

# p21-Activated Kinase Signaling Regulates Oxidant-Dependent NF- $\kappa$ B Activation by Flow

A. Wayne Orr, Cornelia Hahn, Brett R. Blackman, Martin Alexander Schwartz

**Abstract**—Disturbed blood flow induces inflammatory gene expression in endothelial cells, which promotes atherosclerosis. Flow stimulates the proinflammatory transcription factor nuclear factor (NF)- $\kappa$ B through integrin- and Rac-dependent production of reactive oxygen species (ROS). Previous work demonstrated that NF- $\kappa$ B activation by flow is matrix-specific, occurring in cells on fibronectin but not collagen. Activation of p21-activated kinase (PAK) followed the same matrix-dependent pattern. We now show that inhibiting PAK in cells on fibronectin blocked NF- $\kappa$ B activation by both laminar and oscillatory flow in vitro and at sites of disturbed flow in vivo. Constitutively active PAK rescued flow-induced NF- $\kappa$ B activation in cells on collagen. Surprisingly, PAK was not required for flow-induced ROS production. Instead, PAK modulated the ability of ROS to activate the NF- $\kappa$ B pathway. These data demonstrate that PAK controls NF- $\kappa$ B activation by modulating the sensitivity of cells to ROS. (*Circ Res.* 2008;103:671-679.)

**Key Words:** endothelial cell dysfunction ■ extracellular matrix ■ fluid shear stress ■ NF- $\kappa$ B ■ reactive oxygen species

Atherosclerosis, a chronic inflammatory disease of the artery wall, is highly affected by risk factors such as hyperlipidemia, smoking, and diabetes. These factors, however, are relatively uniform throughout the vasculature, whereas atherosclerosis occurs mainly at vessel curvatures, branch points, and bifurcations that show disturbances in blood flow.<sup>1,2</sup> Endothelial cells (ECs) in these regions show decreased flow-induced nitric oxide release and enhanced inflammatory gene expression, so called EC dysfunction.<sup>3</sup> Systemic risk factors stimulate these sites to develop into fatty streaks, regions of lipid-laden tissue macrophages, and subsequently into atherosclerotic plaques.

Flow patterns critically regulate EC function in vitro. Applying laminar flow to EC monolayers triggers transient activation of signaling events, including increased integrin affinity and activation of Rho family GTPases, nuclear factor (NF)- $\kappa$ B and c-Jun N-terminal kinase (JNK).<sup>4</sup> However, these events are downregulated at later times as cells adapt. Prolonged laminar flow decreases oxidative stress, EC turnover, and inflammatory gene expression.<sup>5</sup> By contrast, disturbed flow stimulates sustained activation of inflammatory events and endothelial turnover.<sup>6-8</sup>

The NF- $\kappa$ B family of transcription factors is an important component of the endothelial inflammatory response. NF- $\kappa$ B consists of heterodimeric protein complexes, the most studied involving the p65 and p50 subunits (hereafter referred to as NF- $\kappa$ B), that stimulate antiapoptotic and proinflammatory gene expression.<sup>9</sup> Inactive p65 is held in the cytoplasm by inhibitory I $\kappa$ B proteins.<sup>9</sup> When activated, the upstream IKK

kinases phosphorylate I $\kappa$ B, leading to its ubiquitination and degradation, thereby allowing p65 to translocate to the nucleus. IKKs also phosphorylate p65 on a critical serine (S536) that modulates transcriptional activity.<sup>10</sup> Multiple atherogenic stimuli, including disturbed flow, inflammatory cytokines, and reactive oxygen species (ROS) activate NF- $\kappa$ B.<sup>11</sup> Atherosclerosis-prone arterial regions show chronic NF- $\kappa$ B activation and NF- $\kappa$ B-dependent gene expression, including adhesion molecules and inflammatory cytokines.<sup>12,13</sup> Endothelial NF- $\kappa$ B is thereby thought to contribute to atherogenesis by modulating inflammatory gene expression.

The pathway by which flow stimulates NF- $\kappa$ B has been studied extensively. Flow appears to act directly on a complex of proteins at cell-cell junctions, resulting in stimulation of phosphatidylinositol 3-kinase and conversion of integrins to a high affinity state.<sup>14,15</sup> Newly activated integrins bind extracellular matrix (ECM) proteins, which initiates intracellular signals that include activation of the small GTPase Rac.<sup>14</sup> Rac activates the NADPH oxidase complex to produce ROS,<sup>16</sup> which stimulates NF- $\kappa$ B-inducing kinase (NIK) and IKK $\beta$ ,<sup>17,18</sup> critical kinases in the classic NF- $\kappa$ B activation pathway. All of these components are required for NF- $\kappa$ B activation by flow.<sup>14,17,19,20</sup>

The composition of the subendothelial ECM dictates which of the many EC integrins bind ligand following flow-induced activation.<sup>21</sup> In accordance with the above model, the subendothelial ECM also strongly influences signaling in response to flow through the distinct signaling properties of different

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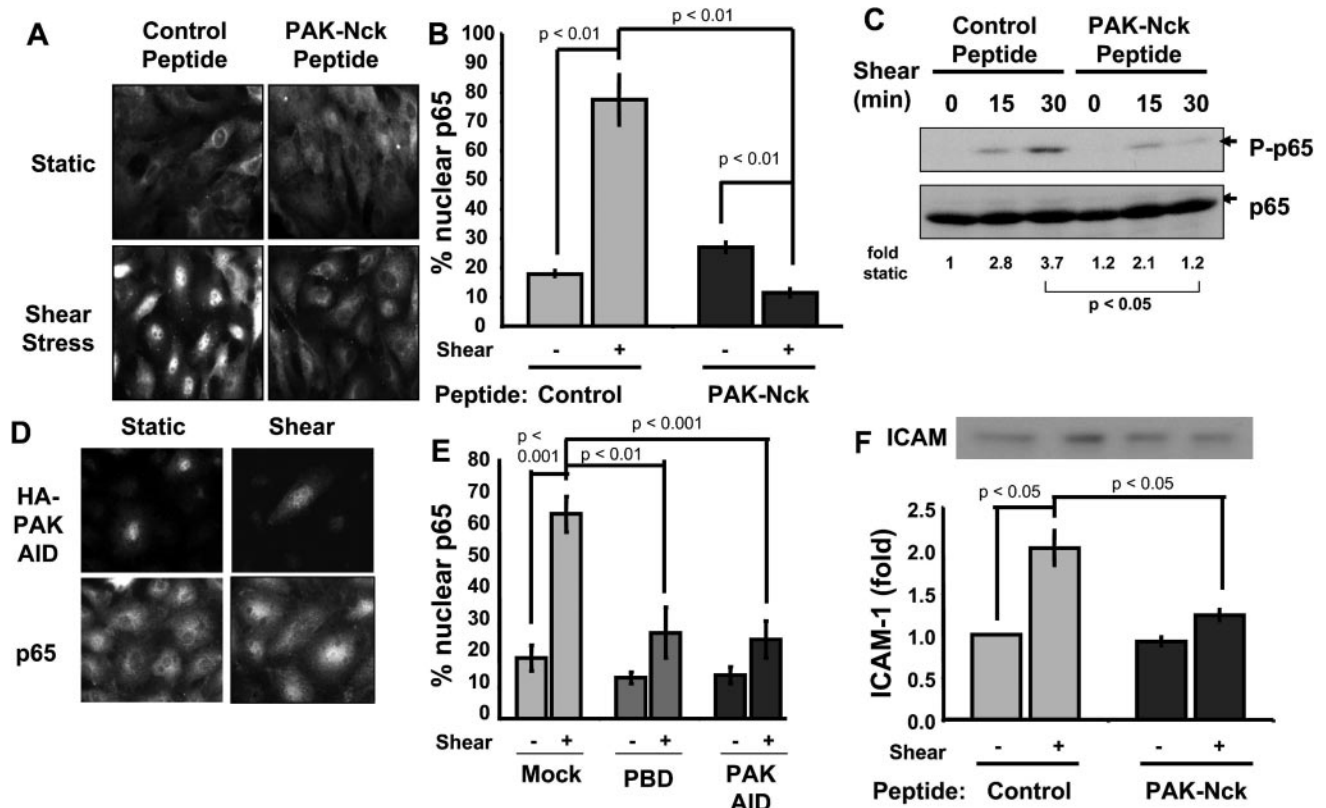
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**Figure 1.** PAK is required for NF- $\kappa$ B activation by onset of flow on FN. ECs plated on FN for 4 hours were pretreated with either control or PAK-Nck inhibitory peptides (20  $\mu$ g/mL for 1 hour) and sheared for 30 minutes. **A**, Cells were fixed and stained for the NF- $\kappa$ B p65 subunit. **B**, Cells from **A** were scored for nuclear p65 (percentage of positive cells). One hundred cells were counted per condition. Values are means  $\pm$  SD (n=3). **C**, Cells were lysed and immunoblotted for p65 phosphorylation on Ser536 or total p65 (n=4). **D**, Flow-induced p65 nuclear translocation was assessed as in **A** in ECs transfected with HA-tagged PAK AID. Cells expressing the AID construct were identified by staining for the HA tag. **E**, Nuclear translocation of p65 was scored (as in **B**) in cells expressing the PAK AID from **D** or with the PAK p21-binding domain (PBD) (n=3). **F**, Cells sheared for 4 hours were lysed and ICAM-1 expression determined by immunoblotting. Values are means  $\pm$  SD (n=3).

integrins. For example, flow activates NF- $\kappa$ B in ECs on fibronectin (FN) and fibrinogen, which are found mainly at sites of injury and inflammation, but not on collagen (Coll) or laminin, components of the normal basement membrane.<sup>22</sup> Importantly, there is little FN or fibrinogen beneath the endothelium in most of the vasculature, but these proteins are found at sites of disturbed flow *in vivo*.<sup>22</sup> This matrix remodeling correlates closely with endothelial inflammatory markers such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Interestingly, deletion of an alternatively spliced domain of FN that reduces its assembly into matrix decreases atherosclerosis in hypercholesterolemic ApoE<sup>-/-</sup> mice.<sup>23</sup> Taken together, these data support a role for matrix remodeling in EC dysfunction and atherosclerosis.

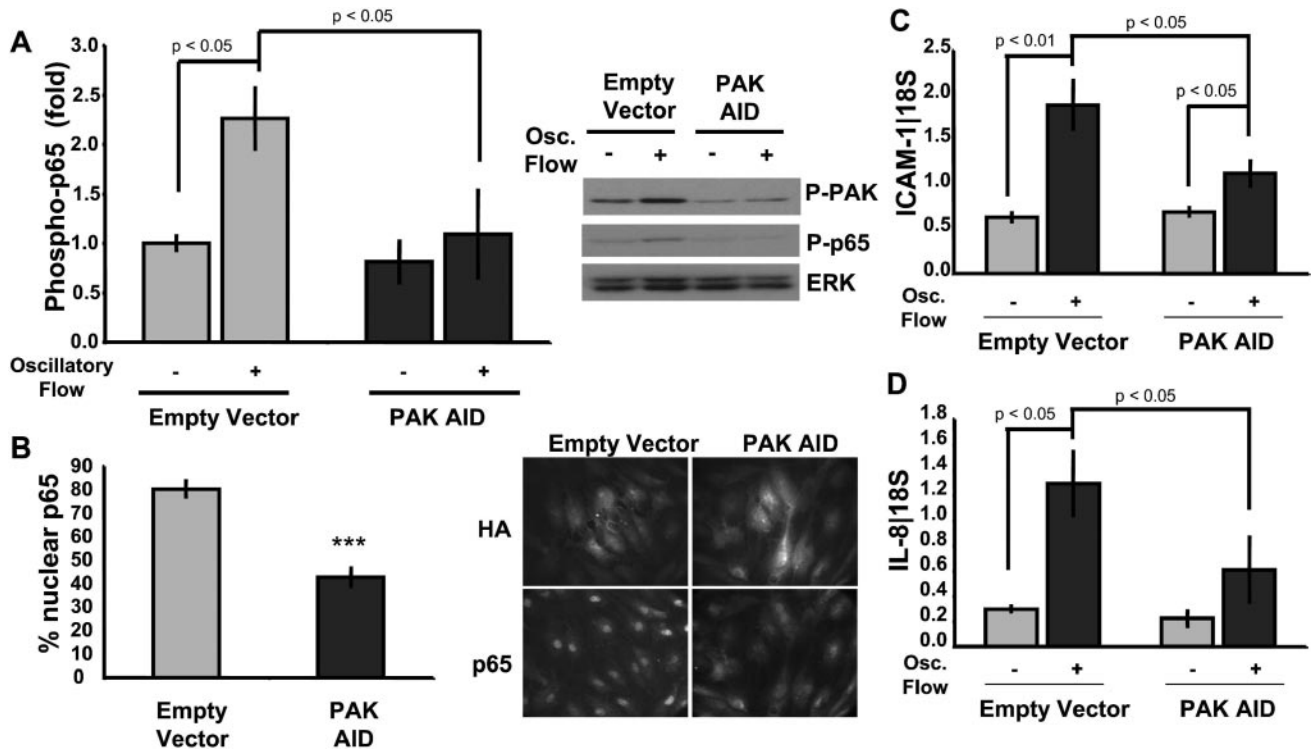
PAK1 to -3 are a group of highly homologous Ser/Thr protein kinases that serve as effectors for Rac and Cdc42.<sup>24</sup> PAK1 and -2 are found in ECs, whereas PAK3 is found largely in the brain. PAK is maintained in an inactive state by its N-terminal autoinhibitory domain (AID), which binds and blocks the kinase domain. Activation results in dissociation of the AID-kinase domain complex and phosphorylation of residues that further block autoinhibition. More than 25 substrates for PAKs have been identified, including many

cytoskeletal proteins, mitogen-activated protein (MAP) kinase pathway components, and regulators of cell survival. PAK also regulates NF- $\kappa$ B activation in a few systems.<sup>25,26</sup> However, this control is by no means universal,<sup>27</sup> and constitutively active PAK does not activate NF- $\kappa$ B.<sup>27,28</sup> Flow activates PAK in ECs, and active PAK regulates junctional integrity and monolayer or vessel permeability.<sup>29</sup> Interestingly, PAK shows the same matrix-dependence as NF- $\kappa$ B, occurring in ECs on FN or fibrinogen but not on Coll or basement membrane protein. Furthermore, PAK activation in mouse arteries correlates with areas of FN deposition and inflammatory gene expression similar to NF- $\kappa$ B.<sup>29</sup>

These findings prompted us to investigate the relationship between matrix-specific activation of PAK and NF- $\kappa$ B in this system. These studies identified a novel role for PAK in matrix-specific NF- $\kappa$ B activation by modulating the ability of ROS to activate NF- $\kappa$ B.

## Materials and Methods

Briefly, our studies used bovine aortic endothelial cells and human umbilical vein ECs treated with various PAK inhibitors and exposed to either laminar flow or oscillatory flow in a parallel plate flow chamber. NF- $\kappa$ B activation was measured by immunoblotting and immunocytochemistry. NF- $\kappa$ B-dependent gene expression was determined by real-time quantitative PCR. Quantification of ROS production was performed using the dye 2,7-dichlorodihydrofluores-



**Figure 2.** PAK is required for sustained NF- $\kappa$ B activation in oscillatory flow. Human umbilical vein ECs transfected with either empty pcDNA3.1 or HA-PAK AID were plated on FN for 4 hours and exposed to oscillatory flow for 18 hours. **A**, Cells were lysed, and phosphorylation of p65 (Ser536) and PAK (Ser141) were determined by immunoblotting using phospho-specific antibodies. Quantified values for p65 phosphorylation were normalized to total protein and shown as fold change compared to static cells. Values are means  $\pm$  SD ( $n=3$  to 4). **B**, Fixed cells were stained for p65 and HA to determine p65 nuclear localization in cells expressing the empty HA vector or HA-tagged PAK AID. The percentage of cells showing p65 in the nucleus was scored. Values are means  $\pm$  SD (100 cells per condition;  $n=4$ ). \*\*\* $P < 0.001$ . **C** and **D**, Inflammatory gene expression was determined by measuring mRNA levels of ICAM-1 (**C**) and interleukin-8 (IL-8) (**D**) using quantitative RT-PCR. Target gene levels were normalized to 18S. Values are means  $\pm$  SD ( $n=3$ ). Results are representative of 3 independent experiments.

cein diacetate ( $H_2$ -DCFDA). In vivo studies were carried out using C57Bl/6J mice, in which effects of PAK inhibition on NF- $\kappa$ B were analyzed by immunohistochemistry.

An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

## Results

### PAK Is Required for NF- $\kappa$ B Activation by Onset of Flow

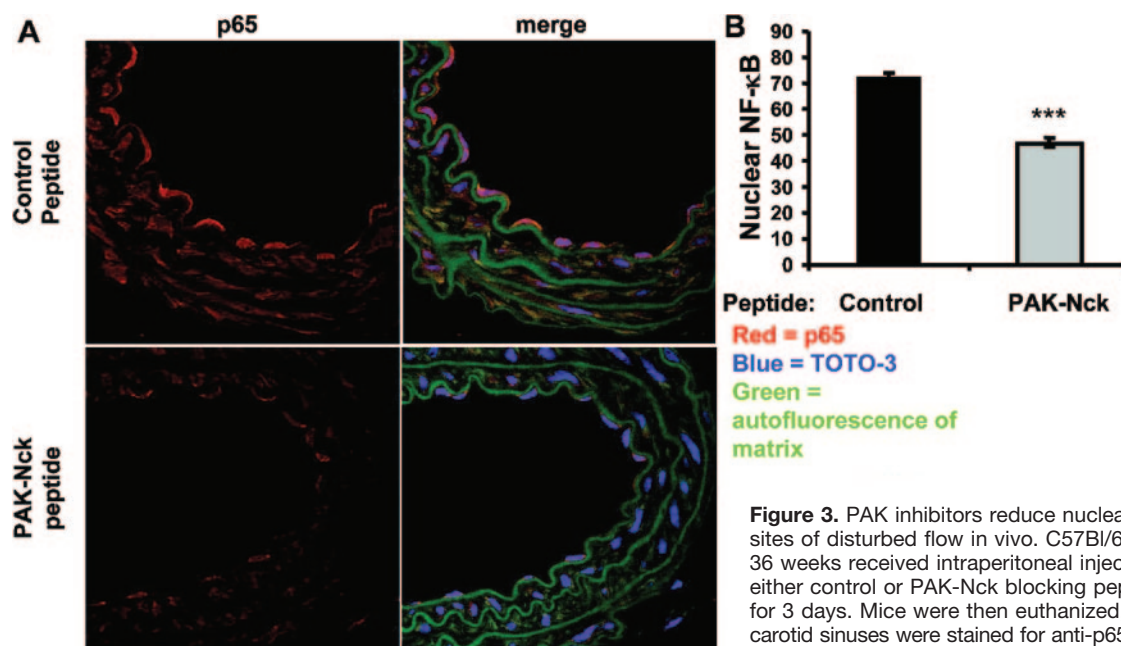
To determine whether PAK is required for flow-induced NF- $\kappa$ B activation in cells on FN, we first used a previously described cell-permeant peptide corresponding to the N-terminal proline-rich sequence of PAK that binds the SH3 adapter protein Nck.<sup>30</sup> This peptide prevents the interaction between PAK and Nck and blocks PAK-dependent changes in endothelial monolayer permeability, migration, and angiogenesis.<sup>29,30</sup> Activation of the classic NF- $\kappa$ B pathway involves IKK-dependent phosphorylation and degradation of the inhibitor I $\kappa$ B, as well as Ser536 phosphorylation and nuclear translocation of p65.<sup>9</sup> Pretreatment of ECs with this peptide completely blocked p65 nuclear translocation in response to flow compared to inactive control peptide (Figure 1A and 1B). Results with control peptide were indistinguishable from untreated cells (not shown). Flow-induced p65 phosphorylation was also substantially reduced by the PAK-Nck peptide (Figure 1C).

To confirm these results, we examined 2 other PAK inhibitors. Expression of a PAK AID construct that blocks kinase activity did not affect basal NF- $\kappa$ B activity but flow-induced p65 nuclear translocation was significantly inhibited (Figure 1D). Transfecting cells with the PAK p21-binding domain, which binds and inhibits the upstream GTPases Rac and Cdc42, also blocked flow-induced NF- $\kappa$ B nuclear translocation (Figure 1E). Finally, the induction of the NF- $\kappa$ B target gene ICAM-1 in response to acute onset of flow was significantly inhibited by pretreatment with the PAK-Nck peptide (Figure 1F).

### Disturbed Flow In Vitro and In Vivo

Both laminar and oscillatory flow initially activate proinflammatory pathways; however, ECs adapt to laminar flow and proinflammatory signals return to baseline or below baseline, although these signals are maintained in disturbed flow.<sup>31</sup> In vitro, both PAK and NF- $\kappa$ B are activated transiently by acute onset of flow<sup>29,32</sup> but in a sustained manner under disturbed flow.<sup>8,29</sup> Both PAK and NF- $\kappa$ B are also activated at sites of disturbed flow in vivo.<sup>12,29</sup> To test whether PAK is required for sustained NF- $\kappa$ B activation by disturbed flow, human umbilical vein ECs transfected with the PAK AID construct ( $\approx 80\%$  transfection efficiency) were plated on FN and exposed to oscillatory flow for 18 hours. The PAK AID completely inhibited both basal (49% reduction,  $P < 0.01$ ) and





the elastic lamina is green. A, Representative images. B, Images were analyzed by confocal microscopy and mean nuclear intensity of p65 staining was compared between the treated and untreated samples ( $n=8$ ).  $***P < 2 \times 10^{-28}$ .

flow-induced PAK activation (67% reduction,  $P < 0.01$ ) (Figure 2A). AID expression also blocked the increase in NF- $\kappa$ B p65 phosphorylation (Figure 2A) and nuclear translocation (Figure 2B). Oscillatory flow-induced expression of the proinflammatory genes ICAM-1 and interleukin-8 were inhibited as well (Figure 2C and 2D). Thus, PAK is required for both transient activation of NF- $\kappa$ B in laminar shear and sustained activation in oscillatory shear.

We next asked whether PAK is required for NF- $\kappa$ B activation at regions of disturbed flow in arteries in vivo. NF- $\kappa$ B activation in regions of disturbed flow colocalizes with FN in the subendothelial ECM,<sup>22</sup> and we previously found that injecting mice with the PAK-Nck peptide reduced vascular permeability at these sites.<sup>29</sup> C57Bl/6 mice have modest PAK and NF- $\kappa$ B activation at locations of disturbed flow in the absence of other markers of atherosclerosis.<sup>12,29</sup> Mice therefore received injections of control or PAK-Nck peptide for 3 days, at which time arteries were examined by immunohistochemistry. Similar to untreated mice,<sup>12</sup> mice treated with control peptide showed nuclear NF- $\kappa$ B in ECs at the expected sites, which was decreased in PAK-Nck peptide treated mice (Figure 3A and 3B). Taken together, these results show that PAK is critical for flow-induced NF- $\kappa$ B activation in vitro and in vivo.

### Rescue by Active PAK

ECs plated on basement membrane proteins, such as Coll and laminin, do not activate either PAK or NF- $\kappa$ B.<sup>22,29</sup> To test whether low PAK activity is rate-limiting for NF- $\kappa$ B activation under these conditions, cells were transfected with WT or active T423E PAK. Active PAK did not directly activate NF- $\kappa$ B in cells on Coll but rescued both p65 nuclear translocation (Figure 4A) and p65 phosphorylation on Ser 536 (Figure 4B) in response to flow, compared to cells

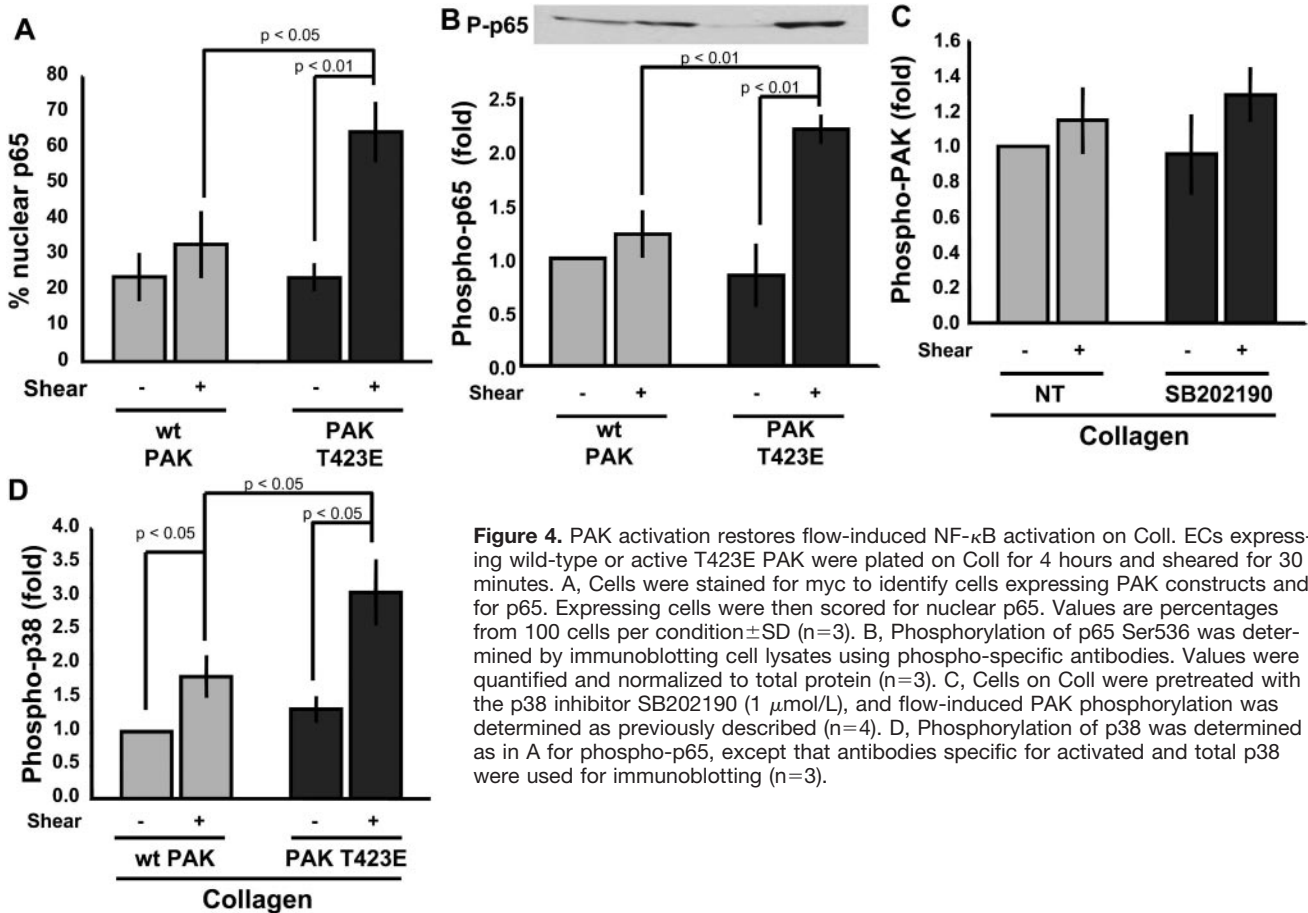
transfected with wild-type PAK. These data provide strong evidence that differential PAK activation mediates matrix-specific NF- $\kappa$ B activation by flow.

### Relationship to p38 MAP Kinase

Previous results demonstrated that p38 MAP kinase was preferentially activated in cells on Coll and that blocking p38 partially restored NF- $\kappa$ B activation by flow.<sup>22</sup> We therefore investigated the relationship between p38 and PAK signaling in this system. Flow does not activate PAK in cells on Coll, suggesting that Coll-specific p38 activation could prevent NF- $\kappa$ B activation by inhibiting PAK. However, inhibiting p38 in cells on Coll did not increase flow-induced PAK activation (Figure 4C). To test the converse hypothesis, that PAK stimulates NF- $\kappa$ B activation in cells on FN by suppressing p38, we transfected cells with active PAK and plated them on Coll. Whereas active PAK is sufficient to rescue NF- $\kappa$ B activation in cells on Coll, active PAK increased rather than decreased p38 activation both with and without flow (Figure 4D). Furthermore, inhibiting PAK signaling in cells on FN did not enhance p38 activation (Figure I in the online data supplement). Thus, PAK does not promote NF- $\kappa$ B by inhibiting p38; rather, the data suggest that the inhibitory effect of p38 cannot overcome the effect of active PAK. Therefore, these results show that the matrix-specific regulation of PAK and p38 are independent events, with PAK being the major determinant of matrix-specific NF- $\kappa$ B activation.

### Role of NF- $\kappa$ B-Inducing Kinase

We next turned our attention to the mechanism by which PAK regulates flow-induced NF- $\kappa$ B activation. We previously found that activation of IKK $\beta$  by flow is matrix-specific, occurring in cells on FN but not Coll.<sup>22</sup> NIK phosphorylates and activates IKK $\alpha$  and IKK $\beta$ ,<sup>33</sup> is activated



**Figure 4.** PAK activation restores flow-induced NF- $\kappa$ B activation on Coll. ECs expressing wild-type or active T423E PAK were plated on Coll for 4 hours and sheared for 30 minutes. A, Cells were stained for myc to identify cells expressing PAK constructs and for p65. Expressing cells were then scored for nuclear p65. Values are percentages from 100 cells per condition  $\pm$  SD ( $n=3$ ). B, Phosphorylation of p65 Ser536 was determined by immunoblotting cell lysates using phospho-specific antibodies. Values were quantified and normalized to total protein ( $n=3$ ). C, Cells on Coll were pretreated with the p38 inhibitor SB202190 (1  $\mu$ mol/L), and flow-induced PAK phosphorylation was determined as previously described ( $n=4$ ). D, Phosphorylation of p38 was determined as in A for phospho-p65, except that antibodies specific for activated and total p38 were used for immunoblotting ( $n=3$ ).

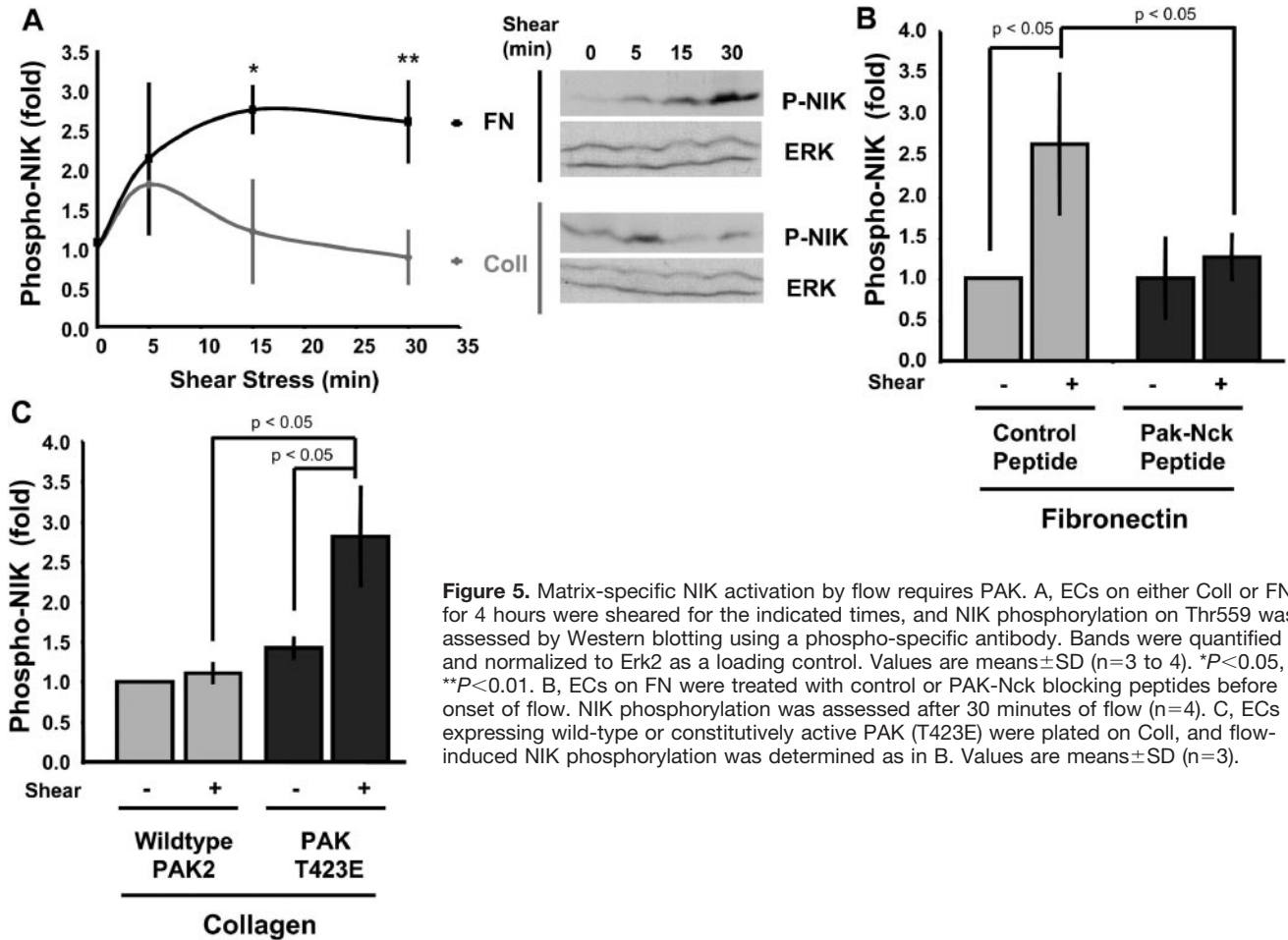
by ROS,<sup>18</sup> and is required for NF- $\kappa$ B activation by both flow<sup>17,19</sup> and constitutively active Rac.<sup>26,28</sup> To test the involvement of NIK, cells plated on Coll or FN were stimulated with flow and NIK activation assayed using a phosphorylation-specific antibody to Thr559, a key phosphorylation site in the activation loop of NIK that regulates NIK kinase activity.<sup>34</sup> In cells on FN, flow induced a 2.5- to 3-fold increase in NIK phosphorylation, whereas cells on Coll showed only a slight and transient response (Figure 5A). Inhibiting PAK in cells on FN with the PAK-Nck peptide reduced flow-mediated NIK phosphorylation (Figure 5B) and expression of constitutively active T423E PAK in cells on Coll rescued flow-induced NIK phosphorylation (Figure 5C). Thus, NIK activation is matrix-specific and PAK-dependent. These data indicate that PAK regulates NF- $\kappa$ B activation either at or upstream of NIK.

### Role of ROS

Flow-induced NF- $\kappa$ B activation depends on the production of ROS, because both antioxidants and genetic deletion of p47phox prevent flow-induced NF- $\kappa$ B activation.<sup>20,35</sup> In neutrophils, PAK regulates the NADPH oxidase complex through phosphorylation of both the p67phox<sup>36</sup> and p47phox<sup>37</sup> subunits. We therefore considered whether PAK might regulate flow-induced ROS production in ECs. To test this idea, we measured ROS production in ECs on either Coll or FN using the cell-permeant redox sensitive compound H<sub>2</sub>-DCFDA. H<sub>2</sub>-DCFDA is oxidized primarily by H<sub>2</sub>O<sub>2</sub>, a metabolite of

superoxide.<sup>38</sup> Surprisingly, the ability of shear stress to increase H<sub>2</sub>-DCFDA fluorescence was matrix-independent (Figure 6A). Additionally, the inhibitory PAK peptide had no effect on flow-induced ROS production in cells on FN (Figure 6B). Static controls were not lysed until the end of the 30 minute time point to ensure that increases in ROS production were not attributable to accumulation of mitochondrial-derived ROS over the course of the assay. Thus, PAK does not act by controlling ROS production.

The lack of flow-induced NF- $\kappa$ B activation in cells on Coll, despite the production of ROS, suggests that matrix regulates cellular sensitivity to ROS. Addition of exogenous H<sub>2</sub>O<sub>2</sub> is sufficient to activate NF- $\kappa$ B,<sup>39</sup> and the H<sub>2</sub>O<sub>2</sub> scavenger catalase blocks both flow-induced inflammatory gene expression<sup>40</sup> and atherosclerosis in vivo.<sup>41</sup> Consistent with these results, polyethylene glycol-catalase inhibited NF- $\kappa$ B activation by oscillatory flow (supplemental Figure II). We therefore tested the sensitivity of cells to ROS by addition of exogenous H<sub>2</sub>O<sub>2</sub>. Cells on FN showed much higher H<sub>2</sub>O<sub>2</sub>-induced p65 phosphorylation compared to cells on Coll (Figure 7A). Similar to flow-induced NF- $\kappa$ B activation, blocking PAK in cells on FN with the inhibitory peptide abolished both H<sub>2</sub>O<sub>2</sub>-induced p65 nuclear translocation (Figure 7B) and phosphorylation (Figure 7C). This effect was not attributable to enhanced antioxidant activity on Coll or in response to the peptide inhibitors because neither treatment affected the oxidation of H<sub>2</sub>-DCFDA by flow. Similar to the



**Figure 5.** Matrix-specific NIK activation by flow requires PAK. A, ECs on either Coll or FN for 4 hours were sheared for the indicated times, and NIK phosphorylation on Thr559 was assessed by Western blotting using a phospho-specific antibody. Bands were quantified and normalized to Erk2 as a loading control. Values are means±SD (n=3 to 4). \**P*<0.05, \*\**P*<0.01. B, ECs on FN were treated with control or PAK-Nck blocking peptides before onset of flow. NIK phosphorylation was assessed after 30 minutes of flow (n=4). C, ECs expressing wild-type or constitutively active PAK (T423E) were plated on Coll, and flow-induced NIK phosphorylation was determined as in B. Values are means±SD (n=3).

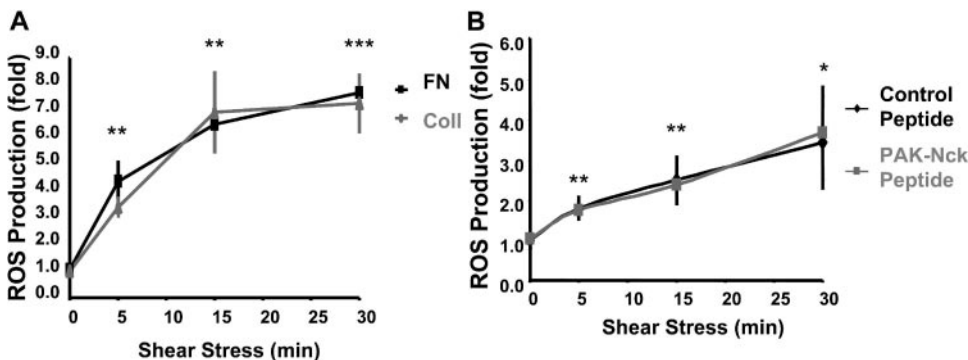
peptide inhibitors, the PAK AID construct also diminished both H<sub>2</sub>O<sub>2</sub>-induced p65 nuclear translocation (Figure 7D) and phosphorylation (Figure 7E) in cells on FN. Finally, expression of the constitutively active T423E PAK construct in cells on Coll rescued H<sub>2</sub>O<sub>2</sub>-induced p65 nuclear translocation (Figure 7F). Although NF-κB activation by exogenous H<sub>2</sub>O<sub>2</sub> required concentrations above that physiologically produced (500 to 750 μmol/L compared to 300 to 400 μmol/L), endogenously produced oxidants can act locally and may therefore be effective at lower doses.

To confirm these results, we also examined NIK activation. ECs on FN showed much higher H<sub>2</sub>O<sub>2</sub>-induced NIK activa-

tion compared to cells on Coll (Figure 8A). The PAK-Nck peptide abolished H<sub>2</sub>O<sub>2</sub>-induced NIK phosphorylation in cells on FN (Figure 8B), as did expression of the PAK AID (Figure 8C). Taken together, these data provide strong evidence that matrix-specific PAK activation regulates flow-induced NF-κB activation by modulating the ability of ROS to activate NIK and NF-κB.

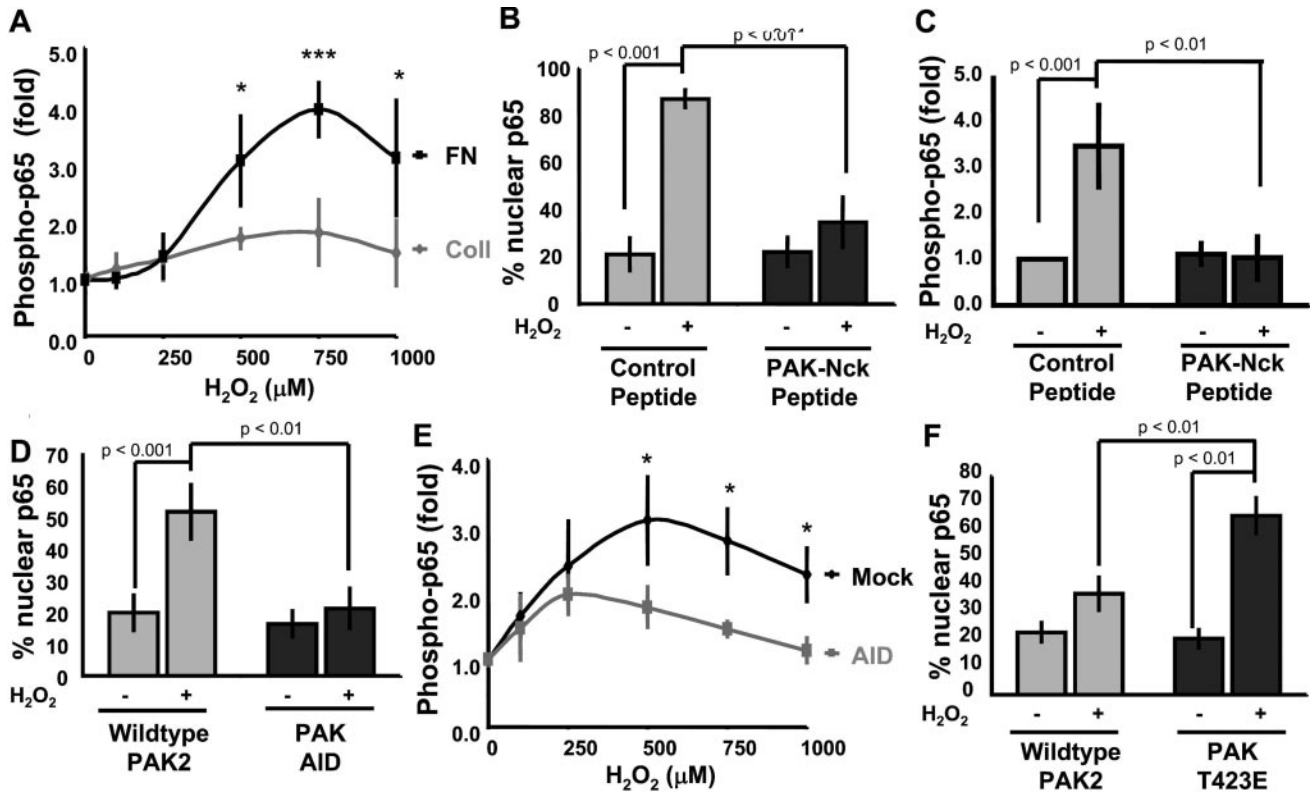
### Discussion

The present work defines PAK as a critical upstream mediator of matrix-specific NF-κB activation by flow. This



**Figure 6.** Neither matrix nor PAK regulate flow-induced ROS production. A, ECs were plated on Coll or FN for 4 hours and loaded with 2,7-H<sub>2</sub>DCFDA (10 μmol/L for 30 minutes). Cells were then stimulated with shear stress for the indicated times and lysed, and fluorescence was measured using a plate reader. Fluorescence was normalized to total protein levels in the lysates. Values are means±SD, normalized to static conditions, (n=3 to 4). \*\**P*<0.01, \*\*\**P*<0.001 compared to static. B, ECs on FN received control or PAK-Nck inhibitory peptide for 1 hour, then loaded with 2,7-H<sub>2</sub>DCFDA (as in A), and stimulated with shear stress for the indicated times. Fluorescence was quantified as in A (n=3 to 4). \**P*<0.05, \*\**P*<0.01 compared to static.

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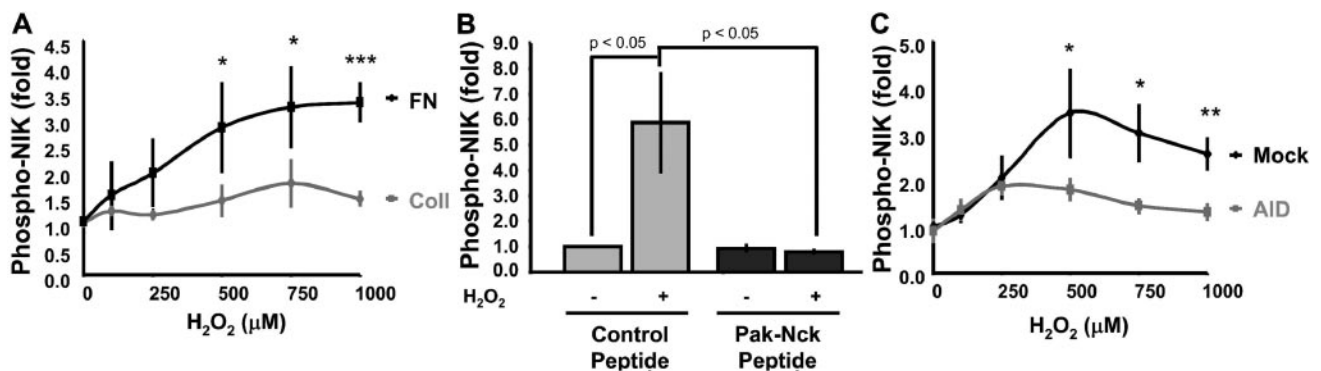


**Figure 7.** Matrix-specific PAK signaling regulates NF- $\kappa$ B activation by H<sub>2</sub>O<sub>2</sub>. A, ECs on Coll or FN for 4 hours were treated for 15 minutes with indicated doses of H<sub>2</sub>O<sub>2</sub>. Phosphorylation of p65 was determined as in Figure 1. Values are means $\pm$ SD, normalized to total p65 (n=4). \**P*<0.05, \*\*\**P*<0.01. B and C, ECs on FN were treated with control or PAK-Nck inhibitory peptide, and p65 nuclear translocation (B) and phosphorylation (C) were determined as in Figure 1 (n=3). D and E, ECs transfected with wild-type PAK or PAK AID were plated on FN for 4 hours, and the ability of H<sub>2</sub>O<sub>2</sub> to induce p65 nuclear translocation (D) and phosphorylation (E) were assessed. Approximately 100 cells were counted for each condition per experiment (n=3). \**P*<0.05. F, ECs transfected with wild-type or T423E PAK were plated on Coll for 4 hours, and the ability of H<sub>2</sub>O<sub>2</sub> to induce p65 nuclear translocation was assessed (n=3).

conclusion is based on results showing that PAK inhibitors blocked NF- $\kappa$ B activation by both acute onset of flow and oscillatory flow in cells on FN; conversely, active PAK restored activation of NF- $\kappa$ B by flow in cells on Coll. Blocking PAK also decreased NF- $\kappa$ B activation in atherosclerosis-prone regions of the mouse carotid sinus in vivo. Active PAK did not, however, affect basal NF- $\kappa$ B activity in the absence of flow. These data suggest that PAK-dependent regulation of NF- $\kappa$ B activation is highly specific and dem-

onstrate that NF- $\kappa$ B activation in this system requires multiple inputs.

Previous work showed that activation of NF- $\kappa$ B by flow<sup>20,35</sup> or Rac<sup>42</sup> requires ROS. PAK can regulate the NADPH oxidase complex in neutrophils, where NOX2 is a critical NADPH oxidase subunit.<sup>36,37</sup> However, neither matrix composition nor PAK inhibition affected flow-induced ROS production in ECs. These cells use mainly NOX1 to generate ROS in response to flow.<sup>40</sup> Thus, a distinct requirement for



**Figure 8.** Matrix-specific NIK activation by H<sub>2</sub>O<sub>2</sub> requires PAK. Cells were lysed and NIK phosphorylation determined by Western blotting as in Figure 1. Values are means $\pm$ SD relative to untreated cells. A, ECs on Coll or FN for 4 hours were treated for 15 minutes with the indicated doses of H<sub>2</sub>O<sub>2</sub> before analysis of NIK phosphorylation (n=3). \**P*<0.05, \*\*\**P*<0.001. B, ECs on FN were treated with control or PAK-Nck inhibitory peptide, and NIK phosphorylation was determined (n=3). C, ECs transfected with wild-type PAK or PAK AID were plated on FN for 4 hours, H<sub>2</sub>O<sub>2</sub> was added, and NIK phosphorylation was assayed (n=3). \**P*<0.05, \*\**P*<0.01.



PAK is not surprising. Instead, we found that activation of both NF- $\kappa$ B and NIK by exogenous H<sub>2</sub>O<sub>2</sub> was higher in cells on FN compared to Coll. Furthermore, the response to H<sub>2</sub>O<sub>2</sub> was decreased by PAK inhibitors in cells on FN and increased by activating PAK in cells on Coll. Whereas H<sub>2</sub>O<sub>2</sub> did not stimulate PAK activation on any matrix, PAK activity was moderately but significantly higher in cells on FN compared to Coll (supplemental Figure III), suggesting that a baseline threshold of PAK activity may be required for this pathway to proceed. Taken together, these data provide strong evidence that PAK modulates the pathway by which H<sub>2</sub>O<sub>2</sub> triggers NF- $\kappa$ B activation.

Relatively little is presently known concerning the role of PAK in the inflammatory response. Migration of leukocytes to CXCL1<sup>43</sup> and CXCL12<sup>44</sup> requires PAK1, and the PAK-Nck inhibitory peptide reduces neutrophil activation and infiltration in lipopolysaccharide-induced lung injury in mice.<sup>45</sup> Active PAK can stimulate the activation of the JNK and p38 MAP kinase pathways, both of which are implicated in proinflammatory gene expression.<sup>46</sup> However, reports of PAK involvement in NF- $\kappa$ B activation have been inconsistent. Constitutively active Rac activates NF- $\kappa$ B through production of ROS,<sup>42</sup> which is blocked by dominant negative NIK and IKK $\beta$ .<sup>26,28</sup> Active Rac mutants incapable of activating PAK still activate NF- $\kappa$ B,<sup>27</sup> and the active T423E PAK construct is insufficient to activate NF- $\kappa$ B.<sup>27,28</sup> Thus, PAK is not a central component of the pathway linking Rac to NF- $\kappa$ B. However, dominant negative PAK inhibits NF- $\kappa$ B activation by some stimuli, including expression of activated Rac.<sup>25,26</sup> These data can be reconciled by a model in which PAK sensitizes the NIK/IKK $\beta$  pathway to activation by ROS. As in other signaling networks, the relative importance of PAK would then depend on both the strength and the nature of the upstream signal.<sup>47</sup>

In addition to flow and atherosclerosis, oxidant-induced activation of NF- $\kappa$ B has been implicated in responses to cigarette smoke, proinflammatory cytokines such as interleukin-1 $\beta$ , aging, ischemia/reperfusion injury, myocardial infarction, cancer, and diabetic renal failure.<sup>48,49</sup> The ability of PAK to regulate oxidant-dependent NF- $\kappa$ B activation may therefore be important in multiple pathologies and suggests that PAK is a potential therapeutic target. However, long-term global PAK inhibition is likely to be deleterious, because strong immunosuppression increases the risk of infection and cancer.<sup>50</sup> Furthermore, PAK3 is important in brain function and PAK inhibition using a different cell-permeable peptide inhibitor results in symptoms resembling Alzheimer's disease in mice.<sup>51</sup> However, multiple endogenous proteins can inhibit PAK signaling, including nischarin, hPIP, POPX1/2, and protein kinase A.<sup>24</sup> These endogenous negative feedback mechanisms, especially those that primarily affect the vasculature, could be useful therapeutic targets in limiting endothelial activation and atherosclerosis.

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### Disclosures

None.

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# Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



## **p21-Activated Kinase Signaling Regulates Oxidant-Dependent NF- $\kappa$ B Activation by Flow** A. Wayne Orr, Cornelia Hahn, Brett R. Blackman and Martin Alexander Schwartz

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## Materials and Methods

*Cell Culture, Transfection, and Shear Stress* – Bovine aortic endothelial (BAE) cells (gift of Dr. Joanne Murphy-Ullrich, University of Alabama-Birmingham) were cultured as previously described<sup>1</sup>. Human umbilical vein endothelial cells (HUVEC) were maintained in DMEM:F12 media containing 10% FBS, 1% bovine brain extract, 60 µg/mL heparin, 10 U/ml penicillin, and 10 µg/ml streptomycin. Endothelial cells were plated onto glass slides and exposed to laminar flow (12 dynes/cm<sup>2</sup>) as previously described<sup>1</sup>. Oscillatory flow was generated using an infusion-withdrawal pump (New Era) combined with a peristaltic pump to superimpose a 1 dyne/cm<sup>2</sup> laminar flow to promote nutrient and gas exchange. Transient transfection of HA-PAK AID, Myc-PAK p21 binding domain (PBD), Myc-PAK2, and Myc-PAK T423E was performed using Lipofectamine 2000 per the manufacturer's instructions. The control and PAK-Nck blocking peptides<sup>2</sup> were produced by EZBiolab.

*Immunoblotting* – Cell lysis and immunoblotting was performed as previously described<sup>3</sup>. Rabbit anti-phospho-Ser536 p65, rabbit anti-phospho-p38 (Cell Signaling Technologies), rabbit anti-p65, rabbit anti-ICAM, rabbit anti-ERK, goat anti-PAK2, and rabbit anti-phospho-NIK (Santa Cruz) were all used at 1:1000 dilutions. Rabbit anti-phospho-Ser141 PAK (Biosource) was used at a 1:5000 dilution.

*Immunocytochemistry* – Cells were processed for immunocytochemistry as previously described<sup>1</sup>. Primary antibodies included rabbit anti-p65 (1:200; Santa Cruz) and mouse anti-HA (1:500; Covance). Primary antibody binding was visualized using Alexa488-



conjugated goat anti-rabbit and Alexa568-conjugated goat anti-mouse secondary antibodies. Coverslips were mounted using Fluoromount G (Southern Biotechnology) and images were taken using the 60X oil immersion objective on a Nikon DiaPhot Microscope equipped with a Photometrics CoolSnap video camera using the Inovision ISEE software program.

*ROS quantification* – BAE cells were preincubated with the dye 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>-DCFDA)<sup>4</sup> (10 μM) for 30 minutes prior to the onset of flow. Shear stress was applied to the cells in the continued presence of dye for varying times. Cells were rinsed with PBS and lysed in PBS containing 0.2% Triton X-100 and 1 mM N-acetylcystein. Fluorescence was measured using the 485 excitation/530 nm emission filter in a Fluorostar plate reader. Fluorescence was normalized to total protein in the lysates (Bradford assay, Pierce).

*Quantitative RT-PCR* – To quantify mRNA levels, we extracted total RNA using TRIzol (Invitrogen) and made cDNAs using the iScript cDNA Synthesis kit (Biorad). Real time RT-PCR was performed using the BioRad iCycler and Sybr Green Master Mix kit. Primers used were as follows: 18S forward 5'-CGGCTACCACATCCAAGGAA, 18S reverse 5'-AGCTGGAATTACCGCGGC, ICAM forward 5'- TGTCCCCCTCAAAAGT CATC, ICAM reverse 5'- TAGGCAACGGGGTCTCTATG, IL-8 forward 5'- CTGCGC CAACACAGAATTTA, IL-8 reverse 5'- TGAATTCTCAGCCCTCTTCAA. Results were normalized to 18S levels and are shown as a ratio of target mRNA to 18S mRNA.

*Animals and Vessel Harvest* – Eight male C57Bl/6 mice from Jackson Laboratories (Bar Harbor, ME), 8-12 weeks old, and weighing 18-20 g were used for this experiment. Mice were maintained on a chow diet for 28 weeks. Mice were injected intraperitoneally with 0.1 ml of either the control or PAK-Nck inhibitory peptide (10 mg/ml) daily for three days. Mice were perfused with 4% paraformaldehyde and the carotid sinuses were excised and processed for paraffin embedding. All experimental procedures were approved by the Animal Care and Use Committee at the University of Virginia and met the guidelines put for by the National Institute of Health.

*Immunohistochemistry (IHC)* – 5  $\mu\text{m}$  sections were cut, deparaffinized and rehydrated, then processed with antigen retrieval solution (Vector Labs). Sections were blocked in either 10% goat serum or 10% donkey serum in PBS/ fish skin gelatin solution for 1h and incubated with anti-p65 (Chemicon, 1  $\mu\text{g}/100 \mu\text{l}$ ) pre-labeled with Alexa-546 (Molecular Probes) overnight in 1% BSA at 4°C. All sections were stained with TOTO-3 (Molecular Probes) and mounted with anti-fading mounting gel.

#### *Analysis of Nuclear NF- $\kappa$ B*

Image analysis was performed to assess the relative intensity of nuclear NF- $\kappa$ B in the endothelium. Confocal images of dual stained NF- $\kappa$ B and TOTO-3 were imported into MetaMorph Imaging software (Molecular Devices). Positive TOTO-3 staining was used to define nuclei. These regions were transferred to the NF- $\kappa$ B stained image and NF- $\kappa$ B intensity was measured for each nucleus.

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## **Supplemental Figure Legends**

### **Supplemental Figure 1. PAK does not suppress flow-induced p38 activation on FN.**

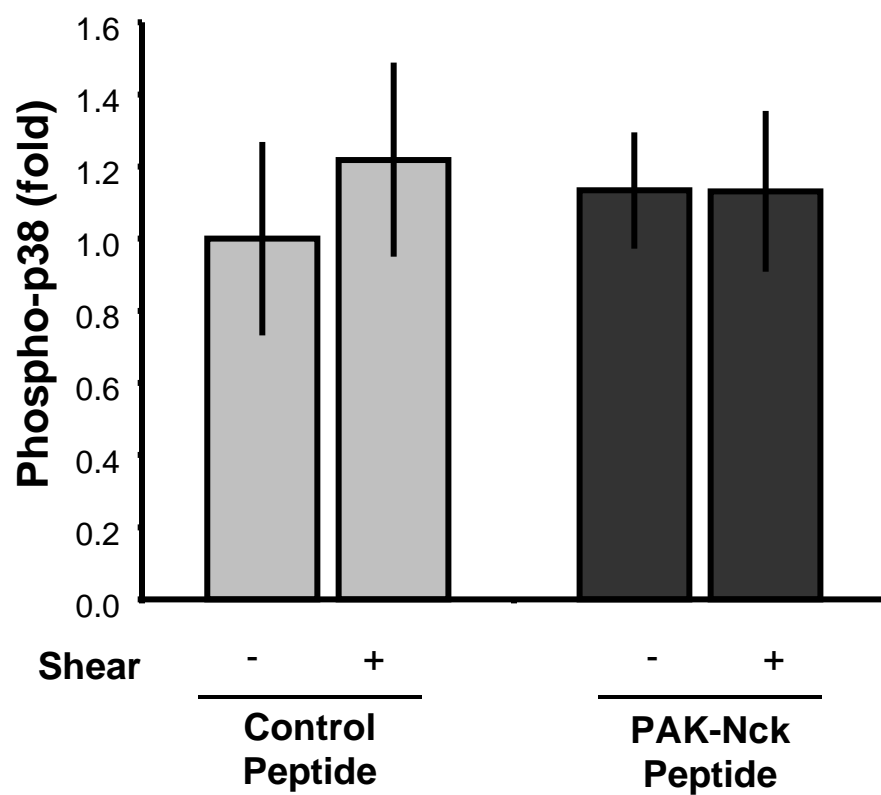
ECs plated on FN for 4h were pretreated with either control or PAK-Nck inhibitory peptides (20  $\mu$ g/ml for 1 h) and sheared for 5 minutes. Cells were lysed and immunoblotted for p38 phosphorylation. Values are means  $\pm$  S.D. after normalizing for total protein. n = 3.

### **Supplemental Figure 2. NF- $\kappa$ B activation by oscillatory flow requires H<sub>2</sub>O<sub>2</sub>.**

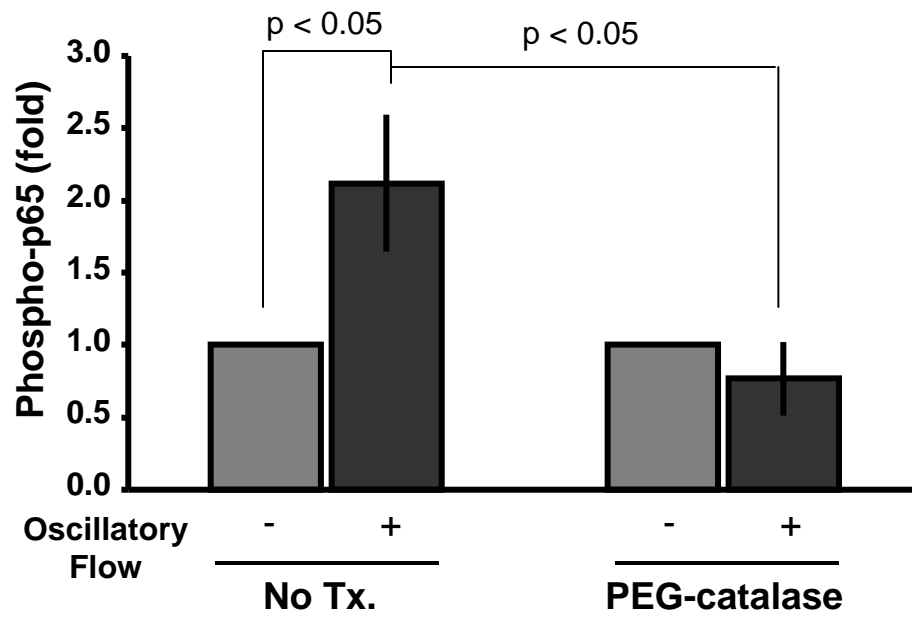
ECs were treated with PEG-catalase (50 U/ml; Sigma) for 24 hours prior to shear to inhibit H<sub>2</sub>O<sub>2</sub> production. Cells were exposed to oscillatory flow for 18 hrs or kept as static controls, and NF- $\kappa$ B activation was measured by Western blotting for phosphorylated p65. Values are means  $\pm$  S.D. after normalizing for total protein. n = 3.

**Supplemental Figure 3. Matrix composition regulates basal levels of PAK activation.** ECs were plated on either MG or FN for 4 hours in serum free media. Cells were lysed and immunoblotted for PAK phosphorylation on Ser141. Values are means  $\pm$  S.D. after normalizing for total protein. n = 5.

## Supplemental Figure I



## Supplemental Figure II





# Supplemental Figure III

