Src Autophosphorylation is an Early Event in Pressure-Mediated Signaling Pathways in Isolated Resistance Arteries

Darian C. Rice, Anca D. Dobrian, Suzanne D. Schriver, Russell L. Prewitt

Abstract—Elevated blood pressure is associated with varying degrees of arterial growth and remodeling. The mechanisms by which mechanical stress is converted into cellular alteration have yet to be fully elucidated. Our laboratory has demonstrated that Src tyrosine kinases and the extracellular signal-regulated kinase subtype of the mitogen-activated protein kinase family mediate pressure-induced c-fos expression in rat mesenteric arteries. Others have reported involvement of integrin and growth factor receptor signaling pathways. Our goal was to determine the role of Src, focal adhesion kinase (FAK), and platelet-derived growth factor (PDGF) receptor signaling in the upstream initiation of these events. Pairs of rat mesenteric arteries were pressurized to 90 mm Hg (control), and then one was raised to 140 mm Hg for 1, 3, or 5 minutes. Western blotting revealed that Src-pY_{418} was elevated 2.4-fold over control values at 1 minute and 2.8-fold at 3 minutes and returned to control at 5 minutes. Significant FAK-Y^{397} phosphorylation was observed only after 3 and 5 minutes of pressure stimulus and was blocked entirely by Src inhibition. Src-pY^{215} activity (associated with PDGF receptor activation) does not seem to be involved at any of the time points tested. These data demonstrate that Src-Y^{418} autophosphorylation is an early event in pressure mechanotransduction and leads to activation of FAK-Y^{397}. This finding suggests that Src may be the messenger that initiates and propagates the cellular growth response to pressure stimulus, and FAK is one of its downstream targets. Src phosphorylation due to PDGF receptor activation does not seem to be involved in the initial response. **(*Hypertension*. 2002;39[part 2]:502-507.)**

Key Words: hypertension, chronic ■ signal transduction ■ arteries ■ remodeling ■ kinase ■ platelet-derived growth factor

Chronic hypertension is associated with varying degrees of hypertrophic and eutrophic arterial remodeling. Arterioles, or "resistance vessels," possess an intrinsic ability to develop myogenic tone and initially vasoconstrict in an attempt to counteract an increase in wall stress due to elevated pressure.[1] Over time, the sustained vasoconstriction will prompt a more permanent structural reduction in lumen diameter through inward eutrophic remodeling.[2] The larger arteries, or "conducting vessels," lack myogenic tone and eventually undergo outward hypertrophy to "tolerate" or counteract the increase in wall stress. Small arteries possess intermediate characteristics and demonstrate both inward and outward hypertrophy.[3,4] Our current hypothesis is that wall stress governs this vascular response.[5] However, the mechanisms by which extracellular mechanical stress is converted into intracellular alterations in signal transduction and gene expression have yet to be fully elucidated.

Cells detect and transmit sensory data from their environment in a variety of ways. In the case of hypertension, cell surface receptors or associated molecules may be the sensors that perceive changes in extracellular forces and act as molecular switches to regulate vascular adaptation. Recent data has suggested a role for integrin and/or growth factor receptor signaling in the initiation of this response.[6]

Integrins are a family of cell surface extracellular matrix (ECM) adhesion receptors. They exist as heterodimers of α- and β-glycoprotein subunits, which associate with such ECM proteins as collagen, fibronectin, laminin, and vitronectin. Integrin interaction with these proteins provides a certain cellular spatial awareness and an ability to sense and react to changes in cell adhesion, stability, shear stress, and mechanical strain.[7–9]

Focal adhesion kinase (FAK) seems to be a central player in integrin signaling. FAK has been shown to associate not only with integrins but also with focal adhesion complexes, cell-to-cell adhesion molecules, and growth factor receptor tyrosine kinases. FAK is a cytosolic protein tyrosine kinase (PTK), which on activation, ie, phosphorylation, has been shown to provide a substrate for association and activation of other cytosolic proteins such as Src, Grb2, paxillin, and $p130^{cas}$. FAK tyrosine residue 397 (Y^{397}) is the major site of FAK autophosphorylation and serves as a docking site for the

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From the Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, Va.

Correspondence to Darian C. Rice, MD, or Russell L. Prewitt, PhD, Eastern Virginia Medical School, Department of Physiological Sciences, Lewis Hall, Room 2013, 700 W. Olney Road, Norfolk, VA 23507. E-mail prewitrj@evms.edu

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Src homology 2/3 (SH2/SH3) domain of Src.[10–12] Src is another family of cytosolic protein tyrosine kinases (PTK), which acts by phosphorylating and activating tyrosine phosphorylation sites. Src-Y^{416} is the major site of autophosphorylation, and Src-Y^{215} phosphorylation has been correlated with activation of the platelet-derived growth factor (PDGF) receptor.[13,14]

PDGF receptors belong to a family of cell surface growth factor receptor tyrosine kinases (RTKs), which are formed by the dimerization of α and/or β receptor subunits to yield $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ receptor subtypes.[15] The key event in the activation of the PDGF receptor is the dimerization of the 2 cytoplasmic tails that juxtapose various tyrosine residues and allow for transphosphorylation of the internal receptor components. The function of autophosphorylation is to regulate the catalytic activity of the receptor kinase and provide docking sites for downstream signal transduction molecules. The tyrosine autophosphorylation sites recognize binding domains such as SH2/SH3. Several SH2-containing signaling molecules have been identified and include the Src family of tyrosine kinases, phosphatidylinositol 3-kinase, phospholipase C-γ, the tyrosine phosphatase SHP-2, and the GTPase activating protein for Ras. Adapter molecules such as Grb2 and Shc are also recruited.[15,16] The growth-promoting behavior of such signaling molecules suggests the potential involvement of the PDGF receptor in the pressure-induced hypertrophic response. Interestingly, Hu et al[17] demonstrated PDGF receptor α activation in vascular smooth muscle cells in response to mechanical stress, in the absence of ligand.

The purpose of the present study was to investigate the potential involvement of FAK, the Src family of cytosolic tyrosine kinases, and the PDGF receptor in the initial vascular smooth muscle response to hypertension.

Methods

Isolated Dual-Vessel Protocol

All experimental procedures were approved by the institutional Animal Care and Use Committee. Male Wistar rats (200 to 410 g) were anesthetized with pentobarbital (60 mg/kg intraperitoneally). After midline laparotomy and perforation of the heart, the mesenteric arcade was then carefully dissected away from the associated length of intestine and placed in cold (4°C) bicarbonate-free physiological saline solution with the following composition (in mmol/L): 141.8 NaCl, 4.69 KCl, 1.59 $MgSO_4$, 0.513 EDTA, 2.79 $CaCl_2$, 10.0 HEPES, 1.18 KH_2PO_4, and 5.0 glucose, adjusted to a pH of 7.37 to 7.4. First-order mesenteric small arteries were then cleared free of surrounding adipose tissue and mounted in a dual-vessel chamber (model CH/2/M; Living Systems Instrumentation). Isolated arteries were cannulated onto tapered glass micropipettes (outside diameter 210 to 250 μm) with the aid of a dissection microscope and were secured using 19-μm nylon filament. Once the vessels were mounted, the chamber was transferred to the stage of an upright microscope (Zeiss), where the transilluminated vessels could be visualized on closed-circuit television. The internal and external diameters were measured and recorded with the aid of video calipers (Texas A&M). The intraluminal pressure was controlled by adjusting the height of a fluid reservoir and was recorded continuously via pressure transducers. The pressure in both vessels was gradually raised in a step-wise manner (15 mm Hg every 15 minutes) to a normal mean arterial pressure of 90 mm Hg, at 37°C. After a 1-hour equilibration at 90 mm Hg, one of the vessels was raised to a hypertensive pressure of 140 mm Hg for 1, 3, or 5 minutes, while the other remained at 90 mm Hg to serve as the experimental control.

To reduce variability, we performed all experiments in a paired manner, and both arteries were isolated from the same rat. For experiments evaluating the role of Src in pressure mechanotransduction, PP1 (10 μmol/L), a specific inhibitor of Src that does not interfere with FAK autophosphorylation,[18] was administered extraluminally in the tissue bath during the 1-hour equilibration period.

Western Blotting

At the endpoint of each experiment, both vessels were removed from the cannula, immediately snap-frozen in liquid nitrogen, and then ground in 50 μL of RIPA protein extraction buffer of the following composition: 50 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 mmol/L EDTA. In addition, the buffer contained the protease inhibitors aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L) and the phosphatase inhibitors sodium orthovanadate (Na_3VO_4; 1 mmol/L) and sodium fluoride (NaF; 1 mmol/L). The total protein content of each sample was determined using the Micro-BCA (bicinchoninic acid) protein assay (Pierce). Protein samples were standardized and prepared with dithiothreitol and bromophenol blue. Equal amounts of protein were then loaded and separated by electrophoresis (45 minutes at 180 to 200 V) using a 7.5% SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to polyvinylidene fluoride membranes (110 V×75 minutes) and then blocked in a 1:1 solution of NAP-Sure Blocker (Geno Technology) and Tris-buffered saline with 1% Tween-20 (T-TBS) for 1 hour at room temperature. Membranes were incubated with the polyclonal phosphorylation-specific 1° antibody (BioSource International) for 2 hours, followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Tropix) for 1 hour. Membranes were then washed in T-TBS followed by Assay buffer before incubation with the developing reagent (CDP-Star, Tropix). The chemiluminescent signal was detected by Kodak Digital Imaging System, and the band signal intensity was quantified using SigmaGel digital software. Prestained rainbow markers (Amersham Pharmacia Biotech) were used as molecular mass standards.

Chemicals

PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-D] pyrimidine), a specific Src inhibitor, was obtained from Biomol Research Laboratories, Inc. All other compounds were purchased from Sigma Chemical Co.

Data Analysis

Results are presented as mean±SEM. GraphPad Instat software was used for determination of mean and SEM, followed by paired *t* test for statistical significance. The null hypothesis was rejected at $P<0.05$, and n depicts the number of paired experiments.

Results

Seventy-six pairs of rat first-order mesenteric arteries were used in this study. During the isolated vessel experiments, the arteries responded to the incremental increases in pressure in a passive manner. Each 15-mm Hg pressure increase resulted in a corresponding increase in lumen diameter and circumferential wall stress. Use of the Src tyrosine kinase inhibitor PP1 (10 μmol/L) did not seem to influence these vessel characteristics. Occasionally, pressures of 90 or 140 mm Hg triggered a moderate myogenic response. As wall stress is believed to be the key determinant in the vascular growth response to hypertension and myogenic tone has been shown to attenuate the pressure-induced c-fos expression, these vessels were not included in the study.[5]

Response of FAK-Y^{397}, Src-Y^{215}, and Src-Y^{418} to Pressure Stimulus

To determine the involvement of FAK and src in the initial events of pressure mechanotransduction, we examined the

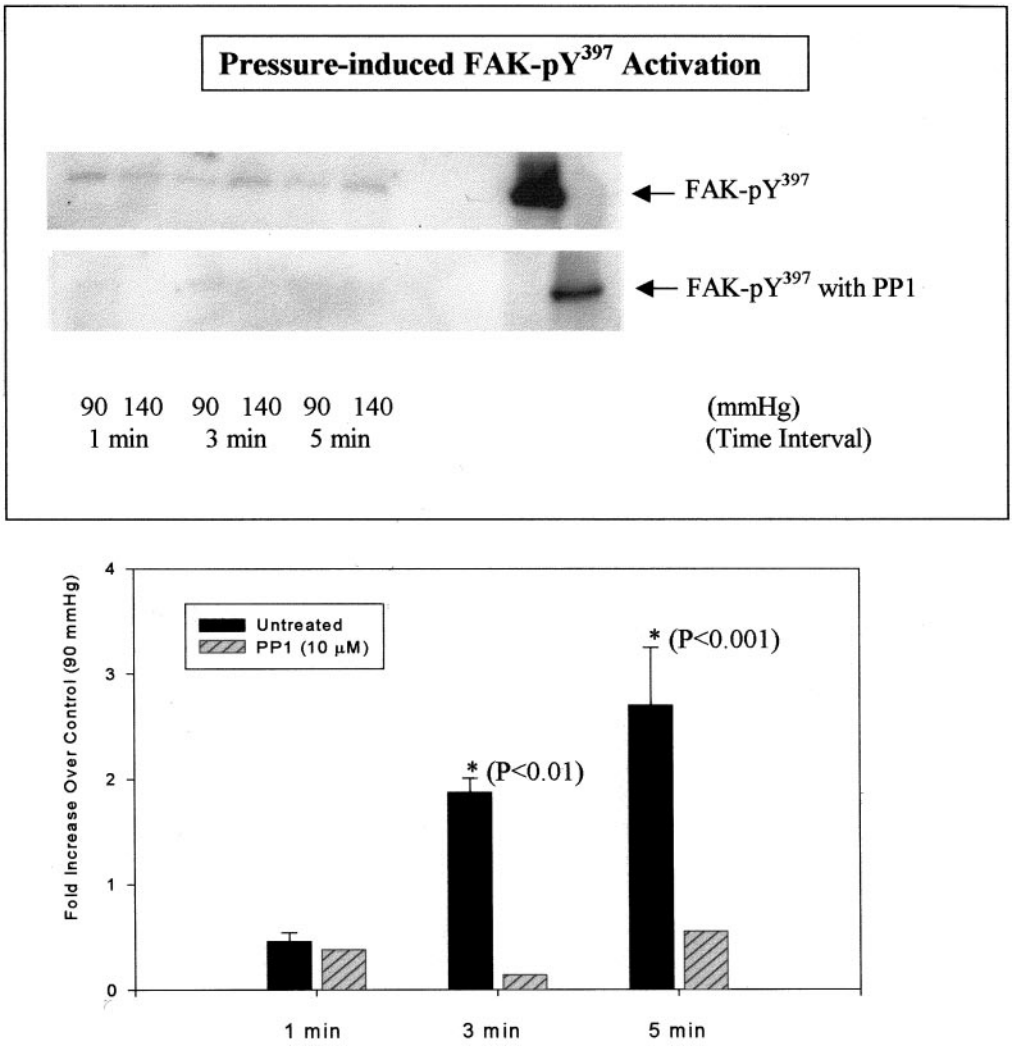


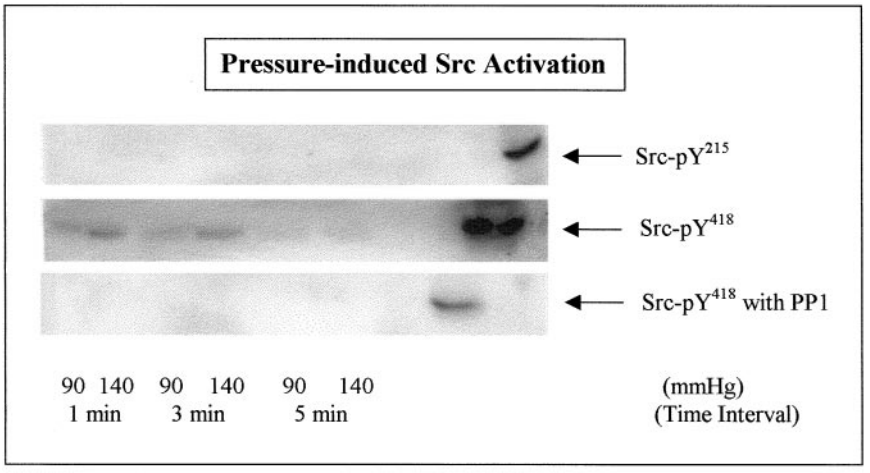
Figure 1. Effect of pressure on FAK-Y^{397} phosphorylation with and without PP1. In untreated vessels, significant FAK-Y^{397} activation occurs after 3 and 5 minutes of pressure stimulus (n=7 per time point; $P>0.05$ at 1 minute, $P<0.01$ at 3 minutes, and $P<0.001$ at 5 minutes). Pretreatment with PP1 completely blocked FAK-Y^{397} phosphorylation (n=3 per time point; $P>0.05$). Results are presented as fold increase over baseline experimental control value (90 mm Hg); *statistical significance.

temporal phosphorylation patterns of these cellular components within the first 5 minutes of acute hypertension. Western blotting with a phosphorylation-specific primary antibody to FAK-pY^{397} was performed on 21 pairs of vessels subjected to pressure stimulus for 1, 3, or 5 minutes. As shown in Figure 1, 1 minute at 140 mm Hg revealed no significant change ($P>0.05$, n=6) in FAK-Y^{397} activation as compared with experimental control vessels at 90 mm Hg. However, 3 minutes at 140 mm Hg increased FAK-pY^{397} 1.9±0.24-fold over control ($P<0.01$, n=6) and by 5 minutes was elevated 2.54±0.33-fold over the 90-mm Hg control ($P<0.001$, n=9). These results show that FAK tyrosine residue 397 is not immediately autophosphorylated by high pressure but becomes significantly activated by 3 and 5 minutes of pressure stimulus.

To unravel cellular events further during the acute phase of hypertension, we investigated the effect of pressure on Src-Y^{215} and Src-Y^{418} phosphorylation. Western blotting using a primary antibody to Src-pY^{215} was performed on vessel homogenates from a total of 25 paired experiments. The results indicate that activation of Src-Y^{215} is not involved in the first 1, 3, or 5 minutes of pressure challenge (Figure 2). However, Src-pY^{418} seems to be a key player in the initial events. Western blotting with a phosphorylation-specific primary antibody to Src-pY^{418} was used to evaluate pressure-induced activation at 1, 3, and 5 minutes. Results based on the analysis of 15 vessel pairs indicate a substantial 2.54±0.16-fold increase ($P<0.001$, n=5) in Src-Y^{418} phosphorylation at 1 minute, 2.86±0.22-fold increase ($P<0.001$, n=5) at 3 minutes, and a return to baseline ($P>0.05$, n=5) after 5 minutes of pressure stimulus. These results clearly demonstrate a significant involvement of Src-pY^{418} in the initial cellular response to acute hypertension.

Effects of Src Tyrosine Kinase Inhibition on FAK-Y^{397} Activation

Src-pY^{418} is significantly activated by 1 minute at 140 mm Hg and is followed by FAK-Y^{397} phosphorylation at 3 and 5



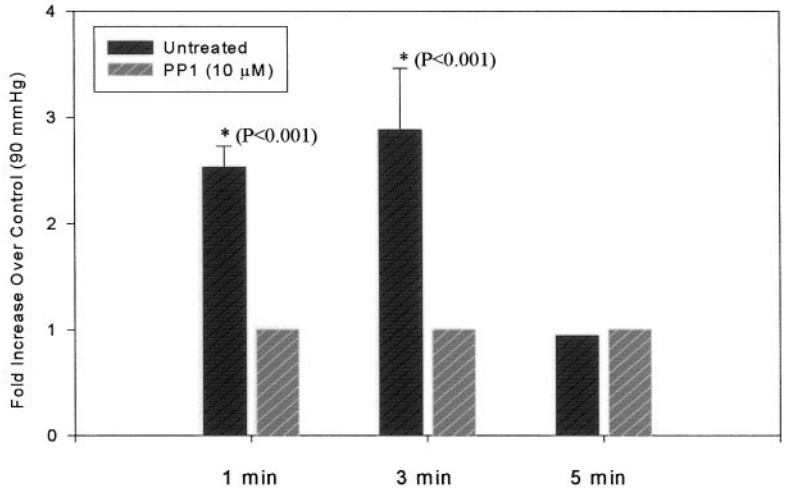


Figure 2. Effect of pressure on Src-Y^{215} and Src-Y^{418} (with and without PP1). In untreated vessels, Src-pY^{215} was not detected at 1, 3, or 5 minutes. However, Src-Y^{418} was significantly activated at 1 and 3 minutes (n=5 per time point; $P<0.001$) and returned to control at 5 minutes of pressure challenge. Pretreatment with PP1 completely blocked Src-pY^{418} activity. Results are presented as fold increase over baseline experimental control value (90 mm Hg); *statistical significance.

minutes. To determine whether FAK-Y^{397} activation is Src-dependent, we performed 9 additional paired experiments on PP1-treated vessels. PP1 was selected for these experiments for its superior ability to inhibit Src activity, based on previous work in our laboratory that compared the efficacy of herbimycin A, PP1, and PP2 in similar vessel experiments.[19] Western blotting using the primary anti–FAK-pY^{397} antibody revealed that PP1 completely blocked FAK-Y^{397} activation at 1, 3, and 5 minutes of pressure stimulus (Figure 1). These results demonstrate the requirement of Src tyrosine kinases in the downstream pressure-induced activation of FAK-Y^{397}.

Discussion

Our long-term goal has been to characterize the pressure-induced hypertrophic growth response in vascular smooth muscle. The extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway has been linked to the cellular growth response. Activated ERK 1/2 has been shown to translocate to the nucleus, where it stimulates ternary complex factors to bind serum response factor. The ternary complex factor–serum response factor complex then binds to the serum response element within the promoter of immediate early proto-oncogenes such as c-fos, c-jun, and c-myc. The newly synthesized fos protein has been shown to then dimerize with jun to form the AP-1 transcription factor, which modulates the expression of key growth regulatory genes.[20–24]

ERK 1/2 seems to be activated by growth factor RTKs, integrin receptor engagement, angiotensin AT-1 receptor stimulation, and mechanical strain. Hu et al[17] demonstrated ERK 1/2 activation after autophosphorylation of RTKs in response to cell stretch, in the absence of growth factors. Li et al[25,26] recently showed that cyclic stretch of aortic smooth muscle cells induces ERK 1/2, JNK, and p38, as well as MAPK phosphatase-1. Mechanical stress may also stimulate ERK 1/2 through the action of c-Src, which is associated with FAK at focal adhesion sites.[27] Wesselman et al[19] demonstrated that ERK 1/2 activity peaks at 5 minutes of pressure stimulus in isolated small mesenteric arteries and subsequently gives rise to an increased expression of the immediate early gene, c-fos. Src inhibition using PP1, PP2, or herbimycin A not only blocked c-fos expression but also inhibited ERK 1/2 activation. Collectively, work in our laboratory has shown that Src tyrosine kinases mediate pressure-induced ERK-MAPK activation and c-fos expression and that this response is correlated to wall stress.[5,19,28,29]

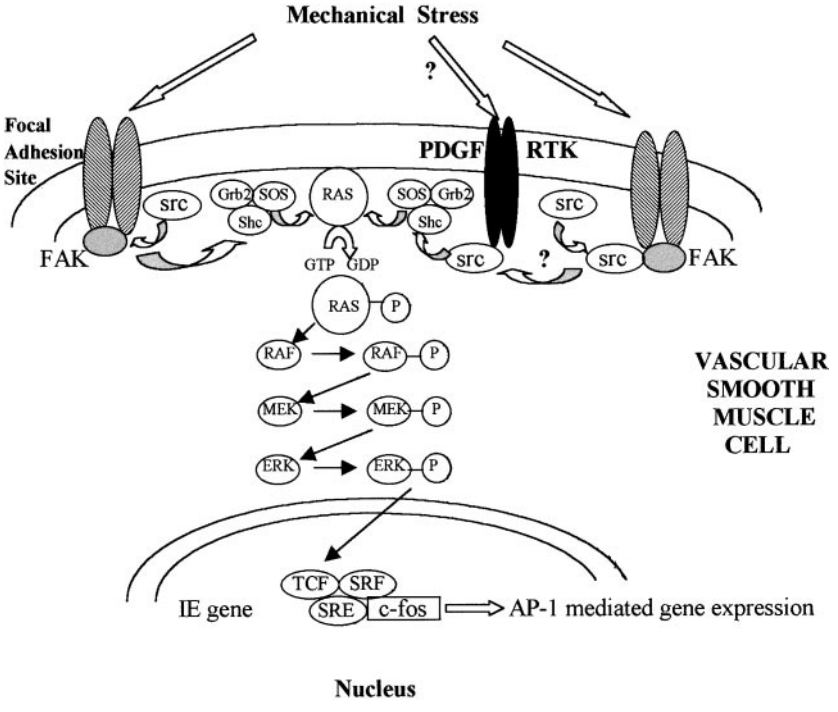


Figure 3. Diagrammatic representation of the theoretical mechanism by which hypertension stimulates vascular smooth muscle hypertrophy. Our findings suggest that increased wall stress triggers the activation of c-Src, which then becomes associated with FAK at focal adhesion sites. Downstream, the ERK-MAPK pathway mediates c-fos expression and subsequent upregulation of critical growth regulatory components. The involvement of growth factor RTKs, such as the PDGF-R, remains to be determined.

The next step in our investigation was to target events upstream of ERK 1/2 activation in an effort to identify the initial cellular trigger of the pressure-induced signaling cascade. Cellular components at or near the plasma membrane are the targets of this investigation. Integrins seemed like an ideal candidate because of their ability to sense and react to changes in cell adhesion, shear stress, and mechanical strain through direct interaction with the ECM. Src and FAK are key components of integrin signaling and are also involved in growth factor RTK pathways. In the present study, we demonstrate that acute hypertension triggers immediate Src-Y^{418} autophosphorylation in intact isolated resistance arteries and is required for downstream activation of FAK-Y^{397}. The involvement of FAK reinforces the growing body of evidence that suggests the potential collaboration among integrins and growth factor RTKs in the pressure response.[6]

Hu et al[17] demonstrated that mechanical strain can activate the PDGF-Rα in the absence of ligand. Interestingly, epidermal growth factor and PDGF receptors are co-localized within focal adhesion sites. Stover et al[14] recently showed that activation of the PDGF receptor triggers phosphorylation of Src at tyrosine residue 215. In the present study, we probed for the presence of Src-pY^{215} in pressurized vessels, and our findings demonstrated an absence of Src-pY^{215} activity at any time point investigated. This suggests that PDGF receptor phosphorylation is not necessary for Src activation.

Overall, the current study provides evidence that Src-pY^{418} may be the messenger that initiates the cascade and propagates the signal to other key players such as FAK. The results of these experiments are incorporated into Figure 3, which depicts a theoretical signaling mechanism by which hypertension stimulates cellular growth in vascular smooth muscle cells. The remaining question is, What activates Src? The mechanism by which Src, a cytosolic component, becomes activated remains unclear, although reactive oxygen species have recently been implicated.[13] Pressure-stimulated production of reactive oxygen species may be the initial trigger that activates Src[30], which in turn phosphorylates and activates FAK, the PDGF receptor, and other mechanically sensitive receptors in pressure mechanotransduction. In addition, activated Src has been shown to transactivate other growth factor receptors, such as the epidermal growth factor receptor, which may amplify the signaling cascade.[30]

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