SUPPLEMENT MATERIAL

Methods

Cell culture **–** Bovine aortic endothelial cells (BAECs) were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (Gibco), and used between passage 6 to 12. Human aortic endothelial cells (HAECs) were grown in Medium 199 containing 10% FBS, 60 μg/ml heparin, 24 μg/ml bovine brain extract, and antibiotics and used between passage 5 and 10. Glass slides were coated overnight with collagen I (40 μg/ml), collagen IV (20 μg/ml), laminin I (20 μg/ml), matrigel (1:50 dilution), fibronectin (10 μg/ml), or fibrinogen (10 μg/ml). Slides were blocked with 0.2% BSA, and cells were plated onto the slides in low serum media (0.2 to 1% FBS). Slides were loaded onto a parallel plate flow chamber and subjected to different flow patterns in plating medium maintained at 37°C and perfused with 5% CO2. Laminar flow (12 dynes/cm^2) was generated by gravity using a two reservoir system and a peristaltic pump as previously described.[1] Oscillatory flow is generated using an infusion-withdrawal pump (±5 dynes/cm2, 1 Hz) with a 1 dyne/cm^2 forward flow superimposed by a peristaltic pump to facilitate nutrient/waste exchange[2]. BAECs were transfected with HA-N17-Rac, GFP-N17-cdc42, Myc-PAK2 AID, mCherry-PKI, empty mCherry vector, or PKA Cα siRNA using Lipofectamine 2000 (Invitrogen).

Immunoblotting

Samples were separated by gel electrophoresis on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in Tris buffered saline containing 0.1% Tween 20 (TBST), then probed with rabbit anti-phospho-PAK (Ser141; Biosource), goat anti-PAK2, rabbit anti-Rac1, mouse anti-GST, rabbit anti-PKA Cα, goat anti-ICAM-1, goat anti-VCAM-1, rabbit anti-ERK1/2 (Santa Cruz), rabbit anti-phospho-CREB (Ser133), and rabbit anti-phospho-NF-κB (p65 subunit, Ser536; Cell Signaling Technology). Densitometry was performed using ImageJ software.

Immunocytochemistry

Cells were fixed in PBS containing 4% formaldehyde, permeabilized in 0.1% Triton X-100, and blocked in PBS containing 10% goat serum. Cells were rinsed in TBST then incubated 2 hours with rabbit anti-NF-κB p65 (Santa Cruz). Cells were rinsed in TBST and incubated for 1 hour with Alexa488-conjugated goat anti-rabbit IgG (Invitrogen). Cells were rinsed in TBST and mounted onto microscope slides with Fluoromount-G (SouthernBiotech) and viewed on a Nikon Eclipse T*i* inverted fluorescent microscope. Images were captured using the Photometrics Coolsnap120 *ES*^{2} camera and the NIS Elements BR 3.00, SP5 imaging software. Greater than 100 cells were counted for nuclear NF-κB.

cAMP quantification – To assay cAMP, cells were lysed in 0.1 N HCl with 0.1% Triton X-100. Levels of cAMP were determined by competitive ELISA (Calbiochem) per the manufacturer's instructions. Values were normalized to total protein.

PKA kinase activity – Cells were lysed in ice cold lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 1% Triton-X100, 0.5% Igepal, 0.1 mM PMSF, 1% protease inhibitor cocktail (RPI), and 1% phosphatase inhibitor cocktail (RPI). Lysates were centrifuged at 15K rpm for 15 minutes to remove insoluble material, precleared for 30 min with 40 μl of Protein A Sepharose beads (GE Healthcare), and immunoprecipitated with rabbit anti-PKA Cα antibody (0.5 μg, Santa Cruz) by incubating for 2 hours at 4°C with agitation, followed by 2 hour with Protein A-Sepharose (40 μl). Beads were washed three times in 500 μl lysis

buffer followed by two washes with 500 µl kinase reaction buffer (50 mM Tris (pH 7.4), 25 mM MgCl, 4 mM EGTA, 150 µM $NaVO_4$, 1 mM DTT, 500 µM ATP). Following the final wash, 0.5 µg/µl recombinant GST-CREB substrate and 50 µl kinase reaction buffer were added and incubated for 30 minutes at 30°C. Reactions were stopped by adding 20 µl 6X Laemmli buffer. Samples were boiled at 95°C for 10 minutes, centrifuged at 15K rpm for 5 minutes, and stored at -20°C prior to analysis by Western blotting for phosphor-CREB.

PKI pulldown – PKA pseudosubstrate affinity pulldowns were performed as described[3]. GST-PKI on Glutathione-Sepharose beads (GE Healthcare) were resuspended in an equal volume of PBS containing 10 mM DTT and 1% Triton X-100. Endothelial cells were lysed in ice cold 25 mM Tris (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-Mercaptoethanol, 1 mM PMSF, and 1% protease inhibitor cocktail (RPI), and centrifuged for 5 min at 13K rpm to remove insoluble material. A small aliquot of the lysate was mixed with 2X Laemmli buffer and reserved as total lysate. Remaining lysates were incubated with 35 µl of GST-PKI beads for 30 minutes followed by 3 washes in 400 µl wash buffer (50 mM Tris (pH 7.4), 100 µM ATP, and 1 mM $MgCl_2$). 2X Laemmli buffer was added, samples boiled at 95°C for 10 min, centrifuged, and frozen at -20°C.

Quantitative Real-Time PCR – mRNA was isolated using TRIZol per the manufacturer's instructions. First strand cDNA synthesis was performed using a BioRad iCycler and the iScript cDNA synthesis kit. Quantitative real-time PCR was performed on using BioRad SYBR Green Master Mix and a BioRad iCycler using a standard curve to quantify the mean starting quantity, and gene expression determined as a ratio between the gene of interest and a housekeeping gene (ex. β2-microglobulin). Primers used include: β2-microglobulin forward, AGCATTCGGG CCGAGATGTCT; β2-microglobulin reverse, CTGCTGGATGACGTGAGTAAACCT; ICAM-1 forward, TGTCCCCCTCAAAAGTCATC; ICAM-1 reverse, TAGGCAACGGGGTCTCTATG.

Animals and immunohistochemistry – Male C57Bl/6J mice (36 to 38 weeks old) fed standard mouse chow were given intraperitoneal injection of iloprost (20 µg; ~ 1 mg/kg). At 2.5 or 24 hr, the carotid arteries were isolated, embedded in paraffin, and cut into 5 µm sections. Alternatively, young male C57Bl/6J mice (10 weeks old) fed standard mouse chow were given retroorbital injection of control saline buffer (100 µl), PKI peptide (24 µg, ~1.2 mg/kg), or PKI and the PAK inhibitory peptide (50 µg, ~2.5 mg/kg). After a 2.5 hr incubation period, the carotid arteries were isolated and processed as previously described. Sections were processed for antigen retrieval and stained with antibodies specific for phosphorylated PAK (Ser141), ICAM-1, or VCAM-1. Staining was visualized using biotinylated secondary antibodies, the Vectastain ABC kit, and DAB (Dako). Sections were counterstained using hematoxylin. The percent of the perimeter that stained positive for the various antigens was determined using either Image Pro Plus or Nikon Elements software.

Statistical analysis – Statistical comparisons between groups was performed with GraphPad Prism software using Student's T-test, one-way ANOVA with Newman-Keuls post-test, or two-way ANOVA with Bonferroni post-tests where indicated. Error bars indicate standard error.

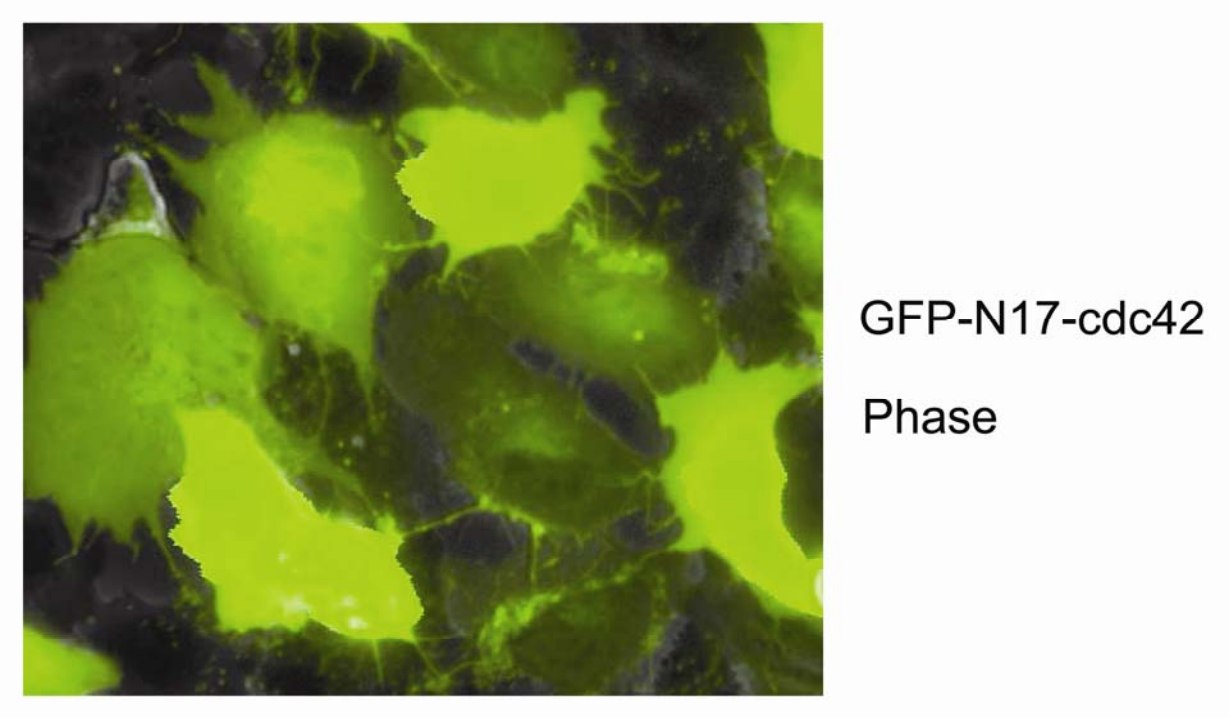
Works Cited

1. Orr AW, Sanders JM, Bevard M, Coleman E, Sarembock IJ, Schwartz MA. The subendothelial extracellular matrix modulates NF-kappaB activation by flow: a potential role in atherosclerosis. *J Cell Biol.* 2005; 169:191-202.

2. Orr AW, Hahn C, Blackman BR, Schwartz MA. p21-activated kinase signaling regulates oxidant-dependent NF-kappa B activation by flow. *Circ Res.* 2008; 103:671-679.

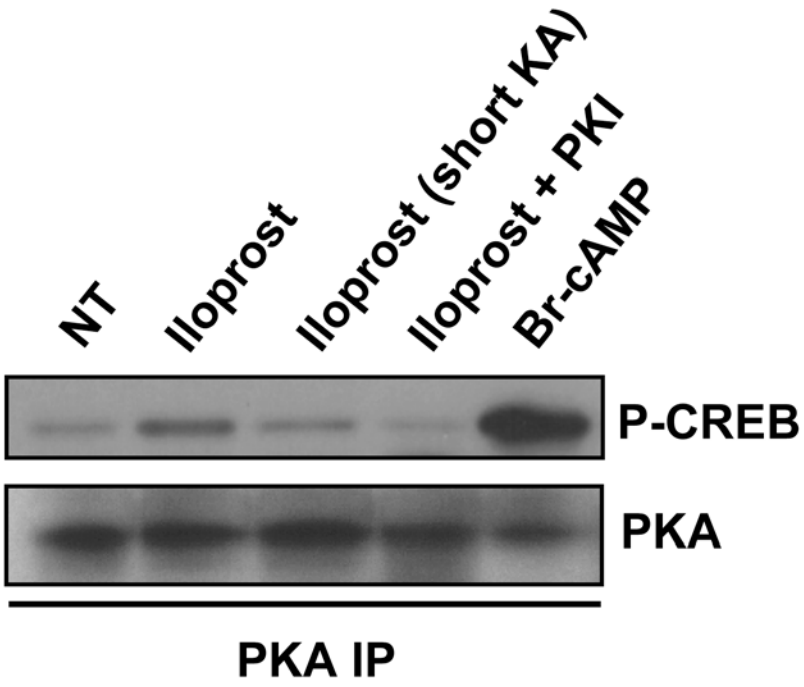
3. Paulucci-Holthauzen AA, O'Connor KL. Use of pseudosubstrate affinity to measure active protein kinase A. *Anal Biochem.* 2006; 355:175-182.

Online Figure I



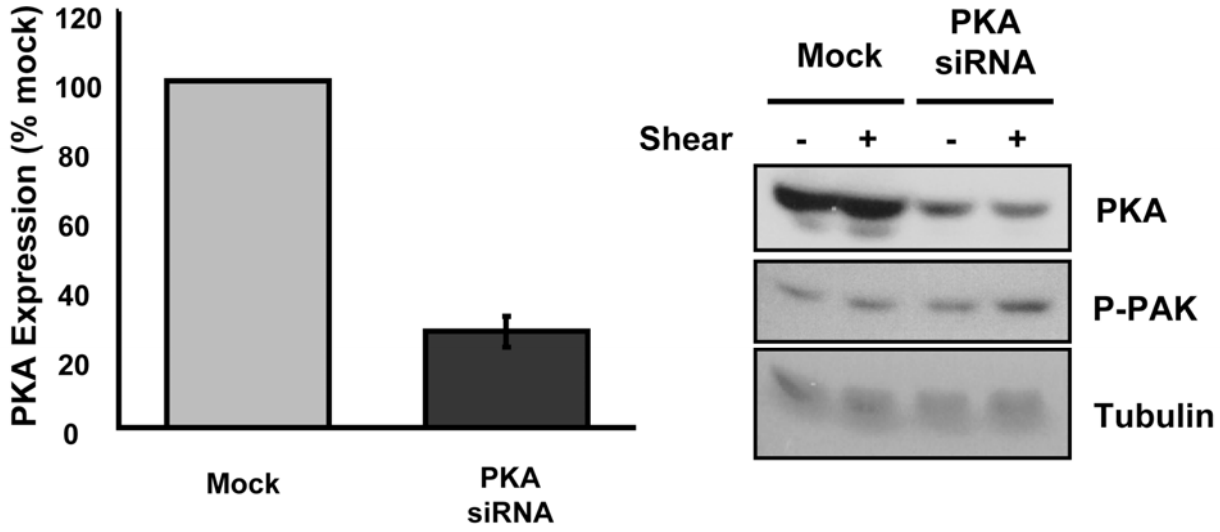
Online Figure I. Demonstration of transfection efficiency. BAECs were transfected with GFP-tagged N17-Cdc42. After 24 hours, the percentage of GFP-positive cells was determined by cell counting to be in the range of 70 to 80%. A representative micrograph is shown. n = 4.

Online Figure II



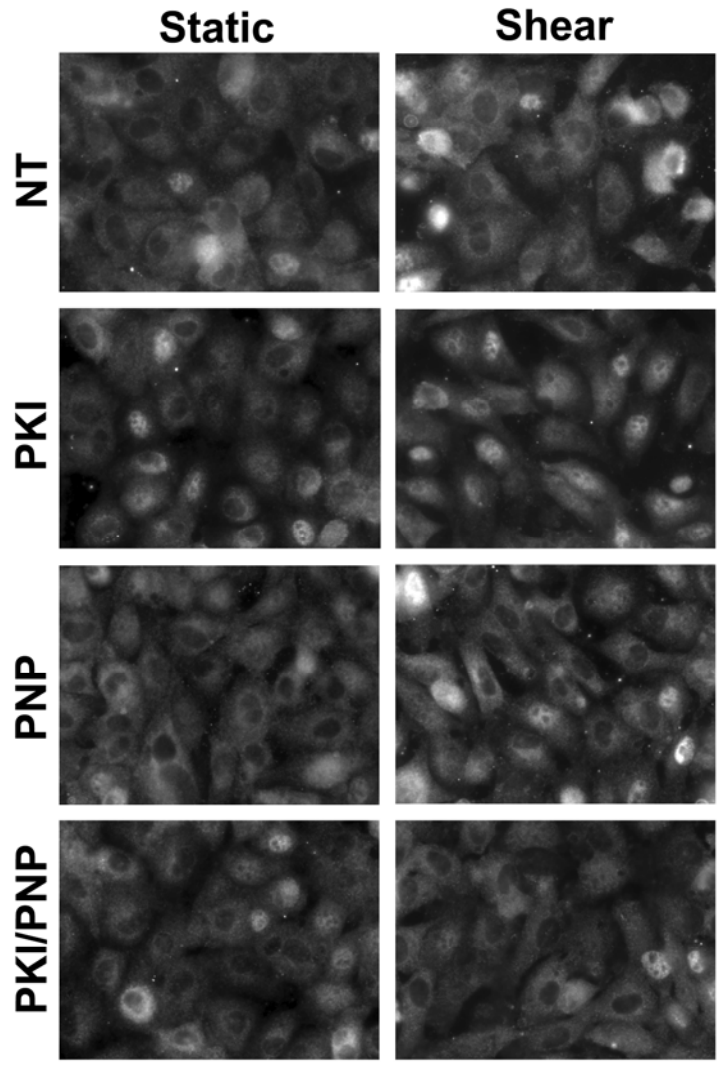
Online Figure II. PKA kinase assay. BAECs were stimulated with iloprost (1 μM) for 15 minutes, and PKA activity was determined by immunoprecipitation and an *in vitro* kinase activity measuring phosphorylation of GST-CREB. Both standard (30 minute) and short (15 minute) kinase assays were performed, and the PKA inhibitory peptide PKI (20 μM) was added to verify specificity of the reaction to PKA. Br-cAMP (250 μM) was added as a positive control to stimulate maximal PKA activation. PKA activity was determined by Western blotting for phosphorylated CREB and normalized to total PKA in the immunoprecipitates. Representative images are shown.

Online Figure III



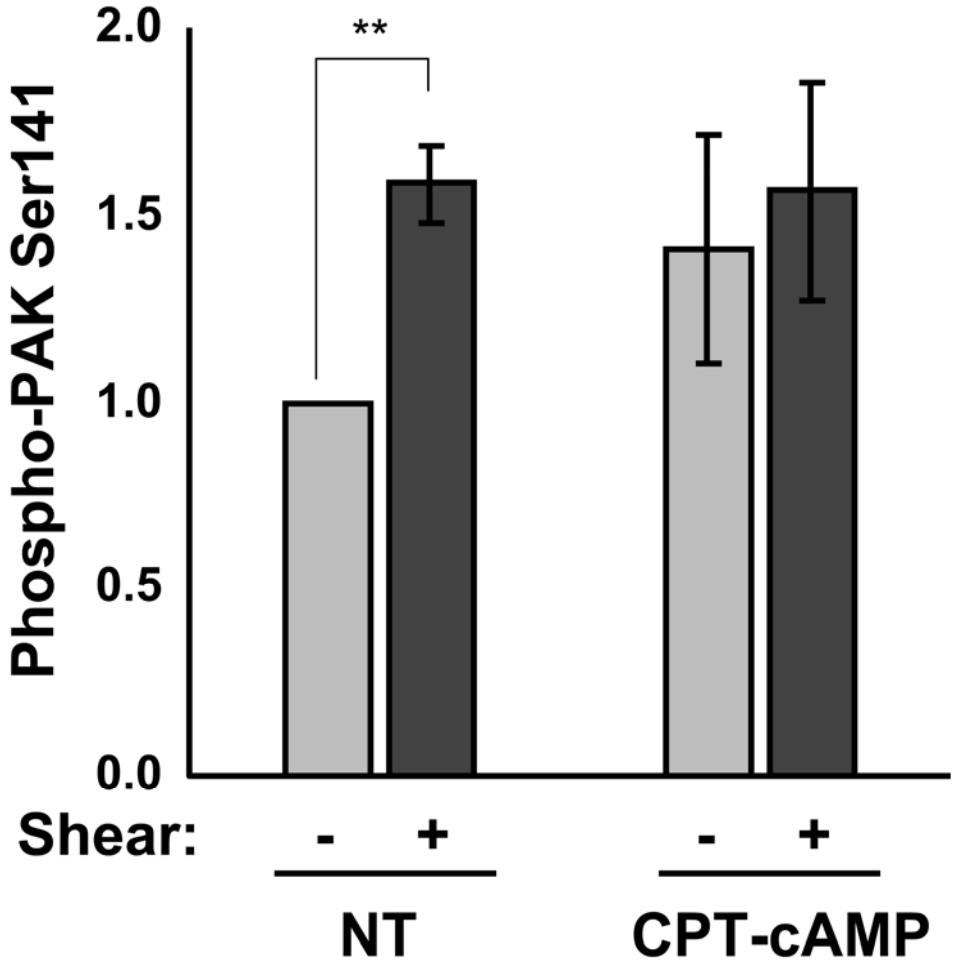
Online Figure III. PKA siRNA efficiency. BAECs transfected with siRNA targeting the PKA Cα subunit were lysed after 24 hrs. Knockdown efficiency was determined to be ~70% by Western blotting for the PKA Cα catalytic subunit. Total PKA Cα levels were normalized to tubulin. n = 3.

Online Figure IV



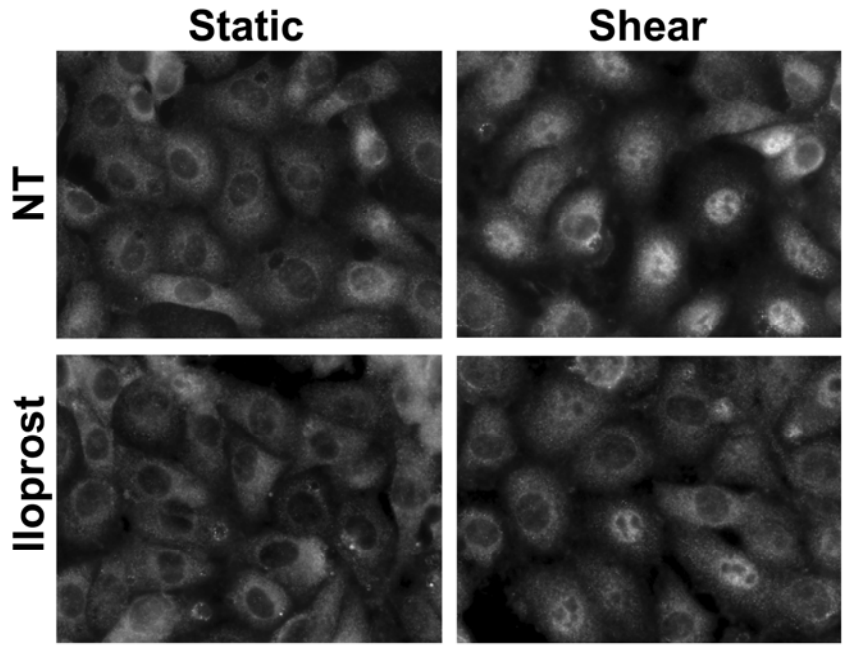
Online Figure IV. PKA inhibitors rescue NF-κB nuclear translocation in cells on matrigel. BAECs plated on matrigel were treated with PKI (20 μM for 15 min.), the PAK inhibitory peptide (PNP; 20 μg/ml for 60 min.) or both. Cells were then sheared for 30 minutes and NF-κB nuclear translocation was determined. Representative micrographs are shown. n = 3.

Online Figure V



Online Figure V. Epac activation does not block flow-induced PAK signaling. BAECs plated on fibronectin were treated with the Epac-specific cAMP analog 8-pCPT-2'-O-Me-cAMP (100 μM) for 15 minutes. Cells were then sheared for 15 minutes and PAK phosphorylation on Ser141 was determined. n =3. ** $p < 0.01$.

Online Figure VI



Online Figure VI. Iloprost inhibits shear-induced NF-κB nuclear translocation. Bovine aortic cells plated on fibronectin were treated with iloprost (1 μM) for 30 minutes. Cells were then sheared for 30 minutes and NF-κB nuclear translocation was determined. Representative micrographs are shown. n =3.