

Fluid Shear Stress Activates Proline-Rich Tyrosine Kinase via Reactive Oxygen Species–Dependent Pathway

Lung-Kuo Tai,* Masanori Okuda,* Jun-ichi Abe, Chen Yan, Bradford C. Berk

Objective—Fluid shear stress (flow) modulates endothelial cell (EC) function via specific signal transduction events. Previously, we showed that flow-mediated tyrosine phosphorylation of p130 Crk-associated substrate (Cas) required calcium-dependent c-Src activation. Because flow increases reactive oxygen species (ROS) production in ECs and because H₂O₂ increases tyrosine phosphorylation of proline-rich tyrosine kinase (PYK2), we hypothesized that flow may activate PYK2 via ROS.

Methods and Results—Exposure of bovine aortic ECs to flow stimulated PYK2 phosphorylation rapidly, with a peak at 2 minutes. The activation of PYK2 and phosphorylation of Cas induced by flow were inhibited by pretreatment with the antioxidant *N*-acetylcysteine. Flow-induced PYK2 phosphorylation was inhibited by BAPTA-AM, an intracellular calcium chelator. Bovine aortic ECs transfected with kinase-inactive PYK2 showed attenuated flow-stimulated Cas tyrosine phosphorylation. Although flow-induced Cas phosphorylation was inhibited by kinase-inactive Src, PYK2 activation induced by flow was not inhibited by overexpression of kinase-inactive Src.

Conclusions—These results show a redox-sensitive pathway for flow-mediated activation of nonreceptor tyrosine kinase activity that requires ROS and intracellular calcium, but not Src kinase. (*Arterioscler Thromb Vasc Biol.* 2002;22:1790-1796.)

Key Words: proline-rich tyrosine kinase ■ calcium ■ reactive oxygen species ■ shear stress

Vascular endothelial cells (ECs), which form the inner lining of the blood vessel wall, play an important role in the regulation of vascular homeostasis. By virtue of their location in the vascular wall, ECs are exposed to the mechanical forces associated with blood flow. Fluid shear stress (flow) modulates vessel structure and function and is one of the most important hemodynamic forces.¹ Among many signal molecules that are activated by flow, members of the mitogen-activated protein kinase family, including extracellular signal-regulated kinase (ERK)1/2,^{2–4} c-Jun NH₂-terminal kinase,⁵ and big mitogen-activated protein kinase/ERK5 are likely to be important for changes in EC gene expression.⁶

manner.⁹ Cas is also associated with FAK and proline-rich tyrosine kinase (PYK2) through SH3 domain interactions. A cooperative interaction between PYK2 and c-Src has been shown to be required for tyrosine phosphorylation of Cas in Cos-7 cells.¹⁰ Therefore, we studied the role of PYK2 in flow signaling.

PYK2, also known as RAFTK/CAK β /CADTK/FAK2, is related to FAK and is regulated by several extracellular stimuli.^{11–13} PYK2 is activated by hormones, G-protein-coupled receptor agonists, stress stimuli, membrane depolarization, and increasing intracellular calcium.^{11,14} Activation of PYK2 has been implicated in the regulation of ion channels,¹¹ cell adhesion and motility,^{10,15} the activity of ERK,^{11,16} c-Jun NH₂-terminal kinase,¹⁴ p70 S6 kinase,¹⁷ and phosphatidylinositol 3-kinase/Akt pathways.¹⁸ It has been found that autophosphorylation of PYK2 on Tyr402 leads to the binding of the Src SH2 domain and subsequent Src activation and PYK2 phosphorylation.¹⁹ The activated Src bound to PYK2 may phosphorylate PYK2 at Tyr579, Tyr580, and Tyr881, which promotes Grb2 (an adaptor protein) binding to PYK2 and enhances PYK2 kinase activity.^{20,21} PYK2 physically associates with cytoskeletal proteins, such as paxillin and Cas, and may increase their tyrosine phosphorylation in response to agonists.^{11,19,22}

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Our laboratory has investigated the early mechanical transduction events stimulated by flow. Results from our laboratory and others have shown that flow activated ERK1/2 in an integrin-dependent manner and that flow activated signal molecules that were localized to focal adhesions.^{7,8} However, we observed that neither focal adhesion kinase (FAK) nor paxillin was rapidly phosphorylated by flow. Crk-associated substrate (Cas) is a potential adaptor protein for integrin-mediated cell adhesion and is phosphorylated by flow in a calcium-dependent

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Previously, our data have shown that c-Src is activated within 2 minutes of flow and that tyrosine phosphorylation of Cas requires calcium-dependent c-Src activation.⁹ Therefore, we hypothesized that flow-mediated activation of PYK2 phosphorylates Cas on the basis of several findings: (1) Flow induces intracellular calcium.²³ (2) PYK2 is a calcium-dependent tyrosine kinase.¹¹ (3) Flow activates Src and induces Cas phosphorylation, and PYK2 is reported to be a Cas tyrosine kinase.^{9,10} In addition, because reactive oxygen species (ROS) production is induced by flow in ECs and because PYK2 is activated by ROS,^{24,25} we examined the interaction between ROS and PYK2. In the present study, we found that PYK2 is an important and proximal target of flow-derived ROS and intracellular calcium in bovine aortic ECs (BAECs) and is also an upstream mediator of Cas in response to flow.

Methods

Materials

A23187, phorbol 12-myristate 13-acetate (PMA), and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma Chemical Co. BAPTA-AM, U73122, cytochalasin D (CD), and PP2 (an Src family kinase inhibitor) were purchased from Calbiochem. Monoclonal anti-PYK2 and p130 Cas antibodies were from Transduction Laboratories. Monoclonal anti-Src (clone GD11) antibody and anti-phosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology. Anti-Myc antibody was from Invitrogen. Anti-hemagglutinin antigen (HA) tag antibodies (12CA5) were purchased from Roche Molecular Biochemicals. Protein A/G agarose beads were from Santa Cruz.

Cell Culture and Transfections

BAECs were harvested from fetal calf aortas by collagenase, as described previously.²⁶ Cells were grown in M199 (Life Technologies, Inc) supplemented with 10% FCS (Hyclone Laboratories) at 37°C in a 5% CO₂/95% air atmosphere. The wild-type (WT) CADTK/PYK2 and kinase-deficient CADTK/PYK2 cDNA constructs tagged with Myc peptide were kindly provided by Dr Li (University of North Carolina, Chapel Hill).¹⁴ The expression vector encoding p130^{CAS} was obtained from Dr Vuori (The Burnham Institute, La Jolla, Calif)²⁷ and was subcloned into another vector with the HA tag. Transient transfections were performed by using LipofectAmine (Invitrogen Life Technologies) according to the manufacturer's directions.

Shear Stress Protocol

BAECs were grown to confluence on gelatin-coated dishes and were serum-starved for 3 to 6 hours. Before the experiment, cells were rinsed free of culture medium with HEPES-buffered saline solution (130 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, and 20 mmol/L HEPES, pH 7.4) plus 10 mmol/L glucose and were either maintained in static condition or exposed to flow in a cone-and-plate viscometer at 37°C, as described previously.²⁸

Immunoprecipitation and Western Blotting

After treatment, cells were washed with cold PBS and lysed in lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 2 mmol/L benzamidine, and 10 μg/mL leupeptin). The lysates were immunoprecipitated, and immune complexes were recovered by the addition of protein A/G agarose beads. The beads were washed 3 times with lysis buffer. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose (Hybond-ECL, Amersham Pharmacia Biotech), as described previously.²⁹ The membranes were blocked and

incubated with primary antibody for 2 hours, followed by incubation for 1 hour with secondary antibody.

Adenoviral Transfection

BAECs were grown on 60-mm tissue culture dishes. On subconfluence, cells were incubated with M199 supplemented with 10% FCS containing Ad.KI-Src (where Ad indicates adenovirus and KI indicates kinase inactive) or Ad.LacZ for 2 days. The construction and preparation of Ad.KI-Src, the characterization of transfection efficiency, and control for cell toxicity have been described previously.⁹

Statistics and Densitometric Analysis

Data are presented as mean ± SE. All experiments were performed at least 3 times. Significant differences were determined by the Student *t* test (*P* < 0.05). Analysis of densitometry was performed by using NIH Image 1.60. Tyrosine phosphorylation was normalized first to the amount of protein on the basis of Western blot density. Results were then normalized for comparison among different experiments by arbitrarily setting the densitometric value of control cells or uninfected cells to 1.0.

Results

Time-Dependent Phosphorylation of PYK2 by Shear Stress in BAECs

To find the kinase upstream from Cas stimulated by shear stress, we studied tyrosine phosphorylation of PYK2. BAECs were exposed to flow for varying times and harvested for analysis of PYK2 phosphorylation. PYK2 was immunoprecipitated, and the immunoprecipitated protein complexes were immunoblotted with 4G10, an anti-phosphotyrosine antibody. Tyrosine phosphorylation of PYK2 occurred as early as 0.5 minutes, peaked at 2 minutes (1.8 ± 0.09-fold increase), and returned to near baseline by 40 minutes after stimulation (Figure 1A, top, and Figure 1B). There was no significant change in PYK2 protein level during these experiments (Figure 1A, bottom).

Shear Stress–Induced PYK2 Phosphorylation Is Dependent on Intracellular Calcium and PLC Activity in BAECs

We and other groups have shown that shear stress stimulates a rapid increase in EC intracellular calcium that is dependent on the magnitude of shear stress and have found that flow-induced Cas phosphorylation is dependent on calcium.⁹ Increasing intracellular calcium by treatment of BAECs with 10 μmol/L A23187 (a calcium ionophore) for 5 minutes stimulated PYK2 phosphorylation (Figure 2). The magnitude of PYK2 phosphorylation in response to shear stress was similar to that observed with A23187 treatment (Figure 2A). We used BAPTA-AM to chelate intracellular calcium and EGTA to chelate extracellular calcium. Flow-induced PYK2 phosphorylation was inhibited by 30 μmol/L BAPTA-AM but not by 2 mmol/L EGTA alone (Figure 2B). These results indicate that increases in intracellular calcium are necessary for PYK2 activation by flow. Previous data had shown that shear stress stimulated phospholipase C (PLC) with increases in inositol trisphosphate formation and diacylglycerol and the subsequent mobilization of calcium from intracellular stores and activation of protein kinase C (PKC).³⁰ In the present study, U73122, an inhibitor of PLC, blocked the phosphorylation of PYK2 induced by flow (Figure 2C). These results indicate that PYK2 activation by flow is mediated through

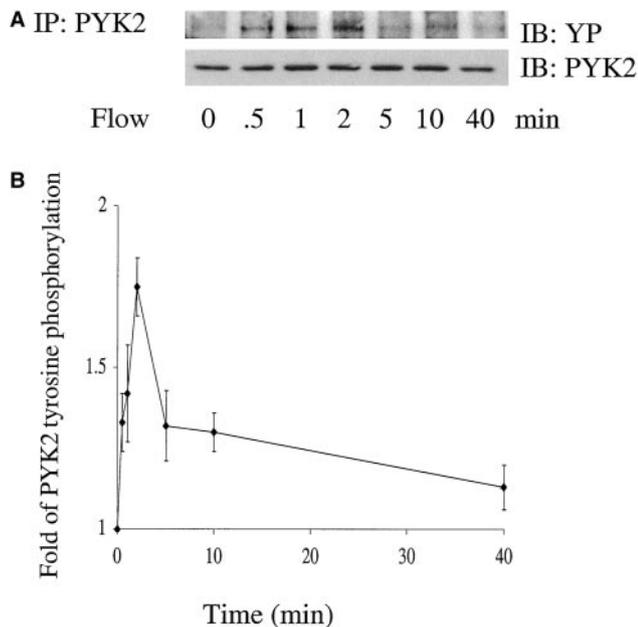


Figure 1. Time course for PYK2 tyrosine phosphorylation by shear stress. Serum-starved BAECs were either kept as static controls (represented by time 0) or were exposed to flow (shear stress 12 dyne/cm²) for the indicated times. A, Cell lysates were subjected to immunoprecipitation (IP) with anti-PYK2 antibody and analyzed by Western blot with anti-phosphotyrosine (YP) antibody (top). The bound antibodies were detected by enhanced chemiluminescence. To assess the reproducibility of PYK2 immunoprecipitation, the membrane was reprobbed with anti-PYK2 antibody (bottom). B, Densitometric analysis of phosphorylated form of PYK2 in BAECs is shown. Results are mean±SE from 3 separate experiments.

PLC, which is known to increase intracellular calcium. Next, we examined whether PKC activity is required for PYK2 phosphorylation by flow. PYK2 phosphorylation was significantly stimulated by treatment with 200 nmol/L PMA for 5 minutes (Figure 2D, lanes 7 and 8). To deplete PKC, BAECs were pretreated with PMA (1 μmol/L) for 20 hours before cells were exposed to flow or PMA. Long-term treatment with PMA inhibited PMA (200 nmol/L)-induced PYK2 activity (lanes 5 and 6) but did not inhibit flow-induced PYK2 phosphorylation (lanes 3 and 4). We also treated cells with 5 μmol/L chelerythrine chloride, a PKC inhibitor, for 30 minutes and found that the inhibitor could not block flow-mediated PYK2 phosphorylation (data not shown). These data show that PYK2 phosphorylation by flow requires PLC activation and mobilization of intracellular calcium, but not PKC activity.

Effect of Cytoskeleton-Disrupting Agents on Flow-Induced PYK2 Phosphorylation

Recent data have shown that disruption of integrin clustering at focal adhesions prevents PYK2 phosphorylation in response to stimuli.³¹ To investigate a possible connection between PYK2 and the actin-based cytoskeleton, cells were pretreated with CD, a specific actin microfilament inhibitor, for 30 minutes before flow. As shown in Figure 2E, we observed that CD had no effect on flow-induced PYK2 phosphorylation, indicating that PYK2 phosphorylation does not require intact actin-based cytoskeleton organization.

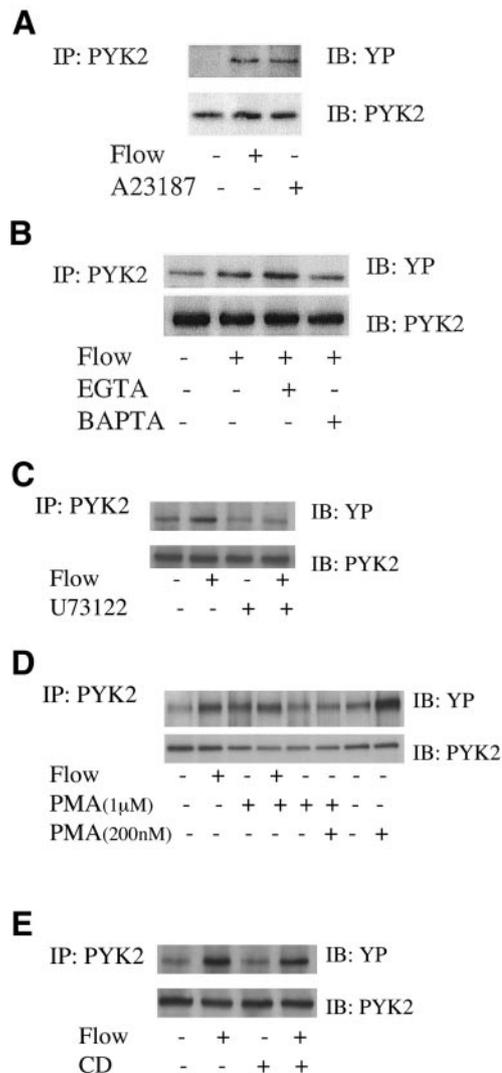


Figure 2. Effects of various inhibitors on flow-induced PYK2 phosphorylation. A, Serum-starved BAECs were exposed to flow (shear stress 12 dyne/cm² for 2 minutes) or treated with A23187 (10 μmol/L for 5 minutes). B, Extracellular calcium was chelated by adding 2 mmol/L EGTA in Ca²⁺-free flow buffer for 30 minutes. To deplete intracellular calcium, BAECs were treated with 30 μmol/L BAPTA-AM for 30 minutes before exposure to flow (shear stress 24 dyne/cm² for 2 minutes). C, Serum-starved BAECs were in the absence or presence of U73122 (5 μmol/L for 30 minutes) before they were exposed to flow (shear stress 24 dyne/cm² for 2 minutes). D, To deplete PKC, BAECs were treated with PMA (1 μmol/L) for 20 hours before exposure to flow (shear stress 24 dyne/cm² for 2 minutes) or PMA (200 nmol/L for 5 minutes). To stimulate PYK2 activity, BAECs were treated with PMA (200 nmol/L for 5 minutes). E, Serum-starved BAECs were in the absence or presence of CD (0.5 μmol/L for 30 minutes) before they were exposed to flow (shear stress 24 dyne/cm² for 2 minutes). PYK2 phosphorylation and protein were analyzed as described above.

Shear Stress-Induced PYK2 Phosphorylation Is Dependent on ROS

Previously, we and other groups have observed that shear stress increases ROS and that this leads to increased calcium.^{23,24} Because PYK2 has been suggested to be a redox-sensitive kinase, we determined the effect of NAC on flow-mediated PYK2 activity. After preincubation of BAECs

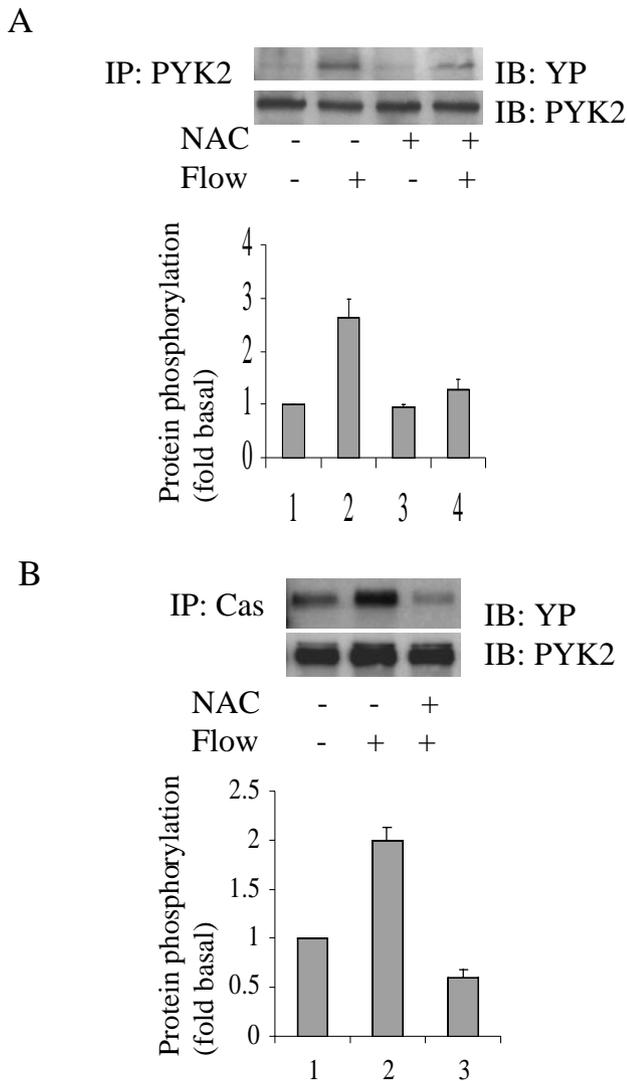


Figure 3. Effect of NAC on PYK2 and Cas phosphorylation by shear stress. A, Serum-starved BAECs were pretreated for 30 minutes with 30 mmol/L NAC. Then cells were exposed to flow (shear stress 12 dyne/cm² for 5 minutes). B, Cells were pretreated for 30 minutes with 30 mmol/L NAC. Then cells were exposed to flow (shear stress 12 dyne/cm² for 10 minutes). Cell lysates were prepared, immunoprecipitated with anti-PYK2/Cas antibody, and analyzed by Western blot with either YP antibody or anti-PYK2/CAS antibody. Graphs at the bottom represent average data expression as fold increase in PYK2/Cas phosphorylation over that in unstimulated cells. Results are mean±SE from 3 separate experiments.

for 30 minutes with 30 mmol/L NAC, BAECs were exposed to flow or maintained in the static condition. As shown in Figure 3A, shear stress-induced PYK2 phosphorylation was inhibited significantly by NAC treatment (47±2.2%). We also examined whether NAC has the same effect on Cas phosphorylation induced by flow. We treated BAECs with 30 mmol/L NAC for 30 minutes, and then cells were exposed to flow for 10 minutes. Treatment with NAC dramatically inhibited Cas phosphorylation by flow (Figure 3B). These data suggest that phosphorylation of PYK2 and Cas are both regulated by ROS.

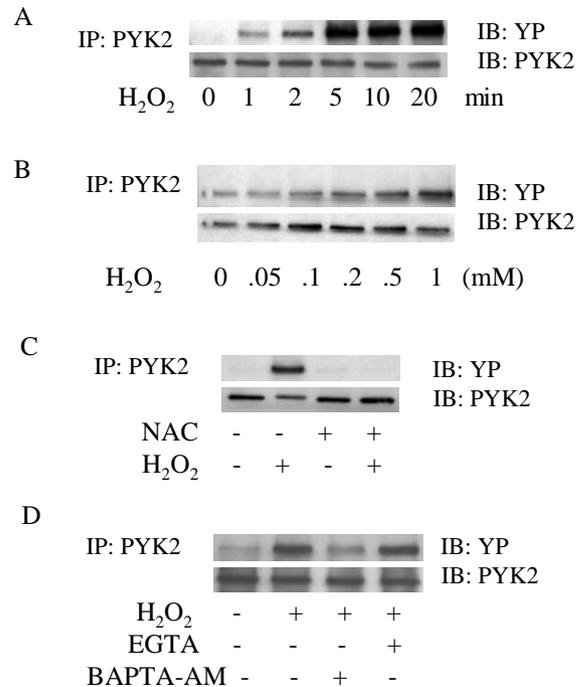


Figure 4. Activation of PYK2 by H₂O₂ was inhibited by pretreatment with NAC and calcium chelators. A, Serum-starved BAECs were stimulated with 500 μmol/L H₂O₂ for the indicated times. B, BAECs were treated with the indicated concentrations of H₂O₂ for 5 minutes. C, BAECs were pretreated for 30 minutes with 30 mmol/L NAC. Then cells were treated with 500 μmol H₂O₂ for 5 minutes. D, BAECs were pretreated for 30 minutes with 30 μmol/L BAPTA-AM or 2 mmol/L EGTA in Ca²⁺-free flow buffer. Then cells were treated with 500 μmol H₂O₂ for 5 minutes. PYK2 phosphorylation and protein were analyzed as described in Figure 1.

H₂O₂ Stimulates PYK2 Activity in BAECs, Which Was Inhibited by NAC

To evaluate the role of ROS in shear stress-induced PYK2 phosphorylation in BAECs, we used 2 approaches: (1) We tested the ability of exogenous ROS to induce phosphorylation of PYK2. (2) We assessed the effect of antioxidants on H₂O₂-induced PYK2 activation. We found that H₂O₂ time- and dose-dependently increased PYK2 phosphorylation. H₂O₂ (500 μmol/L) dramatically increased tyrosine phosphorylation of PYK2, with a peak at 5 minutes, and was sustained for 20 minutes (Figure 4A). H₂O₂ at a concentration of 100 μmol/L stimulated PYK2 phosphorylation, and 1 mmol/L H₂O₂ maximally stimulated PYK2 phosphorylation (Figure 4B). There was complete inhibition of H₂O₂-induced PYK2 phosphorylation at concentrations of 30 mmol/L NAC (Figure 4C), similar to results obtained with shear stress. Taken together, these results suggest that PYK2 is a redox-sensitive kinase and that H₂O₂ may mimic the effect of shear stress.

H₂O₂-Induced PYK2 Phosphorylation Is Calcium Dependent

To determine further the role of ROS in flow-mediated activation of PYK2, the calcium dependence of H₂O₂-induced PYK2 activation was examined. We observed that intracellular calcium chelation with 30 μmol/L BAPTA-AM for 30

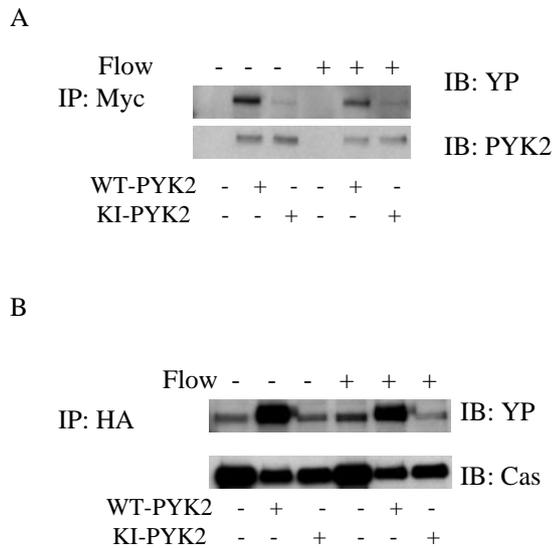


Figure 5. The effect of WT- and KI-PYK2 on shear stress-induced phosphorylation of Cas. Epitope-tagged HA-Cas (1 μ g) was cotransfected with 1 μ g pcDNA 3.1 (Invitrogen) empty vector or epitope-tagged Myc of WT-PYK2 or KI-PYK2 into BAECs. The transfected cells were serum-starved before they were subjected to flow (shear stress 12 dyne/cm² for 5 minutes). Cell lysates were immunoprecipitated with anti-Myc (A) or anti-HA (B) antibody and analyzed by Western blot with anti-YP, PYK2, or Cas antibody.

minutes dramatically inhibited tyrosine phosphorylation of PYK2 by H₂O₂. However, EGTA, which chelates only extracellular calcium, had no effect (Figure 4D).

Effect of WT- and KI-PYK2 on Shear Stress-Induced Phosphorylation of Cas

To characterize further the role of PYK2 in shear stress-mediated phosphorylation of Cas, we cotransfected BAECs with HA-Cas and Myc-tagged WT-PYK2 or KI-PYK2 (Lys457 replaced by Ala), followed by exposure to flow for 5 minutes. The epitope-tagged Myc-PYK2 and HA-Cas were immunoprecipitated by anti-Myc and anti-HA antibodies, respectively. Immunoblots were then probed with anti-phosphotyrosine antibody. Two results were obtained: (1) We found that transfected Myc-WT-PYK2 was highly phosphorylated on tyrosine under basal conditions and was not further phosphorylated by flow (compare lanes 2 and 5 in Figure 5A). (2) KI-PYK2 was not phosphorylated basally and could not be stimulated by flow (Figure 5A; compare lanes 3 and 6), suggesting that autophosphorylation is essential for PYK2 activation by flow. As shown in Figure 5B, we observed that overexpression of WT-PYK2 dramatically increased HA-Cas phosphorylation basally (compare lanes 1 and 2), whereas KI-PYK2 had no effect (compare lanes 1 and 3). In cells transfected with WT-PYK2, flow-mediated tyrosine phosphorylation of HA-Cas was enhanced (compare lanes 4 and 5). Transfection of KI-PYK2 significantly inhibited flow-induced HA-Cas phosphorylation (compare lanes 5 and 6). Thus, KI-PYK2 acts as a dominant-negative kinase in the flow-mediated pathway. These results show that PYK2 is an upstream mediator of Cas tyrosine phosphorylation in response to flow.

Shear Stress-Induced Tyrosine Phosphorylation of PYK2 Is Not Dependent on Src Kinase

c-Src has been shown to be activated rapidly by flow in BAECs, and flow-induced Cas phosphorylation requires c-Src activity.⁹ Because we found that PYK2 is a Cas kinase stimulated by flow (Figure 5B), we studied the role of c-Src activity in flow-induced PYK2 phosphorylation. We pretreated BAECs with 10 μ Mol/L PP2 for 30 minutes. PP2 inhibited the basal phosphorylation of PYK2 (Figure 6A; compare lanes 1 and 3 [$80 \pm 3.5\%$]). In contrast, flow-induced PYK2 phosphorylation was not inhibited when normalized to the baseline (2.0 ± 0.2 - and 2.5 ± 0.49 -fold increases; Figure 6A, lanes 2 and 4). We next tested the effect of overexpression by adenovirus of KI-Src on PYK2 activity. We found that infection of BAECs with KI-Src (multiplicity of infection 1000) inhibited flow-induced Cas phosphorylation (55% inhibition, Figure 6B; compare lanes 4 and 6) but did not inhibit tyrosine phosphorylation of PYK2 by flow (Control: 2.1 ± 0.14 , LacZ: 2.5 ± 0.21 , KI-Src: 2.8 ± 0.23 ; Figure 6C). We also observed that overexpression of KI-Src had no effect on H₂O₂-induced PYK2 phosphorylation (data not shown). Taken together, these data suggest that c-Src kinase activity is not involved in flow-induced PYK2 phosphorylation in BAECs.

Discussion

The major findings of the present study are identification of a redox-sensitive pathway for flow-mediated activation of PYK2 that requires ROS and intracellular calcium, but not Src kinase. Specifically, we found that (1) flow rapidly stimulated PYK2 tyrosine phosphorylation through ROS and intracellular calcium; (2) flow-induced PYK2 phosphorylation required activity of tyrosine kinases and PLC, but not c-Src; and (3) PYK2 is an upstream kinase for Cas.

We anticipated that PYK2 activation by flow would be mediated by ROS in ECs because flow increased ROS production in ECs, because flow-induced PYK2 phosphorylation was inhibited by NAC, and because H₂O₂ stimulated PYK2 activity. Previous studies have shown that ECs generate significant amounts of superoxide and hydrogen peroxide in response to stimuli that increase calcium and PKC activity.^{32,33} Several groups have demonstrated that mechanical forces, such as stretch, oscillatory flow, and steady laminar shear stress, stimulate ROS production in cultured ECs.^{24,34–36} We found that H₂O₂ and flow greatly induced PYK2 phosphorylation (Figures 1A and 4A), which was inhibited by NAC, a well-known precursor of glutathione synthesis (Figures 3A and 4B). Our findings are in good agreement with the observations that H₂O₂ activates PYK2 in cultured vascular smooth muscle cells and that PYK2 can be activated by other ROS generators, such as angiotensin II, platelet-derived growth factor, cytokines, and ultraviolet radiation.^{25,37–40} The mechanism by which ROS are generated in ECs exposed to flow remains unclear but may involve small G-protein-mediated activation of gp91-phox.^{41,42}

PYK2 phosphorylation is calcium dependent in response to various stimuli. There are several pathways to increase calcium concentration. Shear stress activated a pertussis toxin-sensitive G-protein-coupled K⁺ channel, which changes membrane potential. The resulting hyperpolarization, via effects on voltage-sensitive cation channels, may increase calcium influx. Other groups have shown that shear stress activates PLC to form

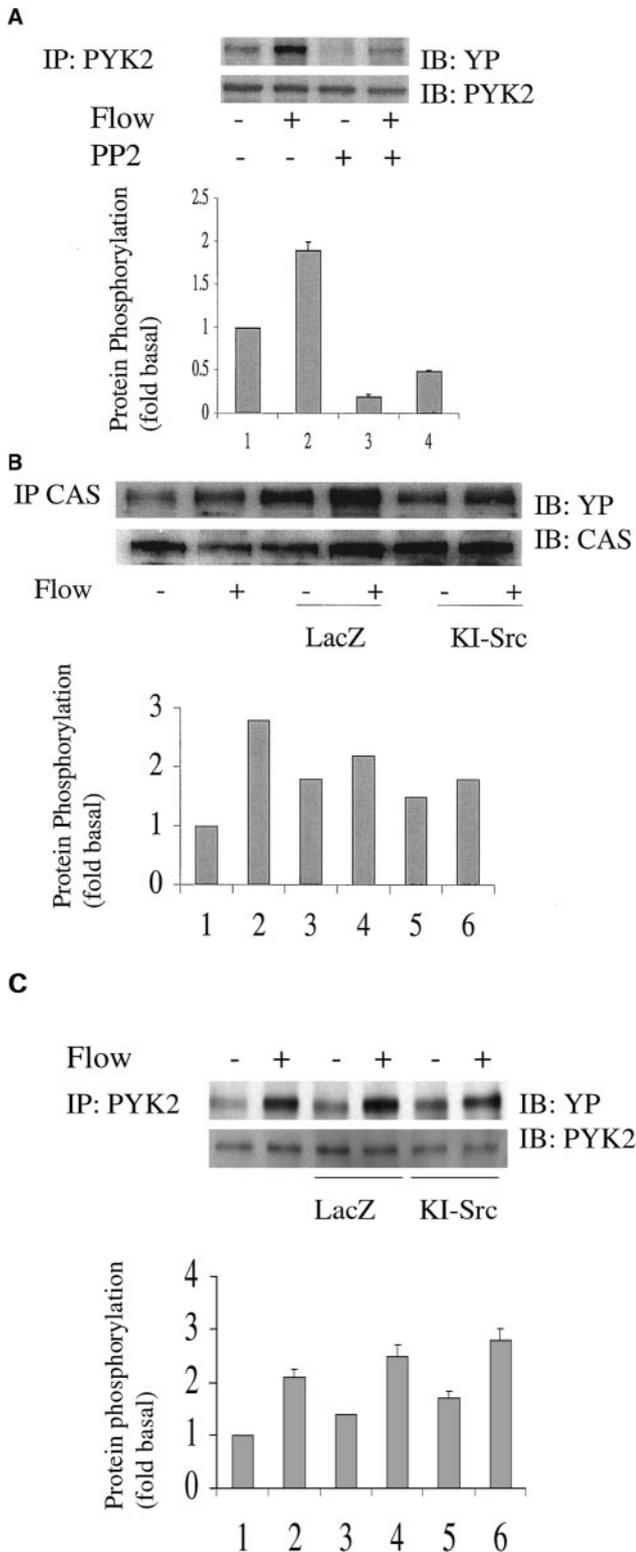


Figure 6. Shear stress-induced PYK2 phosphorylation was not inhibited by KI-Src. A, Serum-starved BAECs were pretreated with 10 $\mu\text{mol/L}$ PP2 for 30 minutes, followed by exposure to flow (shear stress 24 dyne/cm^2 for 2 minutes, $n=4$) B and C, BAECs were infected with adenoviral containing KI-Src or Ad.Lac Z (Lac Z) for 48 hours. The infected cells were exposed to flow (shear stress 12 dyne/cm^2 for 5 minutes). Cas/PYK2 phosphorylation and protein were analyzed as described in Figure 1 ($n=1$ in panel B and $n=4$ in panel C).

inositol trisphosphate and increase calcium concentration from intracellular stores.³⁰ Chen et al⁸ have reported that receptor tyrosine kinases, such as flk-1 and integrins, can serve as mechanosensors in ECs in response to flow. PLC γ has been shown to be recruited to flk-1. Presumably, flow induced the phosphorylation flk-1, which then associated with SH2-containing proteins, such as PLC γ , increasing intracellular calcium. Recently, we observed that PLC γ was phosphorylated in response to shear stress (data not shown). We found that flow-induced PYK2 phosphorylation was inhibited by BAPTA-AM, but not by EGTA (Figure 2B), indicating that intracellular calcium is more important than extracellular calcium in flow activation of PYK2. It is possible that a receptor tyrosine kinase is involved in this pathway. The role of flk-1 in flow-induced PYK2 activation will be examined in the future.

The role of Src family kinases in PYK2 phosphorylation is complex. We found that PP2, a Src family tyrosine kinase inhibitor, did not inhibit flow-induced PYK2 phosphorylation but did inhibit the basal level of PYK2 phosphorylation. Overexpression of KI-Src inhibited flow-induced Cas phosphorylation in BAECs (Figure 6B) and human umbilical vein endothelial cells⁹ but had no effect on flow-induced PYK2 activity (Figure 6C). Thus, we believe that Src is likely involved in PYK2 phosphorylation basally but not in response to flow. However, other groups observed that lysophosphatidic acid-induced activation of PYK2 was normal in Src $^{-/-}$ fibroblasts but decreased in Src $^{-/-}$ Yes $^{-/-}$ Fyn $^{-/-}$ fibroblasts in response to lysophosphatidic acid, bradykinin, carbachol, and angiotensin II.⁴³ It is likely that different members of the Src family kinase bind to PYK2 and phosphorylate PYK2 in response to different stimuli in various cell types. Alternatively, a tyrosine kinase other than Src family kinase may be involved, as suggested by the failure of PP2 to block the flow-mediated increase in PYK2 phosphorylation.

We speculate that integrin-mediated events are important in shear stress-induced signal transduction, because in response to flow, paxillin changes its alignment and FAK is phosphorylated.^{44,45} We found that PYK2 activation was not attenuated by disruption with CD (Figure 2E), suggesting that PYK2 activation in response to flow is via a signaling pathway separate from actin microfilament integrity. This observation is similar to our previous findings showing that actin microfilament integrity is not required for flow-induced big mitogen-activated protein kinase and ERK1/2 activity.⁷

In summary, the present study is the first to demonstrate that shear stress stimulates PYK2 tyrosine phosphorylation through ROS and intracellular calcium in ECs. Cas is a downstream target of PYK2 in response to flow in ECs. This shear stress-induced PYK2 pathway is important in EC function related to focal adhesions, such as migration and cell shape change, inasmuch as PYK2 and Cas are colocalized to focal adhesions.

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