Cyclic Strain Activates Redox-sensitive Proline-rich Tyrosine Kinase 2 (PYK2) in Endothelial Cells*

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Proline-rich tyrosine kinase 2 (PYK2), structurally related to focal adhesion kinase, has been shown to play a role in signaling cascades. Endothelial cells (ECs) under hemodynamic forces increase reactive oxygen species (ROS) that modulate signaling pathways and gene expression. In the present study, we found that bovine ECs subjected to cyclic strain rapidly induced phosphorylation of PYK2 and Src kinase. This strain-induced PYK2 and Src phosphorylation was inhibited by pretreating ECs with an antioxidant N-acetylcysteine. Similarly, ECs exposed to H₂O₂ increased both PYK2 and Src phosphorylation. An increased association of Src to PYK2 was observed in ECs after cyclic strain or H₂O₂ exposure. ECs treated with an inhibitor to Src (PPI) greatly reduced Src and PYK2 phosphorylation, indicating that Src mediated PYK2 activation. Whereas the protein kinase C (PKC) inhibitor (calphostin C) pretreatment was shown to inhibit strain-induced NADPH oxidase activity, ECs treated with either calphostin C or the inhibitor to NADPH oxidase (DPI) reduced strain-induced ROS levels and then greatly inhibited the Src and PYK2 activation. In contrast to the activation of PYK2 and Src with calcium ionophore (ionomycin), ECs treated with a Ca²⁺ chelator inhibited both phosphorylation, indicating that PYK2 and Src activation requires Ca²⁺. ECs transfected with antisense to PKCα, but not antisense to PKCε, reduced cyclic strain-induced PYK2 activation. These data suggest that cyclic strain-induced PYK2 activity is mediated via Ca²⁺-dependent PKCα that increases NADPH oxidase activity to produce ROS crucial for Src and PYK2 activation. ECs under cyclic strain thus activate redox-sensitive PYK2 via Src and PKC, and this PYK2 activation may play a key role in the signaling responses in ECs under hemodynamic influence.

PYK2¹ (also known as RAFTK, CAKβ), a protein-tyrosine kinase related to focal adhesion kinase (FAK), is a recently identified non-receptor tyrosine kinase (1). Studies suggest that PYK2 acts as a common mediator of signaling by a number of stimuli including growth factors (2), lipids (3, 4), and G-coupled receptors (5, 6). PYK2 also serves as a cellular mechanism for convergence between integrins and signaling cascades (7, 8). PYK2 is activated by signals that increase intracellular Ca²⁺ concentration (9). Other intracellular signaling pathways including tyrosine kinase Src physically associates with PYK2 and activates PYK2 through phosphorylation (5). PYK2 was shown to act as one of the signaling mediators required for the G protein-coupled receptor to the MAPK pathway (10–12). However, the downstream effects of PYK2 and how PYK2 plays a role in cellular responses have not been fully elucidated. PYK2 has been shown to trigger several signaling pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), or p38 MAPK (2, 10, 13). The adaptor protein Grb2/Sos complex has been shown to associate PYK2 to ERK activation, whereas the adaptor protein p130Cas and Crk link PYK2 to the JNK pathway (14). Recent studies indicate that PYK2 activation is redox-sensitive (15). How the intracellular redox status affects PYK2 activation and whether PYK2 plays a role in signaling mechanisms in ECs under oxidative stress have not been addressed.

Endothelial cells (ECs) are constantly under rhythmic distension because of pulsatile flow. This rhythmic distension-induced cyclic strain plays an important role in modulating endothelial gene expression. Studies including this group (16–18) have shown that ECs under hemodynamic forces transmit mechanical forces into secondary messengers and subsequently gene alteration. Secondary messengers involved include the activation of protein kinase C (PKC) and calcium mobilization (19, 20). Our studies have shown that the Ras/Raf/ERK pathway is involved in cyclic strain-induced early growth factor-1 (Egr-1) expression in ECs (16). Our most recent study further showed that PKC isozymes α and ε are required for sustained ERK activation in ECs under cyclic strain (17). In addition to ERK, JNK and p38 are also activated in ECs by cyclic strain (16, 21). Among the secondary messengers involved in cellular responses, reactive oxygen species (ROS) have been shown to play a pivotal role in various growth factor- or cytokine-induced cellular responses and gene expression (22, 23). We previously showed that intracellular ROS induced by hemodynamic forces including cyclic strain consequently stimulate the expression of various genes including Egr-1, c-Fos, monocyte chemotactic protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) (16, 24–26). However, the initial cellular responses, including the alteration of redox status induced by hemodynamic forces are complex and remain to be further defined. Since PYK2 is a non-receptor kinase related to FAK, and FAK is known to act as a signal transducer for mechanical forces (27, 28), this redox-sensitive PYK2 may play a role in endothelial response to mechanical forces. In the present study, we examined the role of PYK2 in ECs under cyclic strain and found that
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PYK2 is rapidly activated via Ca\(^2^+\) and PKC\(\alpha\)-dependent mechanisms. This PYK2 activation is redox-sensitive and is mediated via the tyrosine kinase Src. Our findings indicate that cyclic strain to ECs activates PKC\(\alpha\)-dependent NADPH oxidase activity that is crucial for PYK2 activation via Src. This cyclic strain-induced PYK2 activation may play an important role in signaling cascade during endothelial responses to hemodynamic forces.

EXPERIMENTAL PROCEDURES

Materials—Bovine aortic ECs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. The cultured medium was changed with identical medium except with the addition of 0.5% fetal bovine serum overnight before being subjected to cyclic strain. Antibodies against phosphorylated PYK2 (PY402) and phosphorylated Src (PY418) were obtained from BIO SOURCE (Camarillo, CA). Antibodies against Src and phosphotyrosine (PY20) were obtained from Transduction Laboratories (Lexington, KY). Calphostin C and [1,2-bis-(o-anisooxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl)ester] (BAPTA/AM) were obtained from Calbiochem (La Jolla, CA). A specific Src kinase inhibitor PP1 was obtained from Biomol (Plymouth Meeting, PA). NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), was obtained from Sigma. All other chemicals of reagent grade were obtained from Sigma.

In Vitro Cylindrical Strain on ECs—The strain unit Flexcell FX-2000 (Flexcell, McKeenest, PA) consisted of a vacuum unit linked to a valve controlled by a computer program. ECs cultured on a flexible membrane base were deformed by a sinusoidal negative pressure that produced an average strain of 12% at a frequency of 1 Hz.

Chemiluminescence Assay of Superoxide Production—Superoxide production was measured by lucigenin-amplified chemiluminescence as previously described (29). Briefly, ECs were lysed immediately after cyclic strain treatment with a lysis buffer containing lucigenin (500 \(\mu\)mol/liter). Readings were begun immediately upon addition of lysis buffer. Each reading was recorded as single photon counts using a microplate scintillation counter (Topcount, Packard Instrument Co., Meriden, CT).

NADPH Oxidase Activity Assay—NADPH oxidase was measured as previously described (30). In brief, ECs were scraped into ice-cold phosphate-buffered saline buffer containing 1 mmol/liter EGTA and centrifuged for 10 min with 750 \(\times\) g at 4 °C. The pellet was resuspended in lysis buffer (20 mmol/liter potassium phosphate, 1 mmol/liter EGTA, 10 mmol/liter aprotinin, 0.5 mmol/liter phenylmethylsulfonyl fluoride, 0.5 mmol/liter leupeptin) and sonicated. The protein concentration was adjusted to 2 mg/ml. Total cell suspension with a volume of 250 \(\mu\)l was mixed with 250 \(\mu\)l of HBSS containing 500 \(\mu\)g/ml lucigenin and kept at 37 °C for 10 min. NADPH oxidase activity assay was initiated by adding 10 \(\mu\)l of NADPH (100 mmol/liter) as substrate. The photon emission was measured, and the respective background counts were subtracted. Neither the cellular fraction alone nor NADPH alone evoked any lucigenin production.

Immunoblot Analysis—Proteins were extracted in SDS buffer and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred onto a nitrocellulose membrane, antigens were analyzed by specific antibody. Antibody-antigen complexes were detected using horseradish peroxide-labeled rabbit anti-mouse IgG and an ECL detection system (Pierce).

Immunoprecipitation—ECs were lysed with buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture. Cells were disrupted by repeated aspiration through a 21-gauge needle. After removing cellular debris, the same amount of protein from each sample was incubated with monoclonal antibody to Src. To rule out the nonspecific-binding proteins in immune complexes, the immunoprecipitation reaction was carried out with mouse nonspecific IgG as a negative control. The immune complex was incubated with protein A/G-agarose for 1 h, and this agarose was then resuspended in the sample buffer after wash. This immune complex was then subjected to immunoblot analysis.

DNA Plasmid Transfection—For the antisense studies, phosphorothioate oligonucleotides corresponding to bovine PKC\(\alpha\) or PKC\(\gamma\) were synthesized by PerkinElmer Life Sciences and used as an antisense as previously described (17). In brief, ECs were transfected with respective antisense (2 \(\mu\)mol/liter) for 6 h. Transfection was performed using the LipofectAMINE method (Invitrogen). Two days after transfection, endogenous PKCs or PKC\(\gamma\) isofoms were significantly reduced (17).

Transfection, ECs were incubated with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and then seeded onto flexcell plates for further treatment.

Statistical Analysis—Statistical analyses were performed using the Student’s \(t\) test. Data are expressed as mean \(\pm\) S.E. Statistical significance was defined as \(p < 0.05\).

RESULTS

Cyclic Strain to Endothelial Cells Induces Phosphorylation of PYK2 and Src—Activation of PYK2 requires phosphorylation of tyrosine residue 402 (3). ECs after cyclic strain were examined for the tyrosine phosphorylation of PYK2 and Src by using respective phosphospecific antibodies (Fig. 1). As shown in Fig. 1, cyclic strain to ECs induced a rapid phosphorylation of PYK2 and Src. This PYK2 or Src activation was shown to be sustained as cyclic strain continued (Fig. 1).

Endothelial Cells Exposed to \(H_2O_2\) Increase Phosphorylation of PYK2 and Src—ROS have been shown to be able to induce PYK2 phosphorylation (15). Our previous studies have demonstrated that ROS are involved in the modulation of cyclic strain-induced signaling pathways and gene expression. In our earlier studies (25, 26), ECs treated with an antioxidant enzyme catalase decreased intracellular reactive oxygen species and resulted in an attenuation of hemodynamic force-induced gene expression. In the present study, the role of ROS on cyclic strain-induced PYK2 phosphorylation was examined. Pretreatment of ECs with an antioxidant, N-acetylcysteine (NAC), inhibited cyclic strain-induced PYK2 and Src phosphorylation (Fig. 2A), suggesting that ROS were involved. To further confirm that PYK2 phosphorylation is redox-sensitive, ECs were treated with \(H_2O_2\) (1–200 \(\mu\)M) for 10 min. As shown in Fig. 2B, ECs exposed to \(H_2O_2\) increased tyrosine phosphorylation of PYK2 and Src. This phosphorylation occurred at 1 \(\mu\)mol/liter of \(H_2O_2\) exposure and reached a peak at 20 \(\mu\)mol/liter, followed by a decline of phosphorylation in ECs exposed to higher \(H_2O_2\) concentrations (Fig. 2B). These data suggest that cyclic strain-induced phosphorylation of PYK2 or Src is a redox-sensitive mechanism mediated by ROS generated in ECs under cyclic strain.

Cyclic Strain Induces PYK2-Src Complex Formation—It has been shown that autophosphorylation of tyrosine residue 402 is required for PYK2 association with Src (3). To evaluate whether PYK2 associates with Src in cyclic strain-
treated ECs, endothelial Src was immunoprecipitated with anti-Src antibody and analyzed for its PYK2 association with anti-PYK2 antibody. In unstimulated control cells, little association between PYK2 and Src was observed. ECs after cyclic strain increased PYK2-Src complex formation. Similarly, ECs after H2O2 exposure induced the association of PYK2 with Src. In contrast, there was no PYK2 association when nonspecific mouse IgG was used for immunoprecipitation (Fig. 3A). To verify whether the activation of PYK2 by cyclic strain was Src-dependent, ECs were pretreated with an Src inhibitor (PP1). As shown in Fig. 3B, cyclic strain-induced Src and PYK2 phosphorylation was greatly reduced by treating ECs with PP1. These results demonstrate that cyclic strain-stimulated PYK2 phosphorylation is mediated via Src.

NAD(P)H Oxidase Is Involved in Cyclic Strain-induced ROS Generation—Recent evidence suggests that NADPH oxidase or similar enzymes is a source of superoxide generation in ECs under stimulation (31). We have also demonstrated that Rac-dependent NADPH oxidase plays a role in ROS production in ECs under cyclic strain (32). Furthermore, PKC has been reported to regulate ROS generation via activation of NADPH oxidase (33). When ECs were pretreated with a PKC inhibitor (calphostin C), it showed an inhibition of cyclic strain-induced NADPH oxidase activity (Fig. 4A). This indicates that PKC plays a role in the regulation of NADPH oxidase activity. To further demonstrate the role of NADPH oxidase on ROS generation by cyclic strain, intracellular ROS levels were measured by the fluorescent intensity of lucigenin. As shown in Fig. 4B, ECs after cyclic strain increased ROS levels (~2.8-fold), which were greatly attenuated when ECs were pretreated with an inhibitor to NADPH oxidase (DPI). Similarly, ECs treated with PKC inhibitor decreased ROS levels. This indicates that cyclic strain induces NADPH oxidase activity that results in an increase of intracellular ROS levels that may be crucial for PYK2 and Src activation. Taken together, our results indicate that cyclic strain to ECs stimulates PKC, which results in the activation of NADPH oxidase and ROS generation.

Cyclic Strain-induced PYK2 and Src Phosphorylation Is Mediated Via PKC and [Ca2+]:—We demonstrated earlier that PKC was involved in cyclic strain-induced signaling pathways and gene expression (17, 34). To explore whether PKC activation contributed to PYK2 and Src phosphorylation, ECs were

![Fig. 2](image2.png)

**Fig. 2.** Cyclic strain-induced PYK2 activation is redox-sensitive. **A**, ECs were incubated with a ROS scavenger, N-acetylcysteine (NAC; 10 mM), for 2 h followed by cyclic strain (S) for 10 min. Phosphorylation of PYK2 (pPYK2, PY402) and Src (pSrc, PY418) was determined by immunoblot. **B**, ECs were treated for 10 min with H2O2 at the indicated concentrations. Cell lysates collected were immunoblotted with anti-pPYK2 (PY402) or anti-pSrc (PY418) antibody. The lower panel shows equal amounts of PYK2 and Src applied to each lane.

![Fig. 3](image3.png)

**Fig. 3.** Cyclic strain induced PYK2-Src complex formation. **A**, ECs were subjected to cyclic strain (S) or treated with H2O2 (20 μM/liter) for 10 min. The cell lysate was immunoprecipitated with either anti-Src antibody or a mouse-nonspecific IgG, and Western analysis was performed with antibody to PYK2. Equal amounts of Src protein applied to each lane are shown. Results are representative of three experiments. **B**, ECs were pretreated with a specific Src inhibitor (PP1, 50 μM/liter) for 30 min followed with cyclic strain for 10 min. Total cell lysates were collected and analyzed by Western blot using anti-pPYK2 and anti-pSrc antibodies. PYK2 and Src are shown to indicate that equal amounts of protein were added to each lane. Results shown are representative of three different experiments.

![Fig. 4](image4.png)

**Fig. 4.** NAD(P)H oxidase is involved in cyclic strain-induced ROS generation. **A**, ECs after cyclic strain were lysed and immediately followed with NADPH oxidase activity assay. In some studies, calphostin C (Cal, 250 μM/liter) was added 30 min prior to cyclic strain. ECs after cyclic strain were lysed and immediately followed with superoxide assay by lucigenin method. In some studies, ECs were treated with DPI (10 μM/liter) or calphostin C (Cal) for 30 min followed by cyclic strain. Results are shown as mean ± S.E. from four separate studies. *, p < 0.05 versus control ECs. #, p < 0.05 versus strained ECs.
pretreated with a PKC inhibitor, calphostin C. This treatment abolished the PYK2 and Src phosphorylation induced by cyclic strain and H₂O₂ exposure (Fig. 5A). Since ROS were involved in the modulation of PYK2 and Src phosphorylation, NADPH oxidase may play an important role in mediating phosphorylation of PYK2 and Src. When ECs were pretreated with DPI, cyclic strain-induced phosphorylation of PYK2 and Src were attenuated (Fig. 5B). To investigate whether the elevation of [Ca²⁺], in strain-treated ECs is involved in PYK2 activation in ECs, ECs were pretreated with a Ca²⁺ chelator (BAPTA/AM) followed with cyclic strain. As shown in Fig. 5C, BAPTA/AM treatment of ECs decreased cyclic strain-induced phosphorylation of PYK2 and Src. Furthermore, ECs incubated with a potent calcium ionophore (ionomycin) greatly induced phosphorylation of PYK2 and Src. These results indicate that PKC activation and [Ca²⁺], mobilization are crucial for cyclic strain-induced phosphorylation of PYK2 and Src.

**PKCa but Not PKCe Is Involved in Acute PYK2 Phosphorylation by Cyclic Strain**—We previously demonstrated that PKCa and PKCe were sequentially activated and were involved in cyclic strain-induced ERK1/2 activation and gene expression in ECs (17). We further explored whether a specific PKC isoform is involved in PYK2 activation by cyclic strain. Similar to the results as previously described (17), ECs transfected with antisense to PKCa or PKCe greatly reduced their endogenous PKC isoform. Transfected ECs were then subjected to cyclic strain. As shown in Fig. 6A, ECs transfected with antisense to PKCa inhibited PYK2 phosphorylation. In contrast, transfection of ECs with either scrambled oligonucleotides or antisense to PKCe did not show a significant effect on strain-induced PYK2 activity (Fig. 6B). These data clearly indicate that Ca²⁺-dependent PKCa but not PKCe activation contributes at least in initial PYK2 activation in ECs under cyclic strain.

**DISCUSSION**

ECs subjected to cyclic strain increase intracellular ROS that are involved in the regulation of cellular responses and gene expression. The increased ROS modulate the signaling pathway of Ras/Raf/ERK in ECs by cyclic strain. Mechanical force-induced endothelial responses thus appear to be redox-sensitive (16, 26, 35). In the present study, we found that non-receptor tyrosine kinase PYK2 is sensitive to redox changes in cyclic strain-treated ECs. This cyclic strain-induced PYK2 activation was inhibited by an antioxidant NAC pretreatment. This redox-sensitive PYK2 activation was also observed in ECs treated with H₂O₂. Our observation of this redox-sensitive PYK2 activation is consistent with the previous finding of PYK2 activation in vascular smooth muscle cells (15). This redox-sensitive PYK2 activation by cyclic strain is PKC- and calcium-dependent. We showed earlier that PKCa and PKCe are involved in cyclic strain-induced ERK1/2 activation and gene expression (17). PKCa is involved in the early response to cyclic strain caused by calcium influx immediately after the onset of cyclic strain. The majority of PKCa returned to the cytosolic fraction 6 h after continuous cyclic strain. The initial response of this PKC- and calcium-sensitive PYK2 activation is consistent with the pattern of PKC activation after cyclic strain. The inhibition of PYK2 activation in ECs treated with antisense oligonucleotides to PKCa further confirms the role of PKCa in PYK2 activation. The participation of PKCa in PYK2 activation clearly supports the importance of specific PKC isoforms in cellular responses to mechanical forces. This study demonstrates that calcium-dependent PKCa contributes to the initial response of ECs to cyclic strain, and this PKCa activation is required for the activation of Src and PYK2. Due to the rapid response of PKCa and a redox-sensitive nature of this tyrosine phosphorylation to cyclic strain, the PKCa activation may not be sufficient to maintain the steady state level of tyrosine phosphorylation.
phosphorylation. The redox status altered by H2O2 after activation of NADPH oxidase appears to be required for the sustained activation of Src and PYK2 under cyclic strain.

Shear stress that induced Src kinase activity in ECs was reported previously (36). In the present study, we found that cyclic strain activates Src kinase activity, a PP1-sensitive step, leading to an increase of PYK2 association and activation. This phenomenon is similar to the response of H2O2 (treatment of ECs (Fig. 2). This Src kinase activity induced by cyclic strain, similar to PYK2 activation, is also a redox-sensitive step. This is consistent with earlier report that Src activation was sensitive to antioxidant treatment in angiotensin II-activated smooth muscle cells (37). Cyclic strain to ECs increases intracellular ROS with an alteration of redox status and results in gene induction (16, 26). The main source of ROS generation during cellular activation has not been well defined. However, NADPH oxidase has been reported as a major source of ROS in smooth muscle cells after angiotensin II stimulation (38). NADPH oxidase was suggested to be mainly responsible for ROS production in ECs under various stimulations including cyclic strain (35). In the present study, NADPH oxidase appears to be involved in cyclic strain-induced PYK2 activation because ECs pretreated with an NADPH oxidase inhibitor, DPI, greatly reduced PYK2 activation. NADPH oxidase was well studied in neutrophils containing several components including Rac, a Rho family GTPase. Recent studies indicate that a gsp11- phospho-containing NADPH oxidase is selectively expressed in ECs (31, 39). The assembly of these components including Rac is essential for NADPH oxidase activity and superoxide production in ECs (40). We earlier indicated that Rac-dependent NADPH oxidase contributed to cyclic strain-induced ROS production, and that ECs pretreated with DPI reduced this strain-induced ROS generation resulting in a decrease of MCP-1 expression (32). These results support the notion that cyclic strain-induced PYK2 is a redox-sensitive response via a NADPH oxidase-dependent mechanism. This is also in agreement with a recent study indicating that ROS generation by cyclic strain is mediated via NADPH oxidase in endothelial cells (35). NADPH oxidase was shown to be activated by PKC via phosphorylation (41, 42). PKCs are one among those PKC isoforms involved in the phosphorylation of p47phox one of the cytosolic components of NADPH oxidase (43). In this study, ECs treated with a PKC inhibitor reduced their cyclic strain-induced NADPH oxidase activity, resulting in a decrease of PYK2 phosphorylation. We previously showed that cyclic strain to ECs increased the activity of PKC, mainly through the sequential activation of PKCα and PKCε (17). Cyclic strain may increase the calcium influx that results in the activation of PKCα that then leads to NADPH oxidase activation. In the present study we also showed that H2O2-induced phosphorylation of Src and PYK2 was inhibited by calphostin C pretreatment, indicating that PKC is also important for the effects of ROS generated by NADPH oxidase. Although the mechanism of ROS effects involving PKC activation is not clear, H2O2 generated in the cells may activate PKC by tyrosine phosphorylation as previously demonstrated (44). PKCα is one of those PKC isoforms that were tyrosine phosphorylated by H2O2. A protein tyrosine kinase (c-Abl) was shown to phosphorylate PKC in the presence of H2O2 (45). Moreover, intracellularly produced H2O2 may exert its effect via the global inhibition of protein tyrosine phosphatase as previously indicated (46, 47). It is likely that a tyrosine kinase upstream of PKC is activated via the inhibition of phosphatase by H2O2. The inhibition of tyrosine protein phosphatases by H2O2 may also interfere the signaling pathways via altering the heme or thiol redox status in molecules as indicated previously (48–50). Despite this ambiguity, our study clearly shows that ROS generation from NADPH oxidase is involved in the phosphorylation of Src and PYK2 induced by cyclic strain.

The physiological role of PYK2 is not yet clear. PYK2 has been shown to play a role in ERK, JNK, and p38 activation. The association of adaptor protein p130Cas with PYK2 has been reported to link the PYK2 activation to the JNK pathway (14). A decrease of c-Jun promoter activity was observed in ECs transfect with PYK2 kinase inactive mutant,2 supporting this PYK2/JNK pathway. This PYK2 mutant transfection also leads to a decreased activation of a transcriptional factor, Elk-1, one of the JNK substrates.2 Various studies including ours have shown that both ERK and JNK are involved in hemodynamic force-induced cellular signaling mechanisms (16, 18, 36). Antioxidant pretreatment of ECs attenuates gene induction (24, 25) showing that cyclic strain- or shear stress-induced gene expression is redox-sensitive. Cyclic strain-induced PYK2 phosphorylation and the subsequent downstream signaling pathway are consistent with the notion that ECs under hemodynamic forces induce genes that are Src- and redox-sensitive.

PKC-dependent activation of Src has been reported in cells after stimulation (51–53). Sequential activation of PKC and Src was indicated (54). In the present study, PKCα activation appears to be required for the subsequent tyrosine phosphorylation of Src and PYK2. The inhibition of protein tyrosine phosphatase by H2O2 intracellularly produced may also be required for the sustained activation of Src and PYK2 under cyclic strain. It has been reported that Src kinases are critical for activation of PYK2, and Src forms a complex with PYK2, which in turn phosphorylates the epidermal growth factor receptor (EGFR) (5). Src-dependent PYK2 activation has been shown to be involved in the osteoclastic activation during adhesion (55). These are consistent with our present finding that cyclic strain-induced PYK2 activity is mediated via Src. Activation of PKC by phorbol ester results in a focal adhesion targeting of PYK2 and its tyrosine phosphorylation in an integrin clustering-dependent manner (56). EGFR has been shown to be involved in mechanical stretch-induced responses (57). Mechanical forces triggering integrin-dependent cellular responses have been well documented (58, 59). Recent studies further showed the involvement of PYK2 in outside-in signaling by forming a complex with p130Cas and paxillin in human ECs (60). For those ECs under cyclic strain, an increased association of PYK2 with paxillin was also observed.2 Thus, cyclic strain-triggered PYK2 activation may play an important role in modulating the early signaling pathways in ECs subjected to mechanical forces.

In conclusion, our study demonstrates that cyclic strain to ECs activates redox-sensitive PYK2 via Src. This cyclic strain-induced PYK2 activity is mediated via calcium-dependent PKCα that increase NADPH oxidase activity. The ROS thus generated appears to be crucial for Src and PYK2 activation. This PYK2 activation may play an important role in regulating endothelial responses. Because PYK2 phosphorylation is rapid and redox sensitive, its signaling role in regulating vascular gene expression and maintaining vessel integrity remains to be elucidated and warrants further investigation.

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