Load-induced focal adhesion kinase activation in the myocardium: role of stretch and contractile activity

PRISCILA P. DOMINGOS, PRISCILA M. FONSECA, WILSON NADRUZ, JR., AND KLEBER G. FRANCHINI
Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, São Paulo 13081-970, Brazil

Received 24 June 2001; accepted in final form 10 October 2001

Domingos, Priscila P., Priscila M. Fonseca, Wilson Nadruz, Jr., and Kleber G. Franchini. Load-induced focal adhesion kinase activation in the myocardium: role of stretch and contractile activity. Am J Physiol Heart Circ Physiol 282: H556–H564, 2002; 10.1152/ajpheart.00534.2001.—We investigated the influence of stretch and contractile activity on load-induced activation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) in isolated rat hearts. Increases of diastolic pressure from ~0 to ~15 mmHg rapidly increased FAK tyrosine phosphorylation (maximum: 2.3-fold) and binding to c-Src (maximum: 2.8-fold) and Grb2 (maximum: 3.6-fold). This was paralleled by activation (maximum: 2.8-fold) and binding of ERK1/2 to FAK. FAK and ERK1/2 were immunolocalized at sarcolemmal sites of cardiac myocytes and in the nuclei, in the case of ERK1/2. Balloon inflation to raise ventricular pressure in hearts perfused with cardioplegic solution also activated FAK and ERK1/2. However, increases in contractile activity induced by increasing calcium concentration in the perfusate (from 0.5 to 5 mM) did not activate the FAK multicomponent signaling complex or ERK1/2 in the myocardium. These results indicate that stretch rather than contractile activity induces FAK and ERK1/2 activation in the myocardium. In addition, the activation and binding of ERK1/2 to FAK suggest that FAK drives the load-induced activation of ERK1/2.

cytoskeleton; cell signaling; rat heart

MECHANICAL INPUT PLAYS A MAJOR ROLE in cardiac adaptive responses to hemodynamic overload (10). In addition to triggering the force-length mechanism and homeometric autoregulation, mechanical input activates signaling mechanisms involved in hypertrophic growth of cardiac myocytes, the hallmark of the myocardial structural adaptation in response to sustained hemodynamic overload (11, 27). How mechanical input is converted to biochemical signals that induce the myocardial hypertrophic response is still not completely understood. However, increasing evidence supports the notion that living cells transduce and transmit forces into biochemical signals through specialized focal sites of the membrane, whereby integrins connect the cytoskeleton to the extracellular matrix (6, 16, 17, 23). This includes cardiac and skeletal myocytes, where the costameres, the counterpart of the focal adhesion complex, connect the sarcolemma to sarcomere Z lines through cytoskeletal proteins (4, 12, 15, 25, 33, 38). Such structures provide a continuous path for mechanical signal transfer from the extracellular matrix to the sarcomere, nucleus, and internal organelles. In addition, many signaling molecules are immobilized and have their function dependent on the anchorage to the cytoskeleton, which provides a physical basis for mechanobiochemical transduction (6, 14, 17).

Among the various signaling molecules involved in integrin-mediated signaling, focal adhesion kinase (FAK) has received much attention because experimental evidence indicates that it plays a key role in the cellular effects elicited by the integrin/cytoskeletal system, such as cell growth and gene expression (6, 14). The precise mechanism that links integrins to FAK activation is unknown, although it is clear that integrin clustering mediates FAK autophosphorylation, predominantly at Tyr397 (7, 29). After autophosphorylation, additional tyrosine residues of FAK are phosphorylated through the action of c-Src, which binds to FAK at Tyr397 via its SH2 domain (7, 29). This leads to the binding of other SH2 domain proteins such as phosphatidylinositol-3-kinase (PI3K) (14) and the Grb2/Sos complex (30), which in turn activate, among others, the Akt and Ras/mitogen-activated protein (MAP) kinase signal transduction cascades, respectively (30, 36). FAK activation has been demonstrated to occur in isolated cardiac myocytes subjected to pulsatile mechanical stretch and in overloaded feline and rat myocardium (13, 18, 20, 32). With the use of an experimental preparation in which the in situ rat heart was subjected to controlled pressure overload, we were able to show that pressure overload elicits rapid activation of the multicomponent signaling complex associated with FAK in the rat heart (13). The close relationship among increased load and FAK/c-Src activation, FAK/PI3K/Grb2 association, and the concurrent activation of Akt and extracellular signal-regulated kinase

Address for reprint requests and other correspondence: K. G. Franchini, Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Cidade Universitária “Zefherino Vaz,” 13081-970 Campinas SP, Brazil (E-mail: franchini@obelix.unicamp.br).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
(ERK)1/2, two potential downstream effectors of the FAK multicomponent signaling complex, indicated that it may play a role in the earlier myocardial responses to increased workload. Although these results provide evidence that FAK is activated in response to hemodynamic overload, the fact that they were obtained in an “in situ” preparation precluded a better understanding about the nature of the mechanical forces involved in this process.

Thus the present study was designed to examine, in an isolated perfused heart preparation, the separate influences of mechanical stimulus, tension development, and contractile activity on FAK activation during increases in myocardial workload. In addition, ERK1/2 activation, a downstream effector of FAK, was studied by using an anti-phospho-specific antibody against ERK1/2. The relative importance of myocardial passive tension and contractile activity on load-induced FAK and ERK1/2 activation were examined by perfusing isolated hearts with cardioplegic solution and by increasing calcium concentration (Ca0) in the perfusate, respectively. Furthermore, the localization of FAK and ERK1/2 in cardiac myocytes were confirmed by immunohistochemical analysis of myocardial sections. Our findings demonstrate that mechanical stretch, rather than contractile activity, triggers FAK and ERK1/2 activation during increased myocardium workload.

METHODS

Antibodies and Chemicals

Antibodies against FAK, c-Src, Grb2, ERK1/2, phosphorylated (Thr202/Tyr204)-ERK1/2, and phosphotyrosine were purchased from Santa Cruz Biotechnology. Affinity-purified rabbit anti-mouse IgG was from Dako. 125I-labeled protein A ([125I]protein A), anti-rabbit IgG-biotin, and streptavidin-horseradish peroxidase were from Amersham. Protein A-Sepharose 6MB was from Pharmacia. All other reagent grade chemicals were from Sigma.

Isolated Perfused Heart Preparation

All animals received care in compliance with the principles of laboratory animal care formulated by the university’s Animal Care and Use Committee. Wistar rats weighing ~200 g were anesthetized with pentobarbital sodium (50 mg/kg ip). After intravenous administration of heparin (500 IU/kg), the rats’ chests were opened, and the hearts were rapidly excised and mounted on a nonrecirculating Langendorff perfusion apparatus. Retrograde perfusion was established at a pressure of 70 mmHg with oxygenated normothermic HEPES buffer (pH 7.4) containing (in mM) 20 HEPES, 137 NaCl, 1.2 MgSO4, 5 KCl, 1.5 CaCl2, and 16 d-glucose. In experiments performed to analyze the influence of changes in contractile activity on FAK activation, Ca0 of HEPES buffer was increased from 0.5 mM to 1.5, 3.0, and 5.0 mM. To evaluate the contribution of passive tension-stretch on FAK activation, the hearts were perfused with a cardioplegic solution containing (in mM) 20 HEPES, 127 NaCl, 15 KCl, 1.2 MgSO4, 1.5 CaCl2, and 16 d-glucose; pH 7.4. A small fluid-filled latex balloon connected to a polyethylene-50 tube was positioned into the left ventricular chamber and connected to a pressure transducer calibrated to a mercury manometer for ventricular pressure monitoring. The pressure signal was amplified (GP4A General Purpose Amplifier, Stemtech), digitized using an analog-to-digital converter, and visualized on a personal computer loaded with WinDaq software (DATAQ). Data were stored on a disk for off-line analyses. Pressure was recorded at different intraventricular volumes according to the protocols described in Experimental Design.

Tissue Homogenization

After the pressure recording session, the heart was rapidly removed from the perfusion apparatus, and the left ventricle was minced coarsely and homogenized in ~10 volumes of solubilization buffer [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin] at 4°C with a Polytron operated at maximum speed for 30 s. The extract was centrifuged at 10,000 g at 4°C for 30 min, and the supernatant was used for the assay. Protein concentration was determined with the Bradford dye-binding method. The supernatant was treated with Laemmli sample buffer containing 100 mM dithiothreitol heated in a boiling water bath for 4 min and then resolved on SDS-PAGE (8% bis-acrylamide) in a Bio-Rad mini-gel apparatus (Mini-Protean, Bio-Rad Laboratories; Richmond, CA). Equal amounts of total protein were used for all samples.

Protein Analysis by Immunoblotting

Aliquots of whole extracts or immunoprecipitated proteins with specific antibodies and protein A-Sepharose were treated with Laemmli sample buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE (8% bis-acrylamide) in a Bio-Rad mini-gel apparatus (Mini-Protean). Electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 90 min at 120 V (constant) in a Bio-Rad mini-gel transfer apparatus (Mini-Protean). Non-specific protein binding to the nitrocellulose membrane was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 100 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membrane blot was incubated with anti-FAK, anti-Src, anti-phosphotyrosine, anti-Grb2, anti-ERK1/2, or anti-phosphorylated ERK1/2 antibodies diluted in 10 ml of blocking buffer (3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min in blocking buffer without milk or BSA. The blots were subsequently incubated with 3 mCi [125I]protein A (30 Ci/mmol) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. [125I]Protein A bound to the specific antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak; Rochester, NY) with Cronex Lightning Plus intensifying screens (DuPont; Wilmington, DE) at ~80°C for 24 h. Band intensities were quantified by optical densitometry (model GS 300 densitometer, Hoefer Scientific Instruments; San Francisco, CA) of the developed autoradiographs.

Tissue Preparation for Morphometry and Immunohistochemistry

The ventricles were fixed by overnight immersion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed to inclusion in Histotek (Merck). Sections used for
morphometric analysis of cardiac myocytes were stained with hematoyxlin-eosin. Sections for immunohistochemistry (5 μm thick) were transferred to poly-l-lysine-coated glass slides. The endogenous peroxidase activity was blocked by treatment with 0.03% H2O2 in 0.1 M phosphate-saline buffer (PBS) at room temperature, for 30 min. The sections were preincubated in blocking buffer (5% nonfat dry milk on 0.1 M PBS) for 45 min at room temperature, followed by overnight incubation with the primary antibodies anti-FAK (1:100 dilution) and anti-ERK1/2 (1:75 dilution) at 4°C. The sections were extensively rinsed in 0.05 M PBS and incubated with biotin-conjugated secondary antibodies (1:300 dilution) for 2 h at 25°C. After sections were washed as above, they were incubated with streptavidin-peroxidase complex (1:500 dilution) and anti-ERK1/2 (1:75 dilution) at 4°C. The sections were then counterstained with hematoyxlin and treated with lithium carbonate. Secondary antibody specificity was tested in a series of positive and negative control measurements. In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining.

Experimental Design

Group 1: diastolic-systolic pressure relationship. After the baseline pressure-recording period (15 min), the volume of the balloon positioned into the left ventricle of each beating heart was increased to raise the diastolic pressure from ~0 mmHg to ~5, 10, or 15 mmHg for 10 min. In control hearts, the balloon pressure was maintained at the baseline level for 10 min more. After this period, the hearts were rapidly removed and processed for Western blotting and histology.

Group 2: perfusion with cardioplegic solution. In this protocol, the hearts were perfused with a cardioplegic solution. The balloon positioned into the left ventricle was filled to maintain its pressure at ~0 mmHg for 15 min. After this period, the balloon was further inflated to raise the filling pressure to ~5, 10, or 15 mmHg for 10 min more before the hearts were removed and homogenized for Western blotting.

Group 3: changes in Ca2+. In this group, the hearts were prepared as described for group 1, but they were perfused with buffers containing Cao of ~0.5, 1.5, 3.0, and 5.0 mM. Diastolic pressure was maintained at ~0 mmHg along the 25-min experimental period. After this period, the hearts were rapidly removed and processed for Western blotting.

Statistical Analysis

Data are presented as means ± SE. Differences between mean values were tested with one-way ANOVA for repeated measures and Bonferroni's multiple-range test. P < 0.05 was considered significant.

RESULTS

Increases in Diastolic Pressure Activate FAK Multicomponent Signaling Complex and ERK1/2 in Rat Myocardium

The functional parameters and the diameters of cardiac myocytes of perfused hearts in which diastolic pressure was raised from ~0 to ~15 mmHg are shown in Fig. 1A and Table 1. Increases of diastolic left ventricular pressure produced the expected increases of left ventricular systolic pressure, with no significant effect on spontaneous heart rate or on the rate of systolic pressure increase or decrease. The increases in left ventricular diastolic pressure were paralleled by reductions in the diameter of cardiac myocytes, indicating that these cells were progressively stretched by the increasing balloon inflation (Table 1). Moreover, the increases in the left ventricular diastolic and systolic pressures induced by the left ventricular balloon inflation were accompanied by increases in the tyrosine phosphorylation of FAK (Fig. 1B). Increases in diastolic pressure from ~0 to ~5 mmHg, which increased the systolic pressure by ~15 mmHg and reduced the cardiac myocytes diameter by ~15%, were accompanied by a twofold increase in the amount of tyrosine-phosphorylated FAK (Fig. 1B). Modest additional increases of FAK tyrosine phosphorylation were observed with balloon inflation to increase diastolic pressure to ~10 and ~15 mmHg.

FAK autophosphorylation at Tyr397 recruits and activates c-Src, which is responsible for further tyrosine phosphorylation of FAK at residues Tyr577–577, Tyr576, and Tyr925 (6, 9, 14, 29, 30). This elicits further recruitment of signaling molecules such as Grb2, PI3K, and paxillin, resulting in the formation of a multicomponent signaling complex linked to various cellular functions. As shown in Fig. 1C, the load-induced FAK tyrosine phosphorylation was accompanied by an increase in the amount of c-Src communoprecipitated with FAK, indicating a load-induced binding of c-Src to FAK. This was also paralleled by increases in the binding of Grb2 to FAK (Fig. 1D).

Grb2 potentially links the FAK signaling complex to the Ras/ERK1/2 pathway, related to gene regulation and cell growth. The load-induced activation of ERK1/2 was demonstrated by consistent increases in the amount of ERK1/2 detected with a specific antibody against phosphorylated (Thr202/Tyr204)-ERK1/2 in the homogenates of hearts subjected to increased diastolic pressure (Fig. 2A). The link between FAK and ERK1/2 activation was further demonstrated by the load-dependent communoprecipitation of FAK and ERK1/2 and phosphorylated ERK1/2 shown in Fig. 2B.

Immunohistochemical analysis indicated that myocardial FAK protein was localized mainly in cardiac myocytes (Fig. 3A). At higher magnification (Fig. 3B), FAK immunoreactivity appeared along the sarcomerma of cardiac myocytes and also in spots regularly distributed at the sarcolemma, resembling the distribution of T-tubules. Immunohistochemical staining was also performed to determine the localization of ERK1/2 in the myocardium. As shown in Fig. 3, C and D, ERK1/2 protein was localized at the nuclei of cardiac myocytes, but a consistent sarcolemmal and sarcomplasmic localization was also detected. The distribution pattern of ERK1/2 in the cardiac myocytes, resembling the FAK distribution pattern, suggests the colocalization of FAK and ERK1/2 both at the sarcoplasmic and sarcolemmal sites.
Effect of Myocardial Passive Tension/Stretch on FAK and ERK1/2 Activation

Because increases in left ventricular diastolic pressure increased systolic pressure, it was not possible, in beating hearts, to distinguish between myocardial stretch and contractile activity as the major determinant of load-induced FAK and ERK1/2 activation. To test for the ability of increases in passive tension/stretch to activate these enzymes, we performed experiments in arrested hearts. As indicated in Fig. 4A, raising intraventricular pressure in these hearts still increased FAK tyrosine phosphorylation as well as the binding of c-Src (Fig. 4B) and Grb2 (Fig. 4C) to FAK. Moreover, myocardial stretch was followed by ERK1/2 activation (Fig. 5A) and binding to FAK (Fig. 5B).

Effects of Increases in Contractile Activity on FAK and ERK1/2 Activation

To examine whether increases in myocardial contractile activity alone are able to activate myocardial FAK and ERK1/2, experiments were performed in which the isolated hearts were perfused with different \( C_{\text{a0}} \) in the perfusate buffer. As shown in Fig. 6A, increases in the perfusate \( C_{\text{a0}} \) from 0.5 to 5 mM induced progressive increases in systolic pressure. This occurred while the diastolic pressure was maintained.

Table 1. Functional parameters and diameters of cardiac myocytes of perfused hearts in which diastolic pressure was raised from \(-0\) to 15 mmHg

<table>
<thead>
<tr>
<th>Diastolic Pressure, mmHg</th>
<th>Systolic pressure, mmHg</th>
<th>Diastolic pressure, mmHg</th>
<th>Heart rate, beats/min</th>
<th>(+dP/dt, \text{ mmHg/s})</th>
<th>(-dP/dt, \text{ mmHg/s})</th>
<th>Myocyte diameter, (\mu\text{m})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>81 (\pm) 4</td>
<td>1 (\pm) 0.5</td>
<td>265 (\pm) 17</td>
<td>1,717 (\pm) 92</td>
<td>1,152 (\pm) 53</td>
<td>9 (\pm) 0.4</td>
</tr>
<tr>
<td>5</td>
<td>98 (\pm) 5*</td>
<td>4 (\pm) 0.7*</td>
<td>275 (\pm) 15</td>
<td>1,816 (\pm) 89</td>
<td>1,428 (\pm) 92</td>
<td>7.9 (\pm) 0.5*</td>
</tr>
<tr>
<td>10</td>
<td>112 (\pm) 6*</td>
<td>10 (\pm) 1*</td>
<td>268 (\pm) 12</td>
<td>1,973 (\pm) 114</td>
<td>1,378 (\pm) 62</td>
<td>7.4 (\pm) 0.7*</td>
</tr>
<tr>
<td>15</td>
<td>120 (\pm) 5*</td>
<td>14 (\pm) 1*</td>
<td>250 (\pm) 20</td>
<td>2,068 (\pm) 120</td>
<td>1,400 (\pm) 80</td>
<td>5.8 (\pm) 0.6*</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE. \(+dP/dt\) and \(-dP/dt\), rate of left ventricular pressure rise or decrease, respectively. *\(P < 0.05\) compared with control values.
at 0 mmHg. Western blot analysis of FAK tyrosine phosphorylation showed that increases in contractile activity were not accompanied by FAK activation (Fig. 6B). In addition, no change could be demonstrated in the amount of c-Src and Grb2 (Fig. 6C) associated with immunoprecipitated FAK in the homogenates of hearts perfused with different Ca\textsubscript{0}. Besides the lack of FAK multicomponent signaling complex activation, increases in contractile activity alone could not activate ERK1/2, as indicated by the absence of changes in the

**Fig. 3.** Myocardial distribution of FAK and ERK1/2. A and B: localization of FAK protein (arrowheads) as seen in longitudinal and transversal myofiber sections. FAK staining was detected predominantly at sarcolemmal sites, but spots are also observed regularly distributed in the sarcoplasma. C and D: ERK1/2 protein localization (arrowheads and arrows) in longitudinal and transversal myocardial sections. The staining for this protein was detected in the myocyte nuclei, but it was also found at sarcolemmal and sarcoplasmic sites in a distribution similar to that of FAK. Magnification, \( \times 1,150. \)
amount of ERK1/2 detected by the phospho-specific antibody (Fig. 6D).

DISCUSSION

The present study examined the mechanical factors contributing to load-induced activation of the FAK multicomponent signaling complex in the myocardium of isolated perfused rat hearts. The results showed that the activation of the Frank-Starling mechanism induced by increases in left ventricular diastolic pressure elicited activation of the FAK multicomponent signaling complex, as indicated by the load-induced FAK tyrosine phosphorylation and binding to c-Src and Grb2. Immunohistochemical analysis of myocardial sections confirmed that most of the myocardial FAK was localized in cardiac myocytes. The relative influences of increases in passive tension/stretch and contractile activity on the activation of the FAK multicomponent signaling complex were assessed by comparing the effects of increases in passive tension/stretch in

Fig. 4. Effect of increases in left ventricular pressure in isolated rat hearts perfused with a cardioplegic solution in the activation of the FAK multicomponent signaling complex. A: representative blot (top) and average values (bottom; n = 6) of FAK tyrosine phosphorylation detected with anti-αPy in the homogenates of arrested hearts subjected to increasing pressure by the balloon inflation. B: representative blot (top) and average values (bottom; n = 6) of the amount of c-Src coimmunoprecipitated with FAK from homogenates of arrested hearts subjected to increasing passive tension. C: representative blot (top) and average values (bottom; n = 6) of the amount of Grb2 coimmunoprecipitated with FAK from homogenates of arrested hearts subjected to increasing passive tension. *P < 0.05 compared with the values observed in hearts maintained with diastolic pressure of ~0 mmHg (control values).

Fig. 5. Effect of increases in left ventricular pressure in isolated rat hearts perfused with a cardioplegic solution in the activation of ERK1/2. A: representative blot (top) and average values (bottom; n = 6) of the amount of activated ERK1/2 detected by anti-P-ERK1/2 (Thr202/Tyr204) in the homogenates of arrested hearts subjected to increasing passive tension. B: representative blots (top) and average values (bottom; n = 6) of P-ERK1/2 coimmunoprecipitated with FAK from homogenates of rats subjected to increasing passive tension. *P < 0.05 compared with control values.
hearts perfused with a cardioplegic solution with those of increases in contractile activity induced by raising Ca\textsubscript{0} in the perfusion buffer. Increases in the myocardial passive tension/stretch activated the FAK multicomponent signaling complex to the same levels as the ones observed in beating hearts after the activation of the Frank-Starling mechanism. However, increases in contractile activity induced by increases in Ca\textsubscript{0} were unable to activate this signaling system. In addition, it was shown that load-induced FAK activation was accompanied by a simultaneous activation of ERK1/2 and binding of ERK1/2 and activated ERK1/2 to FAK. These effects were also related to increases in myocardial passive tension/stretch rather than to its contractile activity. These results indicate that FAK activation drives, at least partially, the load-induced activation of ERK1/2. This notion was strengthened by the demonstration that FAK and ERK1/2 are similarly distributed at sarcolemmal and sarcoplasmic sites of cardiac myocytes.

**Mechanical Stress and FAK Activation**

Activation of the FAK multicomponent signaling complex induced by increases in mechanical input to myocardial cells might be mediated by a number of factors, namely, the engagement and activation of cytoskeletal proteins associated with the cytoplasmic domains of integrins, release of autocrine/paracrine factors, or stretch-induced activation of ion channels (6, 17, 21, 28, 40). However, studies in cells in culture showing the strict dependence of FAK activation by mechanical stimuli on integrin engagement and cytoskeletal integrity favor the hypothesis that the integrin/cytoskeletal complex is the dominant mechanism of FAK activation in cells subjected to mechanical stress (31, 35). Accordingly, in our previous study (13), we showed a rapid binding of FAK to β-actin, simultaneous with its activation in the myocardium of rats subjected to acute pressure overload. Moreover, our demonstration here that FAK is chiefly distributed along the sarcolemma, at sites resembling the costameres, further supports the hypothesis that the integrin/cytoskeleton complex is centrally involved in the activation of myocardial FAK in response to mechanical stimuli.

Biochemical events that precede FAK activation might be sensitive to mechanical events related to increases in passive tension, stretch, rate of increases in local tension, or contractile activity. The prevailing hypothesis suggests that integrins may respond to mechanical stress and activate FAK by inducing the exposure of its Tyr\textsuperscript{397} phosphorylation site to its kinase domain. Occupation of integrin receptors by extracellular matrix proteins is one mode of inducing autophosphorylation of Tyr\textsuperscript{397} (22, 34). Otherwise, activation of FAK via growth factors or other mediators is thought to occur via activation of transmembrane receptors (14). Presently, we have shown that FAK activation is related to events occurring when the myocardium is subjected to passive tension/stretch but not to contrac-

---

**Fig. 6. Effect of increasing contractile activity in the activation of FAK and ERK1/2.**

A: graphic showing the average values ($n = 5$) of peak systolic pressure in hearts perfused with HEPES buffer with increasing calcium concentration ($C_{a0}$). Blots (top) and average values (bottom) representing the amount of FAK, its tyrosine phosphorylation (B), Grb2 association with FAK (C), and P-ERK1/2 (D) are shown from hearts perfused with increasing $C_{a0}$. *$P < 0.05$ compared with control values.*
tile activity or the rate of increases in systolic tension. However, the experimental model used in the present study did not allow distinction between the effects of tension per se and stretch as the factor responsible for FAK activation. Mechanical stretch or tension applied directly to the extracellular domain of integrins results in increased protein tyrosine phosphorylation, cytoskeletal stiffening, and the activation of downstream signaling pathways, suggesting that integrins can function as mechanotransducers (31, 35, 42, 43). Studies in tracheal smooth muscle, however, have suggested that muscle length rather than tension is the primary stimulus for mechanosensitive regulation of FAK activity (37).

Cardiac myocytes are normally subjected to cyclic changes in load. In this regard, a recent study (39) has shown differences in the activation of signaling mechanisms in response to load and stretch during systolic and diastolic phases of the cycle. Different phenotypic responses were observed in cardiac myocytes subjected to stretch during the contraction or relaxation phase of the cycle. Possible differences in the efficiency of load-induced FAK activation by stretch of the myocardial cell during systole or diastole were not explored in the present study and remain an open question.

Load-Induced Activation of ERK1/2 Via FAK Multicomponent Signaling Complex

Experiments showing a load-dependent association of FAK to Grb2, ERK1/2, and phosphorylated ERK1/2 to FAK and a similar distribution of FAK and ERK1/2 at sarcoplasmatic and sarcomemmal sites indicated that FAK activation may function as an upstream activator of ERK1/2 in cardiac myocytes. This hypothesis was strengthened by our results showing that load-induced ERK1/2 activation is also dependent on myocardial stretch or increases in passive tension rather than on contractile activity. These results are in accord with the results of previous studies (2, 3, 8, 9, 13, 26, 41, 44) that showed load-induced activation of ERK1/2 in the myocardium and cardiac myocytes. They also agree with recent evidence showing that activation of ERK1/2 may be controlled by cellular adhesion via integrins/FAK activation (1, 24). However, in addition to mechanical stimuli, ERK1/2 can be activated by a wide variety of different stimuli acting through diverse receptor families, including hormones and growth factors, vasoactive peptides, transforming growth factor-β-related polypeptides, inflammatory cytokines of the tumor necrosis factor family, and environmental stresses such as osmotic shock, ionizing radiation, and ischemic injury (19). Alternatively, the integrin/cytoskeletal/FAK mechanism might collaborate in the enhancement of growth factor activation of the ERK1/2 pathway by utilizing the actin network as a scaffold and FAK as a coactivator (1, 24). However, the relative contribution of FAK to ERK1/2 activation via the integrin/cytoskeleton system to load-induced myocardial hypertrophy remains unclear.

ERK1/2 are implicated as important regulators of cardiomyocyte hypertrophic growth in culture. The early activation of ERK1/2 in cardiac myocytes has been suggested to contribute to the reexpression of fetal ventricular genes (e.g., atrial natriuretic factor, β-myosin heavy chain, and skeletal muscle α-actin). More recently, studies (5) have shown that permanent activation of the ERK1/2 pathways in transgenic mice with cardiac-restricted expression of an activated MAP or ERK kinase (MEK1 cDNA developed concentric hypertrophy. This indicates that the MEK1-ERK1/2 signaling pathway stimulates a physiological hypertrophic response associated with augmented cardiac function and partial resistance to apoptosis. The relative importance of integrin/FAK to these mechanisms is unknown.

In conclusion, our results provide further argument for a role of FAK in the early myocardial responses to mechanical stimuli. The demonstration here that stretch or passive tension alone instead of increases in contractile activity can activate FAK and its downstream effectors is compatible with the appealing hypothesis that load-induced FAK activation in myocardial cells could be linked to the integrin/cytoskeletal signaling complex. Furthermore, experiments showing that stretch- or passive tension-induced binding of FAK to Grb2, ERK1/2, and activated ERK1/2, and also a common localization of these enzymes at the sarcolemma and sarcoplasm of cardiac myocytes, indicate a linkage between load-induced FAK and ERK1/2 activation during mechanical overload. Whether this pathway is critical for load-induced hypertrophy in the myocardium deserves further study.

This study was supported by Fundação de Auxílio à Pesquisa do Estado de São Paulo Grant 98/114-7 and Conselho Nacional de Desenvolvimento Científico e Tecnológico Grant 521098/97-1.

REFERENCES

LOADING SIGNAL TRANSDUCTION IN THE MYOCARDIUM


