Differential Regulation of Vascular Focal Adhesion Kinase by Steady Stretch and Pulsatility

Stéphanie Lehoux, PhD; Bruno Esposito; Régine Merval; Alain Tedgui, PhD

Background—In vivo tensile strain in arteries comprises 2 components: steady stretch and pulsatile stretch. However, little attention has been paid to the differential transduction of these stimuli in whole vessels.

Methods and Results—Using rabbit aortas maintained in organ culture for 24 hours, we found that focal adhesion kinase (FAK) was strongly activated by high intraluminal pressure (150 mm Hg), as evidenced by increased phosphorylation ($P<0.01$) of tyrosine residues Tyr-397 (3.06±0.17-fold), Tyr-407 (3.71±0.65-fold), Tyr-861 (1.92±0.33-fold), and Tyr-925 (2.41±0.39-fold), compared with 80 mm Hg controls. Immunohistochemistry showed positive staining for these phosphotyrosines in the endothelium and innermost smooth muscle cell layers. Total FAK phosphorylation was reduced in vessels at 150 mm Hg by treatment with the Src family kinase inhibitor PP2 or with the integrin–extracellular matrix interaction–blocking RGD peptide, attaining 1.75±0.22-fold and 2.00±0.19-fold, respectively ($P<0.05$), compared with 3.07±0.38-fold ($P<0.001$) in untreated vessels. PP2 prevented phosphorylation of Tyr-407 and Tyr-925, whereas RGD peptide abolished phosphorylation of Tyr-397 and Tyr-407. PP2 and RGD peptide also inhibited high pressure–induced binding of FAK with the effector Grb2 and blocked activation of extracellular regulated kinase (ERK) 1/2 in vessels at 150 mm Hg. In contrast, 10% cyclic stretch in aortas did not induce significant FAK phosphorylation relative to nonpulsatile controls. Furthermore, although ERK1/2 was activated in vessels exposed to pulsatility, it was not blocked by PP2 or RGD peptide treatment.

Conclusions—Our results demonstrate that (1) steady and cyclic modes of stretch are transduced differently in the aorta, the former implicating FAK, the latter not, and (2) Src and integrins are involved in steady pressure–induced FAK. **(*Circulation*. 2005;111:643-649.)**

Key Words: mechanics ■ signal transduction ■ remodeling ■ hypertension ■ arteries

Arteries are permanently exposed to a basal stretch established by arterial pressure and paired to a pulsatile component owing to the cardiac cycle. Vascular smooth muscle cells (VSMCs) are able to distinguish between pulsatility and steady stretch, because chronic amplification of these 2 stimuli can have very different repercussions: areas of high pulsatility are particularly prone to atherosclerotic plaque formation,[1] whereas vessels of hypertensive patients acquire a characteristic rigidity due to extensive remodeling.[2]

Vascular cells possess a variety of mechanosensors capable of perceiving and transmitting external signals to the intracellular compartment (reviewed in Lehoux and Tedgui[3]). These include the integrins, which bind extracellular matrix proteins and interact functionally with cytoskeletal elements and associated proteins within the cell. In VSMCs in culture, creation of new integrin–extracellular matrix interactions appears to be a key component of the mechanotransduction process, because the RGD peptide that prevents integrin–extracellular matrix bridging also inhibit the VSMC proliferative response to cyclic strain.[4] Mechanical signaling cascades also include FAK (focal adhesion kinase), which is recruited to focal adhesion complexes formed on integrin stimulation. FAK activation has been shown to occur in endothelial cells or VSMCs exposed to cyclic stretch,[5,6] and in resistance arteries, acute steady stretch induces phosphorylation of FAK at Tyr-397.[7]

Downstream signaling pathways elicited by mechanical factors and transmitted through FAK may include the extracellular signal–regulated kinase (ERK) 1/2 cascade. Indeed, in fibroblasts, cyclic strain induction of ERK1/2 is significantly inhibited if cells are transfected with a mutant form of FAK.[8] Similarly, shear stress activates ERK1/2 in endothelial cells, but this response is attenuated in cells expressing a dominant-negative FAK mutant.[9] Although both cyclic and steady modes of stretch are known to activate ERK1/2 in vessels[10–12] and in VSMCs,[13] the role of FAK in this ERK1/2 activation has not been established. Interestingly, the pathways of vascular ERK1/2 activation by steady and pulsatile stretch appear to differ, the former being sensitive to Src family kinase inhibition,[10] the latter not,[11] so studying FAK

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activity in vessels exposed to these 2 forms of strain may also yield distinct results.

Signaling pathways induced by stretch are dependent not only on the type of matrix on which cells are plated[13] and on specific integrin–matrix interactions[14] but also on the spatial organization of VSMCs in culture (3-dimensional versus a 2-dimensional environment)[15] as well as their differentiation state.[16] Although these specificities are interesting with regard to transduction pathway identification, they also imply that observations made in cultured cells may differ from what occurs in whole vessels. Therefore, in the present study, we evaluated and compared the effects of pulsatile and steady stretch on FAK activity in whole vessels in organ culture. Furthermore, we sought to elucidate how FAK activation processes contribute to the mechanically induced ERK1/2 signaling cascade under these conditions.

Methods

Organ Culture

Male New Zealand White rabbits (2 to 2.5 kg; Charles River Laboratories, Lyon, France) were anesthetized with sodium pentobarbital (30 mg/kg IV). Arterial segments from the descending thoracic aorta were isolated and cannulated under pressure as previously described.[17] Isolated aortic segments were connected to a closed perfusion circuit consisting of a 3-port reservoir, a peristaltic pump (Masterflex, Cole Palmer Instrument Co), and a pressure chamber, which allowed application of controlled, intraluminal hydrostatic pressure. Vessel segments were immersed in a bath filled with culture medium identical to that used in the intraluminal compartment, consisting of Dulbecco's modified Eagle's medium containing antibiotics (100 IU/L penicillin, 100 mg/L streptomycin, and 10 mg/L amphotericin B) and supplemented with 20% fetal calf serum (Boehringer Mannheim). Organ culture of the aortic segments was carried out under sterile conditions in an incubator containing 5% CO_2 at 37°C. Segments used for experiments were maintained in organ culture for 24 hours unless mentioned otherwise. The local ethics committee approved all experiments.

Experimental Protocol

Each aorta was divided into 3 segments that were arbitrarily assigned different culture conditions, such that for each complete aorta there was 1 nonpulsatile segment at 80 mm Hg (the 100% setting for phosphorylation, coprecipitation, or activity assays) and 2 segments either at high pressure (150 mm Hg) or at 10% pulsatility. Pulsatility (0.75 Hz) was applied with a pump that produced a 10% change in vessel diameter at each pulse, as illustrated earlier.[10] At such settings, the pump produced a flow of 40 mL/min, corresponding to a negligible shear stress (≈0.4 dyne/cm^2). In comparison, control nonpulsatile vessels were perfused at 0.2 mL/min to ensure renewal of culture medium within the aorta but to avoid any pulsatility (0.5%) effects. The average pressure in the pulsatile segments was set at 80 mm Hg, but it effectively ranged from 70 to 90 mm Hg at each pulse. In some experiments, RGD peptide (Gly-Arg-Gly-Asp-Ser-Pro, 0.4 mg/mL), RGE peptide (Gly-Arg-Glu-Ser, 0.4 mg/mL), or PP2 (10 μmol/L) was added to the culture medium at the onset of the experiments in both intraluminal and extraluminal compartments. For short-term experiments, vessels were stabilized for 90 minutes at 80 mm Hg, and then intraluminal pressure was elevated to 150 mm Hg and maintained for 5 to 20 minutes at this pressure before rapid removal and processing.

Tissue Extraction

Vessel segments were pulverized in LN_2. The powders were resuspended in ice-cold lysis buffer (20 mmol/L Tris-HCl pH 7.5, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20, and protease inhibitor tablets [Boehringer]) at a ratio of 0.3 mL/10 mg wet weight. Extracts were incubated on ice for 15 minutes and then centrifuged (14 000*g*, 15 minutes, 4°C). The detergent-soluble supernatant fractions were retained, and protein concentrations in samples were equalized by a Bio-Rad protein assay.

Immunoblotting

Lysates containing equal amounts of protein (30 μg) were electrophoresed on polyacrylamide gels and transferred to nitrocellulose membranes. In some experiments, 30-μg protein samples were immunoprecipitated with anti-FAK antibodies (Transduction Laboratories) and protein A/G agarose (Santa Cruz) before electrophoresis. Membranes were probed with anti–phospho-ERK1/2, anti-FAK (Transduction Laboratories), anti-ERK2, anti-Grb2 (Santa Cruz), or anti–phospho-Tyr-397-, Tyr-407-, Tyr-576-, Tyr-577-, Tyr-861-, or Tyr-925-FAK antibodies (Biosource International). An enhanced chemiluminescence system was used as the detection method (Amersham).

FAK Activity

FAK was immunoprecipitated from 30-μg protein samples as indicated earlier. Immunoprecipitates were incubated with paxillin in kinase buffer (50 mmol/L HEPES pH 7.5, 10 mmol/L $MnCl_2$, 10 mmol/L $MgCl_2$, and 2 mmol/L NaF) containing [γ^{32}P]ATP and PP2 (10 μmol/L) for 20 minutes at 30°C. Kinase reactions were stopped by addition of 2× Laemmli buffer, and samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by autoradiography. After autoradiography, gel proteins were transferred onto a polyvinylidene difluoride membrane and probed with anti-FAK to confirm equal loading.

Immunohistochemistry

Vessel segments were embedded vertically in Tissu-Tek (Sakura), and cryosections (10 μm) from the 3 segments of each aorta were processed simultaneously. Sections were incubated with anti–phospho-Tyr-397, Tyr-407, Tyr-861, and Tyr-925 antibodies. Immunostains were revealed by avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit, Vector Laboratories).

Data Analysis

Results, indicating fold increases based on ratios of paired observations within rabbits, are expressed as mean±SE. Data were analyzed by 1-way (for comparison of different intraluminal pressure groups) or 2-way (to evaluate effects of inhibitor treatments) ANOVA. When ANOVA analysis yielded significant results, comparisons were done with Bonferroni's test. Statistical significance was accepted for values of $P<0.05$.

Results

Divergent FAK Phosphorylation Patterns in Vessels Under Steady or Pulsatile Stretch

A first set of experiments was designed to evaluate total FAK phosphorylation, an index of its activation state, in cultured vessels. FAK was immunoprecipitated from aortic lysates and probed for tyrosine phosphorylation. As depicted in Figure 1A, total FAK phosphorylation was greatly enhanced in vessels maintained at 150 mm Hg for 24 hours compared with vessels kept at 80 mm Hg (3.24±0.32-fold, $P<0.001$). On the other hand, segments submitted to 10% pulsatility did not exhibit significantly different levels of total FAK phosphorylation.

A more detailed assessment of FAK activation with the use of site-specific phosphotyrosine antibodies (Figure 1B) revealed that phosphorylation of Tyr-397, Tyr-407, Tyr-861, and Tyr-925 was enhanced in aortic segments kept at 150 mm Hg versus vessels at 80 mm Hg. In fact, FAK

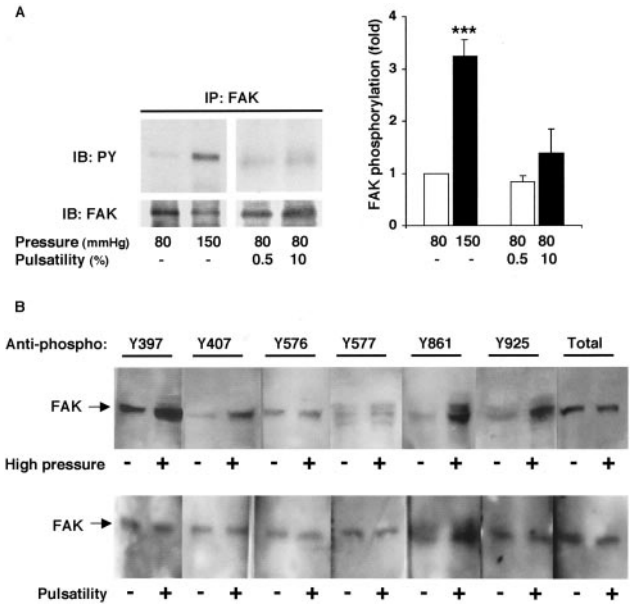

Figure 1. FAK phosphorylation is enhanced in aortas exposed to steady but not pulsatile stretch. A, Aortas were maintained for 24 hours at normal (80 mm Hg) or high (150 mm Hg) pressure in nonpulsatile conditions or at 80 mm Hg with 10% pulsatility. Total FAK was immunoprecipitated from vessel lysates, and its phosphorylation state was assessed with anti-phosphotyrosine (PY) antibodies. FAK phosphorylation was increased significantly only in aortas exposed to high intraluminal pressure (150 mm Hg). Data are mean±SE of 7 or 8 experiments. ***$P<0.001$ vs 80 mm Hg. B, Two segments from same aorta were placed at 80 or 150 mm Hg (top) or at 80 mm Hg, with or without pulsatility (bottom). Vessel lysates were probed with phosphospecific antibodies targeting 6 separate FAK tyrosine (Y) residues or with total FAK antibody. FAK tyrosine phosphorylation was enhanced at 4 sites (Tyr-397, -407, -861, and -925) in aortas at high pressure and at 1 site (Tyr-861) in pulsatile aortas. Representative of 4 separate experiments. All other abbreviations are as defined in text.

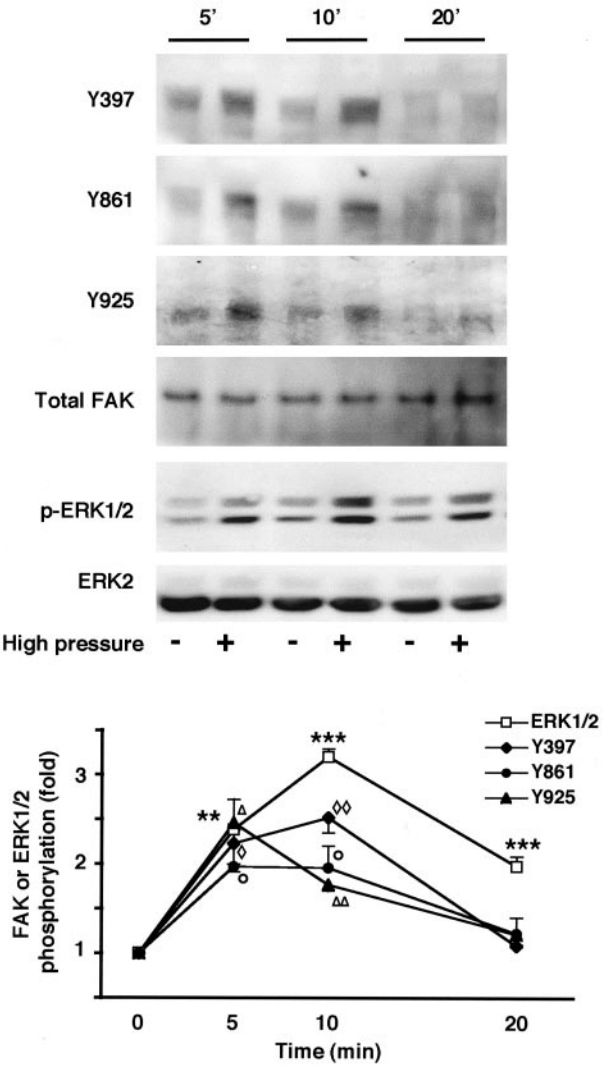

Figure 2. Kinetics of FAK and ERK1/2 phosphorylation induced by pressure. Aortic segments were cannulated and maintained at 80 mm Hg for 90 minutes, and then intraluminal pressure was readjusted to 150 mm Hg in some segments for indicated times. Pressure-induced phosphorylation of Tyr-397, Tyr-861, and Tyr-925 was maximal by 5 minutes and remained high at 10 minutes, returning to baseline thereafter. ERK1/2 phosphorylation was of comparatively slower onset, reaching its peak at 10 minutes and remaining substantial at 20 minutes. Figure representative of 3 separate experiments. *, ○, Δ,◇$P<0.05$; **,ΔΔ,◇◇$P<0.01$; and ***$P<0.001$ vs 80 mm Hg (0 minutes). All abbreviations are as defined in text.

activation was incremental, because vessels maintained at 120 mm Hg also showed enhanced FAK phosphorylation, though to a lesser degree than arteries at 150 mm Hg (Data Supplement Figure I). Furthermore, FAK tyrosine residue phosphorylation was also enhanced in an in vivo model of acute, angiotensin II–induced hypertension (Data Supplement Figure I). Conversely, the phosphorylation state of Tyr-576 and Tyr-577 did not differ between vessels maintained at either pressure condition, nor did total FAK content (Figure 1). Vessels stimulated by cyclic stretch displayed enhanced phosphorylation of Tyr-861 only when matched with nonpulsatile aortas.

Kinetics of FAK Activation

We have previously demonstrated that pressure-dependent ERK1/2 activation is biphasic, characterized by an acute peak and a second, more prolonged induction.[10] Here, we confirmed that ERK1/2 activity was maximal (3.20±0.09-fold, $P<0.001$) 10 minutes after onset of high intraluminal pressure and declined at 20 minutes, corresponding to the early activation phase (Figure 2). Interestingly, the kinetics of FAK phosphorylation slightly preceded ERK1/2 activation: Phosphorylation of FAK on Tyr-397, Tyr-861, and Tyr-925 was maximal within 5 minutes (by 2.23±0.32-, 1.97±0.41-, and 2.46±0.46-fold, respectively; $P<0.05$), remained elevated at 10 minutes, and then fell to baseline levels at 20 minutes. These observations concur with our hypothesis of a stretch-induced signaling pathway involving sequential activation of FAK and ERK1/2.

Integrin–Extracellular Matrix Interactions and Src Family Kinases Participate in Pressure-Induced FAK Phosphorylation

To characterize the pathways of FAK activation induced by steady high pressure, vessel segments were maintained in culture for 24 hours at 150 mm Hg in the presence of a Src

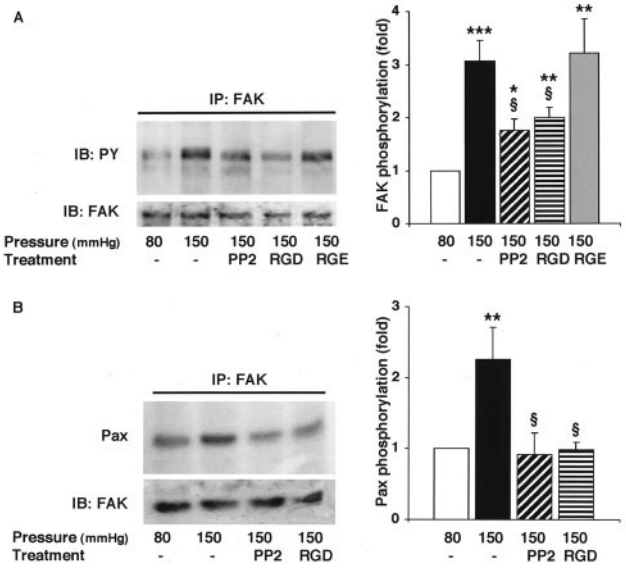

Figure 3. Steady pressure–induced FAK phosphorylation is reduced by Src family kinase or integrin–extracellular matrix interaction inhibitors. Aortas were cultured for 24 hours at 80 or 150 mm Hg without treatment or at 150 mm Hg with PP2 (10 μmol/L), RGD peptide (0.4 mg/mL), or RGE peptide (0.4 mg/mL). A, Total FAK was immunoprecipitated and probed for tyrosine phosphorylation (PY). FAK phosphorylation induced by high pressure was diminished in treated vessels at 150 mm Hg but remained above levels seen in 80-mm Hg controls. B, Activity of immunoprecipitated total FAK was evaluated in kinase assay with paxillin as substrate. Data are representative of 8 (A) or 3 (B) separate experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs 80 mm Hg; §$P<0.05$ vs untreated vessels at150 mm Hg. All abbreviations are as defined in text.

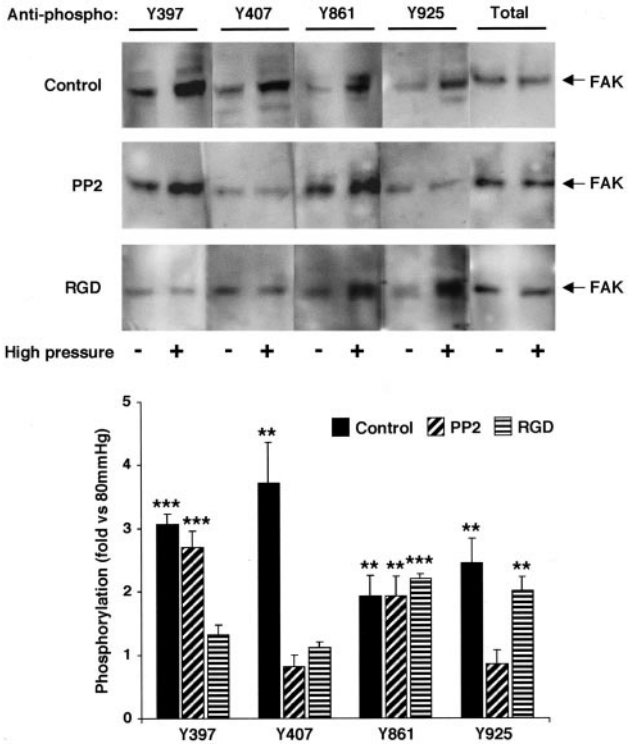

Figure 4. PP2 and RGD peptide affect specific FAK tyrosine phosphorylation sites. Segments from same aorta were maintained at normal (80 mm Hg) or high (150 mm Hg) pressure in absence (control) or presence of PP2 or RGD peptide. Vessel lysates were probed with phosphospecific antibodies targeting 4 tyrosine (Y) residues phosphorylated in vessels at high pressure according to Figure 1. PP2 prevented phosphorylation of Tyr-407 and Tyr-925, whereas RGD peptide blocked phosphorylation of Tyr-397 and Tyr-407. Histogram shows phosphorylation levels of 4 tyrosines in aortic segments at 150 mm Hg as percentage of treatment-matched segments from same aorta kept at 80 mm Hg (100%). Results are mean±SE, representative of 6 (controls) or 3 (treated) separate experiments. **$P<0.01$ and ***$P<0.001$ vs vessels at 80 mm Hg with same treatment. All abbreviations are as defined in text.

family kinase inhibitor (PP2) or RGD peptide. Figure 3A shows that both treatments partially reduced total FAK phosphorylation in vessels; FAK phosphorylation only reached 1.75±0.22-fold of 80 mm Hg controls in PP2-treated aortas and 2.00±0.19-fold in RGD-treated aortas, compared with 3.07±0.38-fold in untreated vessels. Control RGE peptide did not affect total FAK phosphorylation.

Elevated total FAK phosphorylation in vessels at high pressure was correlated with increased kinase activity (2.26±0.45-fold, $P<0.01$), as measured by phosphorylation of paxillin (Figure 3B), despite the fact that residues Tyr-576 and Tyr-577 were not phosphorylated in these conditions, as shown earlier. In comparison, although inhibition of FAK phosphorylation by PP2 or RGD peptide was only partial, these treatments completely prevented FAK activity.

The effects of PP2 and RGD were examined in greater detail by focusing on the tyrosine residues shown in Figure 1 to be specifically phosphorylated in vessels at high pressure. In untreated controls, phosphorylation of Tyr-397, Tyr-407, Tyr-861, and Tyr-925 reached 3.06±0.17-fold ($P<0.001$), 3.71±0.65-fold ($P<0.01$), 1.92±0.33-fold ($P<0.01$), and 2.41±0.39-fold ($P<0.01$) of controls (80 mm Hg), respectively. Phosphorylation of Tyr-407 and Tyr-925 was abolished in vessels at 150 mm Hg treated with PP2, whereas RGD peptide prevented phosphorylation of Tyr-397 and Tyr-407 (Figure 4). Interestingly, neither treatment interfered with phosphorylation of Tyr-861, which remained elevated in all vessels at high pressure. Similarly, pulsatility-induced phosphorylation of Tyr-861 was also impervious to PP2 or RGD peptide treatment (data not shown).

To localize FAK activation sites within the arterial wall, histological sections of vessels were probed with antibodies corresponding to the pressure-dependent phosphotyrosines (Figure 5). As expected, little staining was detected in aortas maintained at 80 mm Hg. However, vessels at 150 mm Hg showed positive staining for Tyr-397, Tyr-407, Tyr-861, and Tyr-925. Staining was most marked in the innermost layer of the aorta, corresponding to the area of greatest strain. In vessels treated with PP2 or RGD peptide, staining for Tyr-407 and Tyr-925 or for Tyr-397 and Tyr-407 was reduced, respectively, in agreement with immunoblot observations, whereas Tyr-861 staining was not abated. Furthermore, only staining for Tyr-861 was enhanced in pulsatile vessels.

FAK Associates With Grb2 in Vessels at High Pressure

To establish a link between FAK phosphorylation and signaling events leading to ERK1/2 activation, the interaction of

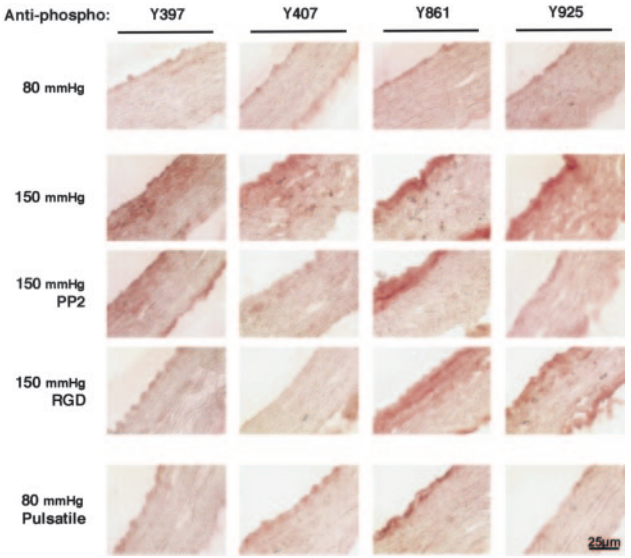

Figure 5. Immunohistochemical localization of phosphorylated FAK in cultured aortas. Sections (10 μm) from vessel segments maintained in culture at different pressure of pulsatility conditions, with or without PP2 or RGD peptide treatment, were stained with antibodies targeting phospho-Tyr-397, -Tyr-407, -Tyr-861, or -Tyr-925. Staining was positive in endothelial cells and in inner layers of SMCs of aortas cultured at 150 mm Hg compared with segments maintained at 80 mm Hg. In vessels at 150 mm Hg, PP2 abated staining for Tyr-407 and Tyr-925, whereas RGD peptide reduced Tyr-397 and Tyr-407 staining, but neither treatment prevented Tyr-861 staining. Phospho–Tyr-861 was also revealed in pulsatile vessels. Representative of 5 (controls), 4 (treated), and 3 (pulsatile) separate experiments. All abbreviations are as defined in text.

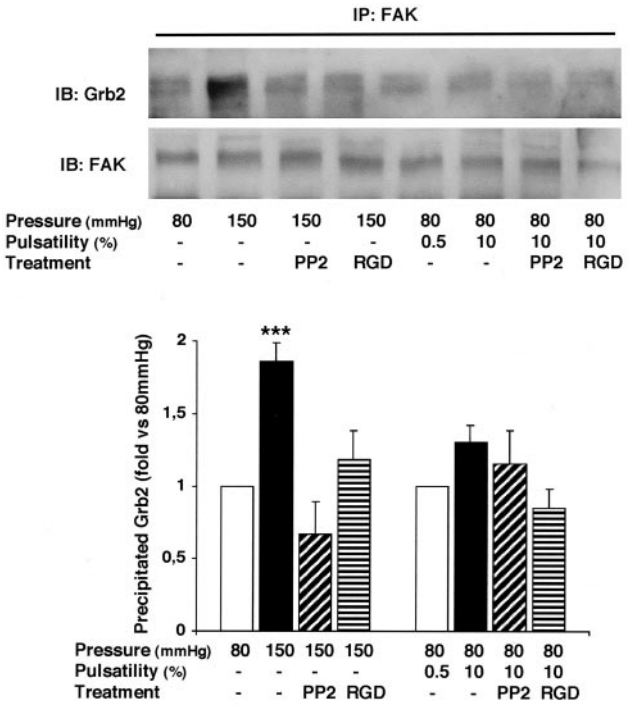

Figure 6. Grb2 interacts with FAK in aortas at high pressure. FAK was immunoprecipitated from lysates of vessels maintained at different pressure or pulsatility conditions, with or without PP2 or RGD peptide treatment, for 24 hours. Precipitated proteins were then probed with anti-Grb2 or anti-FAK antibodies. Grb2 was found to coimmunoprecipitate with FAK in aortas at 150 mm Hg, but this association was abolished in PP2- or RGD peptide–treated vessels. Grb2 and FAK did not coimmunoprecipitate in pulsatile vessels. Data are mean±SE of 6 (controls), 3 (treated, steady stretch), or 4 (treated, pulsatile) experiments. ***$P<0.001$ vs 80 mm Hg. All abbreviations are as defined in text.

FAK with the downstream effector Grb2 was evaluated. As depicted in Figure 6, Grb2 coimmunoprecipitated with FAK exclusively in vessels exposed to steady, high, intraluminal pressure and not in aortas stimulated by pulsatile pressure. In vessels at 150 mm Hg, the amount of Grb2 found to coprecipitate with FAK was nearly doubled (1.86±0.13-fold, $P<0.001$) compared with vessels at 80 mm Hg. Furthermore, the interaction of Grb2 with FAK was no longer observed in vessels at 150 mm Hg treated with PP2 or with RGD peptide.

Differential Activation of ERK1/2 by Steady and Pulsatile Pressure

Finally, in untreated vessels, both high pressure and pulsatility induced ERK1/2, to 2.61±0.23 and 2.00±0.20-fold of controls, respectively ($P<0.001$; Figure 7). ERK1/2 activation by steady pressure was completely abolished in aortas cultured in the presence of PP2 or RDG peptide, whereas the same treatments failed to prevent ERK1/2 activation by pulsatile stretch (Figure 5). Hence, only ERK1/2 activation by high intraluminal pressure required Src family kinases and integrin–extracellular matrix interaction. Moreover, the specificity of this pathway was demonstrated by the failure of PP2 or RGD peptide to prevent pressure-induced nuclear factor-κB pathway activation, measured by inhibitor-κB degradation (Data Supplement Figure II).

Discussion

The present work reveals key differences in signal transduction pathways elicited by steady versus cyclic stretch. Most strikingly, we found a major disparity in the extent of FAK phosphorylation induced by these 2 stimuli, which translated to a divergence observed in ERK1/2 activation pathways. In vessels exposed to steady stretch, ERK1/2 activation was prevented by Src family kinase inhibition and RGD peptide, both of which reduced FAK phosphorylation. Short-term experiments also showed that FAK phosphorylation precedes ERK1/2 activation, substantiating our hypothesis that FAK lies upstream of ERK1/2. On the other hand, ERK1/2 activation in pulsatile aortas was impervious to PP2 or RGD peptide, demonstrating that this process, previously shown to depend on oxygen free radical production,[11] does not implicate Src family kinases or integrin engagement.

Steady pressure–induced signaling has received little attention thus far. In one recent study, resistance arteries exposed to a sudden increment in intraluminal pressure showed increased phosphorylation of Src at 1 and 3 minutes but not at 5 minutes and Src-dependent FAK phosphorylation on Tyr-397 at 3 and 5 minutes.[7] Our findings are in keeping with those results for acute pressure-induced FAK phosphorylation at this site but contrast in the sensitivity of Tyr-397 to Src kinase inhibition, which was absent in our case. This difference may be due to the nature of the vessels studied (resistance versus conductance arteries) or to the discrepancy in kinetics (5 minutes versus 24 hours). One

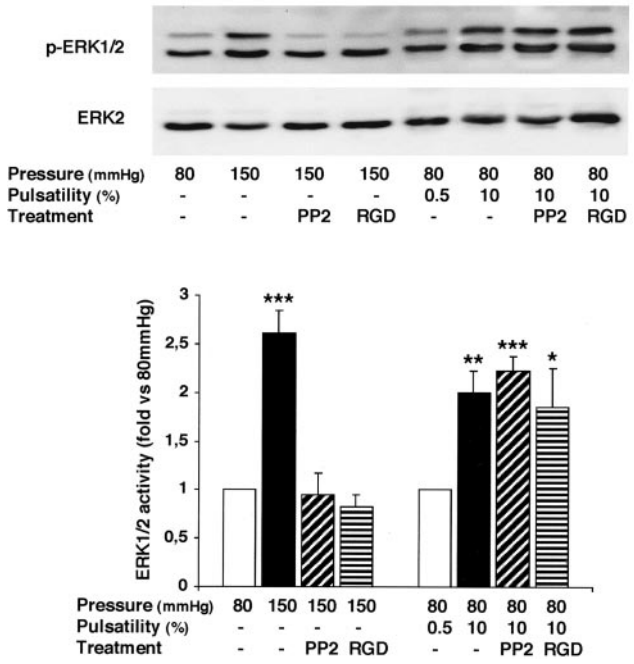

Figure 7. ERK1/2 activation by steady stretch is abolished by PP2 or RGD peptide. Lysates from aortic segments placed in different culture conditions were probed with antibodies targeting phosphorylated ERK1/2 or total ERK2. ERK1/2 was activated by both high pressure and pulsatility, but only effects of high pressure were inhibited by treatment with PP2 or RGD peptide. Results represent mean±SE of 6 (controls), 3 (treated, steady stretch), or 4 (treated, pulsatile) experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs 80 mm Hg. All abbreviations are as defined in text.

particular interest of looking at signal transduction over 24 hours is that it may be a good indicator of pathways involved in long-term vascular remodeling rather than short-term regulatory processes.

Autophosphorylation of FAK at Tyr-397 activates a docking site for Src family kinases, which in turn can phosphorylate several other activation sites on FAK, including Tyr-925. Binding of Grb2 at this latter tyrosine launches a chain of events that culminate in ERK1/2 activation.[18] In the present study, we went on to show that beyond Tyr-397, 3 other tyrosines on FAK are phosphorylated in vessels maintained at 150 mm Hg: Tyr-407, Tyr-861, and Tyr-925. Only Tyr-407 phosphorylation was inhibited by both RGD peptide and PP2, whereas phosphorylation of Tyr-397 was blocked by RGD peptide and that of Tyr-925 inhibited by PP2 only. So our findings imply that pressure-induced FAK phosphorylation involves 2 separate events: (1) integrin–extracellular matrix interaction, driving recruitment of FAK to focal contacts at sites of integrin clustering and autophosphorylation at Tyr-397, and (2) Src family kinase activation, leading to phosphorylation of FAK at 2 more sites, Tyr-407 and Tyr-925. A previous study indicated that phosphorylation of Tyr-397 and Tyr-925 is required for FAK-Grb2 association; the actual Grb2 binding site was reported to encompass Tyr-925 only, but phosphorylation of Tyr-397 initiated the sequential binding of Src to FAK, leading to Tyr-925 phosphorylation.[19] Here, however, Tyr-925 phosphorylation occurred in spite of RGD treatment that blocked Tyr-397 phosphorylation, but apparently phosphorylation of Tyr-925 alone was not sufficient to support FAK-Grb2 association and downstream ERK1/2 activation. Because coprecipitation of Grb2 with FAK was sensitive to both RGD peptide and PP2, as was phosphorylation of Tyr-407, our results suggest that this latter tyrosine may play a previously unsuspected role in transducing mechanical stimuli in the intact vessel. Our observation that steady pressure–dependent activation of ERK1/2 was blocked by RGD peptide and PP2 further substantiates the dual role for integrins and Src in FAK-mediated signaling.

It is interesting that Tyr-576 and Tyr-577 were not phosphorylated in vessels at high pressure. These tyrosines lie in the activation loop of FAK, and their phosphorylation is classically required for full activation of the kinase.[18] Nevertheless, an appreciable level of FAK activity could be detected in vessels at steady elevated pressure, as suggested by the downstream activation of ERK1/2 and confirmed by the activity assay with paxillin as a substrate.

On the other hand, the only FAK residue found to be phosphorylated not only in vessels at steady pressure but also in pulsatile aortas was Tyr-861. Intriguingly, although Tyr-861 was originally shown to be phosphorylated by Src,[20] this did not appear to be the case in pressure-stimulated vessels, because phosphorylation state was unaffected by PP2 or by RGD peptide for that matter. Nevertheless, immunohistological staining revealed that phospho–Tyr-861 distribution did not differ significantly from that of other tyrosines investigated, so that inhibitor penetration cannot be held accountable for this discrepancy. Perhaps differences in experimental models or species are at cause. Further studies will be needed to uncover mechanosensitive Tyr-861 activation pathways and downstream signaling cascades.

The present results outline a pathway for steady stretch–induced ERK1/2 activation involving FAK as an upstream mediator, both in short-term experiments and at 24 hours. Conversely, phosphorylation of Tyr-861 alone is clearly not sufficient to drive ERK1/2 activation in pulsatile vessels. However, in vitro cell cultures exposed to cyclic strain appear to respond differently as regards stretch-activated FAK phosphorylation or integrin–extracellular matrix–dependent ERK1/2 activation. For example, it was recently demonstrated that imposing cyclic stretch on ventricular myocytes induces Src-dependent FAK phosphorylation at Tyr-397 and Tyr-577.[21] In a similar study, cyclic stretch was found to stimulate phosphorylation of FAK at Tyr-397 and Tyr-925 in cultured fibroblasts, along with activation of ERK1/2 and p38. Transfection of cells with inactive FAK mutants completely abolished cyclic stretch–induced p38 and strongly reduced ERK1/2 activation.[22,23] Cyclic strain also triggered total FAK phosphorylation in endothelial cells.[5] In SMCs, ERK1/2 was stimulated by vibrating ferromagnetic beads coated with fibronectin, vitronectin, or β_3-integrin but not by α_2- or β_1-integrin–coated beads,[14] and cyclic stretch induced ERK1/2 in cells cultured on pronectin but not laminin.[13] Differences between whole-vessel and cell culture experiments are not restricted to FAK-ERK1/2 signaling; numerous publications describe cyclic stretch–activated NF-κB in vascular

cells (summarized in Lemarie et al[24]), but presently, although we demonstrated a FAK-independent, steady stretch activation of NF-κB, we found no evidence for activation of this pathway in pulsatile vessels. Several factors could account for the disparities between our observations and the aforementioned studies. Perhaps most important is the fact that vessels comprise cells within a 3-dimensional matrix environment, unlike the 2-dimension cell culture, which can influence vascular cell differentiation state and response to stimuli,[15] as well as focal adhesion organization and FAK phosphorylation.[25] Cells in culture, as used in previous experiments cited earlier, are bound to be dedifferentiated and, in the case of VSMCs, of a synthetic rather than contractile phenotype. Phenotype has been shown to influence ERK1/2 signaling in VSMCs exposed to pulse pressure,[16] and it is likely to explain, at least in part, the contrasting responses to pulsatile stretch encountered in cultured cells and whole vessels. Of further consideration is extracellular matrix composition, which is complex and organized in native vessels and, as such, dictates the nature of new integrin–matrix protein interactions that can occur in response to stretch.

Nevertheless, distinguishing between pulsatile and steady stretch is important because in the whole vessel, these stimuli have very different outcomes. In the aorta, pulsatility is a physiological stimulus, given the rhythmic changes in pressure within this vessel imposed by the cardiac cycle. Hence, ERK1/2 activity observed in pulsatile segments presumably represents a basic level contributing to maintenance of vessel structure and function. In the case of a blood pressure increment, several morphological changes take place in the vessel wall, associated with thickening and rigidification of the vessel. Supplemental activation of ERK1/2 in this context, transmitted through FAK, may tip the balance toward a proliferative/hypertrophic SMC phenotype. The in vitro vascular cell experiments described earlier, typifying SMCs in a wounded vessel (such as after angioplasty), also point to a role for FAK-driven ERK1/2 activation in cell proliferation (driving restenosis). Blocking ERK1/2 would presumably prevent undesired SMC proliferation but may incur other phenotypic changes through inhibition of basal ERK1/2 activity. Our observations suggest that targeting Tyr-407 may be the single most specific way to maintain SMC quiescence in the vessel; on the contrary, it may prove beneficial to avoid hindering FAK phosphorylation at Tyr-861, which could be involved in other signaling pathways unrelated to ERK1/2.

In summary, our results show that high intraluminal pressure and pulsatile pressure are transduced differently in the vascular wall. Although both stimuli activate the ERK1/2 pathway, only in the case of steady stretch is FAK implicated as an upstream mediator; steady stretch is in fact a strong activator of FAK, acting through integrin–extracellular matrix interaction and Src family kinase induction. Pulsatility, on the other hand, has very little impact on FAK phosphorylation in the whole vessel. These results emphasize key differences in vascular response to steady and pulsatile mechanical stresses and underscore the benefit of whole-vessel preparations in elucidating mechanotransduction pathways.

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Differential Regulation of Vascular Focal Adhesion Kinase by Steady Stretch and Pulsatility

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