM-1, and (D) VCAM-1 was determined. n = 3. * $p < 0.05$ by one-way ANOVA.

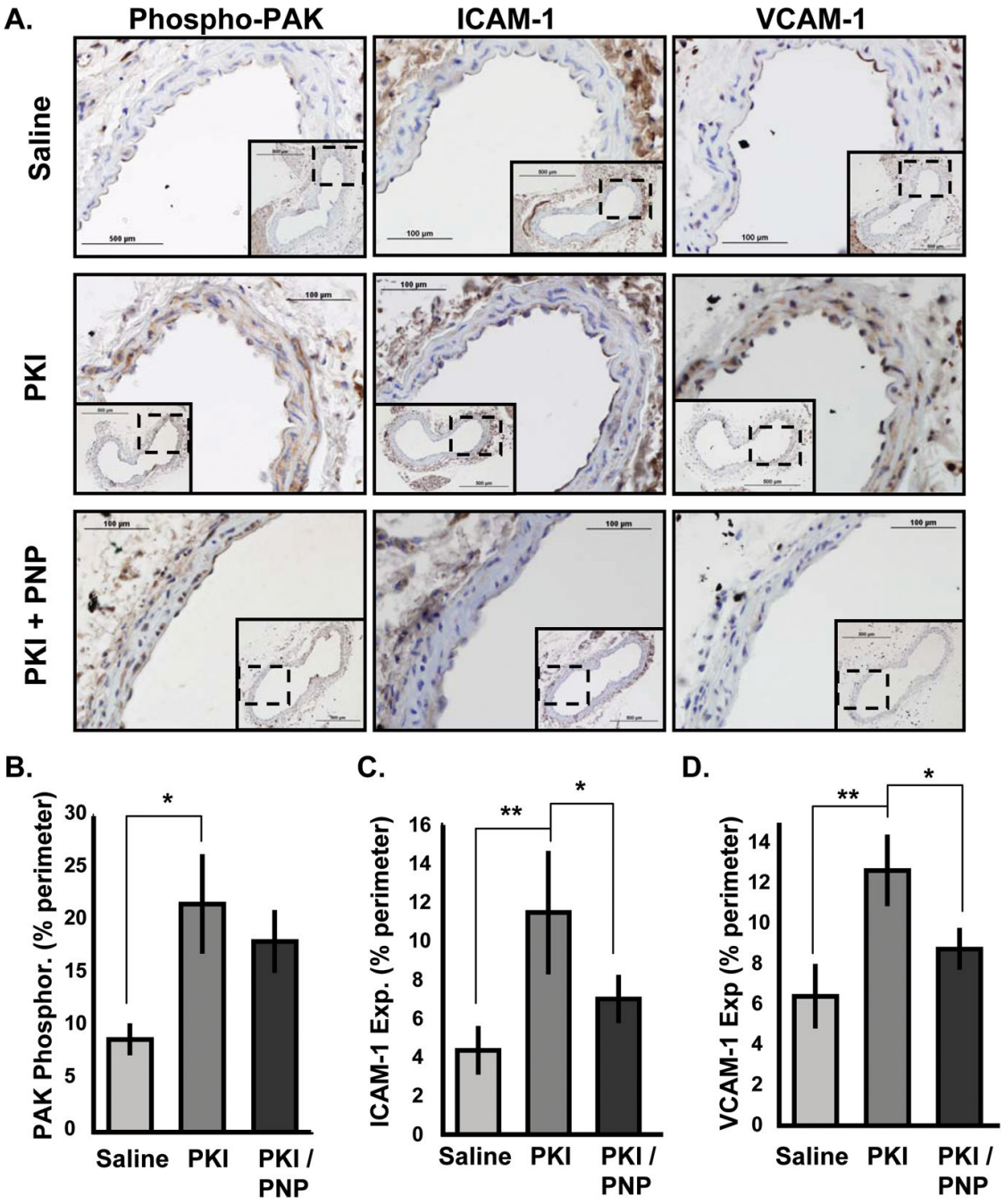

Figure 8. PKA inhibition stimulates PAK activation and inflammation in vivo

(A) C57Bl/6J mice at 10 weeks old received retroorbital injection of saline, PKI (24 μg, ~1.2 mg/kg) or PKI and the PAK inhibitory peptide (PNP, 50 μg, ~2.5 mg/kg). After 2.5 hours, carotid arteries were collected and analyzed for PAK phosphorylation (Ser141), ICAM-1 expression, and VCAM-1 expression by immunohistochemistry. 40X images show the atheroprone region at the outer wall of the carotid sinus. 10X images show the entire artery (insets). (B,C,D) The percent of the vessel lumen staining positive for (B) phosphorylated PAK, (C) ICAM-1, and (D) VCAM-1 was determined. n = 3. * $p < 0.05$ by one-way ANOVA.