Early activation of p160ROCK by pressure overload in rat heart

Adriana S. Torsoni, Priscila M. Fonseca, Daniela P. Crosara-Alberto, and Kleber G. Franchini
Department of Internal Medicine, School of Medicine, State University of Campinas, 13081-970 Campinas, SP, Brazil

Submitted 4 March 2002; accepted in final form 23 January 2003

Torsoni, Adriana S., Priscila M. Fonseca, Daniela P. Crosara-Alberto, and Kleber G. Franchini. Early activation of p160ROCK by pressure overload in rat myocardium. Constriction of transverse aorta, controlled to increase peak systolic pressure of ascending aorta by ~40 mmHg, induced a rapid association of RhoA with Dbl-3 and p160ROCK. The binding of p160ROCK to RhoA was rapidly increased, peaking at 30 min (~3.5-fold), but reduced to lower levels (~1.9-fold) by 60 min of pressure overload. The activity of immunoprecipitated p160ROCK toward myosin light chain increased ~2.5-fold within 10 min but decreased to lower levels (~1.6-fold) after 60 min of pressure overload. Confocal microscopic analysis indicated that pressure overload induced the formation of aggregates of p160ROCK and RhoA along the longitudinal axis of cardiac myocytes. Immunoelectron microscopic analysis showed that pressure overload induced the association of p160ROCK and RhoA to Z-line, T-tubule, and subsarcolemmal areas. The rapid activation of p160ROCK by pressure overload and its aggregation in subcellular structures involved in transmission of mechanical force suggest a role for this enzyme in the mechanobiochemical transduction in the myocardium.

mechanical stress; cell signaling; myocardium

MECHANICAL STRESS has been implicated as a major factor responsible for the functional and structural changes of the myocardium to hemodynamic overload (6). Although the effects of mechanical stress could be mediated by activation of mechanosensitive ion channels or by locally and systemically released growth factors (16, 17, 29), the mechanical input itself may trigger cellular signaling mechanisms via the interaction of cells to the underlying extracellular matrix through the transmembrane integrins (13, 21). Integrins connect to a meshwork of F-actin through bridging proteins such as vinculin, talin, and α-actinin at specialized membrane-bound regions known as focal adhesion complexes (3, 10, 25, 27). This system transmits mechanical stimuli through the elastic coupling to sites, such as plasma membrane, internal organelles, or nucleus (21). In addition, integrin clustering leads to the recruitment and activation of several signaling proteins such as focal adhesion kinase (Fak), c-Src, small G proteins, and MAP kinases to F-actin meshwork (4, 28). These signaling molecules may act as transducers of the mechanical stimuli into intracellular signaling events. Accordingly, our previous studies have shown (7, 8) that either acute pressure overload or stretch induces rapid activation (within 3–5 min) of the multicomponent signaling complex associated with Fak in the myocardium of rats. This effect includes the activation of ERK1/2, involved in cellular functions such as metabolism, gene regulation, and growth (32).

The assembly of focal adhesion complex, a critical step in cellular signaling through Fak, involves multiple steps and pathways and may be regulated by mechanical stimuli or soluble factors, such as growth factors, angiotensin II, and endothelin (3, 10). The small GTPase Rho has been shown to play a central role in the reorganization of F-actin and focal adhesions in response to several different stimuli (11, 15). In cardiac myocytes, RhoA has been shown to be required for phenotypic changes induced by growth factors and stretch (1, 5, 12, 19, 22, 30, 31, 35). However, the signaling mechanisms that mediate these effects are still unclear. Among the putative Rho effectors are a number of protein kinases that bind to and are activated by Rho (15). ROCK, the most extensively characterized Rho effector, stimulates cytoskeletal reorganization in response to various stimuli (15). In particular, ROCK has been shown to stimulate myosin-based contractility by directly and indirectly elevating phosphorylation of the regulatory myosin light chain (2, 18, 34). The resulting activation of myosin triggers myosin filament formation and reorganization of F-actin (20, 33). Despite the fact that Rho/ROCK pathway has been suggested to mediate hypertrophic signals in neonatal cardiac myocytes (12, 19, 36), a clear demonstration that ROCK is activated as well as the identity of the upstream activators in overloaded myocardium is still lacking. Several lines of evidence indicate that Rho-GTPase activity is regulated by proteins known collectively as GAPs (GTPase-activating proteins) and GEFs (guanine nucleotide exchange factors). Although numerous GEFs and enzyme effectors are capable of stimulating nucleotide exchange and medi-
ate the effects of small GTPase RhoA, recent studies (24, 26) have shown that Dbl is the main activator of RhoA/ROCK pathway when the stimulus elicits integrin engagement. In this context, it would be relevant to know whether Dbl is involved in the activation of myocardial RhoA/ROCK pathway in response to increased workload.

Thus the aim of the present study was to examine the activity, expression, and cellular location of p160ROCK in the myocardium of rats subjected to acute pressure overload. Additional experiments analyzed the engagement of the upstream activators Dbl-3 and RhoA on the early activation of p160ROCK in the overloaded myocardium.

METHODS

Antibodies and chemicals. Polyclonal goat antibody against p160ROCK, polyclonal rabbit antibody against Dbl-3, and monoclonal mouse antibody against RhoA were purchased from Santa Cruz Biotechnology. Myosin light chain (MLC) was from Calbiochem. Anti-goat and anti-rabbit IgG biotin-conjugated antibodies, streptavidin-gold 15 nm (Auroprobe), 125I-protein A, [γ-32P]ATP, and protein A-Sepharose 6MB were purchased from Amersham Pharmacia. TRITC-phalloidin, FITC-conjugated anti-goat, and all other reagent grade chemicals were obtained from Sigma.

Experimental animal model. Acute pressure overload was obtained by controlled constriction of the transverse aorta, produced with a micro-Blalock clamp in anesthetized rats as described previously (8). Briefly, the animals were anesthetized with pentobarbital sodium administered via an endovenous catheter placed in the right jugular vein. The heart was perfused with a washing solution or blebs. After isolation, the ventricular myocytes were immediately transferred to poly-l-lysine-coated glass slides and then processed for immunohistochemistry.

Tissue preparation for immunoprecipitation. The ventricles were homogenized in 10 volumes of solubilization buffer (1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 mM NaHCO3, 6 mM KH2PO4, 1.2 mM KH2PO4, 100 mM NaPO4, 100 mM Na3VO4, 2 mM PMFS, and 0.1 mg/ml aprotinin) at 4°C. The extracts were centrifuged at 8,000 g at 4°C for 20 min, and the supernatant was used for the assays. Protein concentration was determined with the Bradford dye binding method. An equal amount of total protein of the supernatants of these tissues was submitted to immunoprecipitation with specific antibodies and protein A-Sepharose 6MB.

Protein analysis by immunoblotting. Aliquots of whole extracts or immunoprecipitated proteins containing an equal amount of total protein were treated with Laemmli sample buffer and run in SDS-PAGE. The nitrocellulose membranes with transferred proteins were incubated with specific antibodies and 125I-protein A. Band intensities were quantified by optical densitometry of the developed autoradiographs.

Immune complex kinase assay. Serine/threonine kinase activity associated with the immune complex of anti-p160ROCK antibody was measured by using MLC as substrate. The immune complex of anti-p160ROCK antibody was immunoprecipitated, washed, resuspended, and incubated with 25 μg of MLC at 30°C for 20 min in 18 μl of kinase buffer (100 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM DTT, and 50 μM Na3VO4) containing 2 μCi [γ-32P]ATP. Adding boiling Laemmli sample buffer to the reaction terminated the reaction. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membrane and the 32P-labeled MLC band (~20 kDa) was visualized by autoradiography and quantified by densitometry.

Immunofluorescence study. Sections of left ventricle and freshly isolated cardiac myocytes from adult rats were double-stained with TRITC-phalloidin and anti-ROCK or anti-RhoA antibodies. The heart was perfused with a washing solution (PBS, heparin, and lidocaine) and then with a 10% sucrose solution in PBS. The left ventricle was removed, frozen, and stored at ~80°C. Cryosections (5 μm) and freshly isolated cardiac myocytes were transferred to poly-l-lysine-coated glass slides and fixed (2% paraformaldehyde in PBS, pH 7.4). Nonreactive sites were blocked with 5% nonfat milk in PBS. The sections or isolated cardiac myocytes were incubated with specific antibodies and TRITC-phalloidin and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence was detected by confocal laser scanning microscopy (CLSM; Carl Zeiss). Double-stained images from FITC and TRITC channels were simul-
taneously acquired from the same area and superimposed. The same sensitivity of the CLSM was used to compare the anti-p160ROCK and anti-RhoA staining in myocardial cryosections of the various groups of rats. As negative controls, cryosections were not incubated with primary antibodies. No specific staining was observed in the negative control.

Tissue preparation for immunogold labeling. Pieces of heart (<1 mm) were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h, washed for 45 min with 0.05 M phosphate buffer (pH 7.4), and treated with 0.1 M glycine for 1 h. After being washed as described above, the pieces were dehydrated through graded concentrations of N,N-dimethylformamide, embedded in LR-White resin using gelatin capsules, and polymerized at ~20°C under UV light for 3 days. Thin sections were cut with a diamond knife on an ultramicrotome (Leica Ultracut-UCT) and collected on 150-mesh nickel grids. Section staining was performed by floating grids, sections down, on droplets of the immunolabeling and washing solutions placed on paraflim. All incubations involving the antibodies were done in moistened chambers. A noncompetitive blocking step was done with 3% BSA in 0.1 M PBS (pH 7.4) for 1 h. The incubation with primary antibody diluted 1:50 in 1% BSA was carried out overnight at 4°C. The sections were then incubated with anti-goat IgG biotin-conjugated antibody diluted 1:300 in 1% BSA for 1 h, followed by an incubation with streptavidin-gold 15 nm for 45 min. After immunolabeling, the sections were stained with 5% uranyl acetate for 5 min. Immunostained sections with anti-p160ROCK or anti-RhoA were examined and photographed in a transmission electron microscope (LEO 906). All the experiments were accompanied by a negative control.

Statistical analysis. The data are presented as means ± SE. Differences between the mean values of the densitometric readings were tested with one-way ANOVA for repeated measures and Scheffe’s test. A value of P < 0.05 indicates significant difference.

RESULTS

Acute effects of aortic constriction on hemodynamics. Acute aortic constriction produced a peak systolic gradient between the ascending (~170 mmHg) and abdominal (~130 mmHg) aorta of ~40 mmHg, stable along the 1-h experimental period (Fig. 1). Peak systolic blood pressure of the anesthetized control rats averaged 131 ± 5 mmHg.

Pressure overload induces the association of RhoA with Dbl and p160ROCK. Coimmunoprecipitation experiments were performed with specific antibodies against Dbl-3, RhoA, and p160ROCK in the myocardial homogenates obtained from control and overloaded hearts. Immunoprecipitates obtained with anti-RhoA and anti-p160ROCK antibodies were resolved by SDS-PAGE and immunoblotted with anti-Dbl-3 and anti-RhoA antibodies, respectively. As indicated in Fig. 2A, pressure overload enhanced the association of Dbl-3 with RhoA. The amount of Dbl-3 detected in the immunoprecipitates of RhoA increased to ~150% at 5 min and to ~250% at 60 min after the beginning of pressure stimulus. Pressure overload also induced a rapid increase in the association of p160ROCK with RhoA (Fig. 2B). The amount of RhoA detected in the immunoprecipitates of p160ROCK increased to 180 and 350% at 5 and 30 min after the beginning of pressure stimulus, respectively. After 60 min of pressure overload, p160ROCK/RhoA association was reduced to lower levels compared with those seen at 30 min but remained significantly increased compared with values for control rats. No difference was observed when values of RhoA detected in immunoprecipitates of p160ROCK at 60 min were compared with those at 5 and 10 min of pressure overload. Parallel immunoblotting with anti-Dbl and anti-RhoA revealed that the amount of these proteins remained unaltered in the myocardium over the 60-min period of pressure overload (Fig. 2, A and B, bottom).

Pressure overload activates p160ROCK. Load-induced activation of p160ROCK in the myocardium was tested with an in vitro kinase assay of the immunoprecipitated p160ROCK toward MLC, a cellular substrate for p160ROCK. As shown in Fig. 3A, pressure overload was followed by a consistent increase of the kinase activity of anti-p160ROCK immune complex. p160ROCK activity peaked at 10 min (to ~260%), remained elevated by 30 min, and reduced to a lower level but was still significantly elevated at 60 min of sustained stimulus. As shown in Fig. 3B, the amount of p160ROCK remained unchanged during the experimental period.

Immunolocalization of p160ROCK in cardiac myocytes of rats. p160ROCK was first localized by laser scanning microscopy in sections of adult rat hearts using double-stained TRITC-phalloidin and anti-p160ROCK antibody. In longitudinal sections of left ventricle from control rats, staining was evident in the cardiac myocytes (Fig. 4A). In these cells, immunostaining of p160ROCK was observed along the longitudinal axis of cardiac myocytes with some areas stained as spots organized regularly in the sarcoplasm (Fig. 4). Pressure overload enhanced the spot-pattern staining along the myocytes (Fig. 4B). To verify our findings in rat left ventricular sections, we also determined the localization of p160ROCK in freshly isolated left ventricular myocytes from adult rat hearts (Fig. 5, A–F).
Isolated myocytes also showed consistent staining with anti-p160\textsuperscript{ROCK} antibody, and the distribution patterns of p160\textsuperscript{ROCK} generally reproduced those seen in left ventricular sections of control (Fig. 5, A–C) and overladen hearts (Fig. 5, D–F), including the enhanced aggregation and the number spots of p160\textsuperscript{ROCK} staining induced by pressure overload.

Experiments were performed with immunogold electron microscopy of myocardial sections to further explore the specific location of p160\textsuperscript{ROCK} in cardiac myocytes. In sections obtained from control hearts, specific labeling with anti-p160\textsuperscript{ROCK} antibody was found to be evenly distributed in the sarcolemma and close to the region of Z lines (Fig. 6A). In the sections obtained from
overloaded hearts, clusters of p160ROCK were found at regions of T tubules and subsarcolemmal areas as early as 5 min after the beginning of the stimulus (Fig. 6B). Moreover, in overloaded hearts, p160ROCK was found more frequently at the Z line and intercalated disk than in sections of control rats as indicated in the representative examples of Fig. 6, C and D.

Immunolocalization of RhoA in cardiac myocytes of rats. In sections of left ventricles from control rats, anti-RhoA antibody consistently stained cardiac myocytes (Fig. 4, C and D). In overloaded myocardium, anti-RhoA staining was seen more frequently as longitudinal aggregates and spots as indicated in the representative example of Fig. 4D. In freshly isolated left ventricular myocytes, anti-RhoA staining was similar to the patterns of left ventricular sections (Fig. 7, A–F) and resembled those seen with anti-p160ROCK antibody. Aggregates and spots were more frequently observed in myocytes from overloaded than in those from control hearts (Fig. 7, D–F).

Fig. 4. Confocal laser scanning microscopy (CLSM) studies showing the immunolocalization of p160ROCK (green) and sarcomeric actin (red; phalloidin) in sections of rat left ventricle. A: left ventricular section from a control heart, showing the distribution of p160ROCK throughout the sarcoplasm of cardiac myocytes. B: representative example of p160ROCK staining in myocardial section of heart subjected to a 30-min period of pressure overload, showing the enhanced spot-pattern staining (arrows) along the myocytes. C: left ventricular section from a control heart, showing the distribution of RhoA throughout the sarcoplasm of cardiac myocytes. D: anti-Rho-A staining in the myocardium of 30-min overloaded rat left ventricle, showing the enhanced spot-pattern staining (arrows) along the myocytes.

Fig. 5. CLSM studies showing the immunolocalization of p160ROCK (green) and sarcomeric actin (red; phalloidin) in isolated adult rat ventricular myocytes. A: phalloidin staining in isolated myocytes from control rats. B: representative example of anti-p160ROCK staining of isolated myocytes from control rats. C: p160ROCK/phalloidin double staining of isolated myocytes from control rats. D: phalloidin staining in isolated myocytes from 30-min overloaded hearts. E: anti-p160ROCK staining. F: p160ROCK/phalloidin double staining of cardiac myocytes isolated from rat heart subjected to 30 min of pressure overload. Arrows indicate aggregates and spots of anti-p160ROCK-specific labeling.
Fig. 6. Immunoelectron micrographs of anti-p160ROCK staining in rat left ventricle. A: representative example of p160ROCK staining in the sarcomere and sarcolemma (arrows) in a myocardial section from a control rat. ECM, extracellular matrix; mit, mitochondrion; S, sarcolemma. Magnification, ×50,600. B: after 3 min of pressure overload, note clusters (arrows) of colloidal gold particles at T-tubule-like structure at the periphery of the cell. Magnification, ×51,700. C and D: staining of p160ROCK in 30-min overloaded hearts. Localization of p160ROCK at Z disks (Z; arrows) and intercalated discs (ID; arrows). Magnification, ×68,500 (C); ×55,000 (D).

Fig. 7. CLSM studies showing the immunolocalization of RhoA (green) and sarcomeric actin (red; phalloidin) in isolated adult rat ventricular myocytes. A: phalloidin staining in isolated myocytes from control rats. B: anti-RhoA staining of isolated myocytes from control rats. C: RhoA/phalloidin double staining of isolated myocytes from control rats. D: phalloidin staining with anti-Rho-A antibody in isolated myocytes from hearts subjected to 30 min of pressure overload. E: anti-RhoA staining. F: RhoA/phalloidin double staining at 30 min of pressure overload. Arrows indicate aggregates and spots of anti-Rho-A-specific labeling.
Immunogold electron microscopy of myocardial sections with anti-RhoA antibody indicated that in hearts from control rats, Rho-A was frequently seen along the sarcolemma and in the Z-line regions (Fig. 8A). In sections obtained from overloaded myocardium, anti-RhoA staining was detected more frequently as clusters at the Z lines and intercalated disks (Fig. 8, B and C) as well as at subsarcolemmal regions (not shown).

DISCUSSION

This study provided evidence that pressure overload induces a rapid activation of p160ROCK in the adult rat myocardium. Because this activation was paralleled by increases in p160ROCK/RhoA and RhoA/Dbl-3 association, our data also indicate that the rapid activation of p160ROCK in the myocardium may be mediated by Dbl-3/Rho-A complex. These functional protein data were extended to include data provided by immunohistochemistry and immunoelectron microscopic analysis on distribution and location of p160ROCK and RhoA in cardiac myocytes. Immunofluorescence confocal microscopic analysis of adult rat left ventricle sections and freshly isolated adult rat cardiac myocytes showed that pressure overload enhanced the appearance of aggregates of p160ROCK as well as of RhoA, regularly arranged on similar regions along the longitudinal axis of cardiac myocytes. The aggregation of p160ROCK and RhoA staining in cardiac myocytes of overloaded hearts were confirmed by the immunogold electron microscopy, which showed the appearance of clusters of p160ROCK and RhoA at specific regions such as the Z-line, T-tubule-like structures, intercalated disk, and subsarcolemmal/sarcolemmal areas. Overall, these data support the conclusion that load induces a rapid assembly and activation of Dbl/RhoA/p160ROCK signaling complex at structures compromised with force transmission in adult rat cardiac myocytes.

The finding here that pressure overload induces p160ROCK as well as RhoA to localize and cluster at specific subcellular structures simultaneously to p160ROCK/RhoA association detected by immunoprecipitation assays suggests that their activation is dependent not only on the interaction with upstream activators but also on their recruitment to a particular subcellular compartment. p160ROCK contains multiple domains, including a kinase domain in the NH2 terminus followed by a long coiled-coil region in the middle, that bind RhoA, and then a pleckstrin homology region and a Cys-rich zinc finger at the COOH terminus, which could target this enzyme to membranes and cytoskeletal actin (14). Studies performed in distinct experimental models have confirmed the target of p160ROCK to cytoskeletal proteins (9, 23). The location at specific subcellular structures might confer to p160ROCK the ability to contribute to signaling mechanisms involving cell membrane and cytoskeleton. Thus one could argue that the load-induced activation and target of p160ROCK and RhoA to the Z line, intercalated disk, and subsarcolemmal area, structures that stand and transmit mechanical forces, might indicate a role for these enzymes in mechanobiochemical transduction in cardiac myocytes.

Earlier studies have implicated RhoA/p160ROCK signaling complex in stretch and agonist-induced activation of gene regulation, sarcomerogenesis, and hypertrophy of cardiac myocytes (1, 5, 12, 19, 22, 30, 31, 35).
Our present data indicating rapid RhoA/p160ROCK association and activation of p160ROCK at specific sites suggest that these enzymes play a role in the initial events triggered by increased workload in cardiac myocytes. Accordingly, p160ROCK has been shown to contribute to several independent features of myocardial cell hypertrophy, including increase in cell size, sarcomere organization, and induction of atrial natriuretic factor and β-MHC expression (36). It remains to be determined, however, whether such pleiotropic effects are mediated indirectly by the influence of p160ROCK on stress-induced cytoskeletal organization or by its direct effect on multiple signaling pathways.

Despite the fact that our findings implicate p160ROCK on the initial events elicited by the load-induced Dbll/RhoA activation, they do not rule out a possible role for other targets or upstream activators of RhoA in myocardial responses to pressure overload. Several targets of RhoA have been identified besides p160ROCK, including citron kinase, protein kinase N, p140mDia, and rhotekin (15). Although the relative contributions of the various RhoA downstream effectors are still unclear, it is possible that they play distinct roles on RhoA-activated signaling mechanisms. In fact, previous studies (22) have demonstrated that protein kinase N regulates atrial natriuretic factor gene transcription in cardiac myocytes through a serum response element. On the other hand, it has also been shown (36) that the Rho/ROCK pathway contributes to cardiac myocyte hypertrophy induced by α1-adrenergic agonist via activation of extracellular signal-regulated kinases and GATA-4, suggesting that the effects of RhoA on gene regulation are mediated by multiple downstream effectors and mechanisms. Interestingly, our present results indicate that the load-induced p160ROCK activation is transient with a peak at 30 min of pressure overload, although Dbll/RhoA association, and presumably RhoA activity, was still increased at 60 min of pressure overload. This finding might indicate that after the initial period, when p160ROCK is the major effector of RhoA, other effectors could be activated and play a role in the effects of RhoA on myocardial responses to pressure overload.

In conclusion, we have shown here that p160ROCK is rapidly and transiently activated in the myocardium in response to pressure overload. Given the potential effects of p160ROCK on multiple aspects of the initial cellular response to mechanical stimuli such as cytoskeletal organization, contractility, and influence on gene expression, the early activation of p160ROCK in overloaded myocardium indicates that this enzyme as well as its upstream activators may occupy a central position in the coordination of the initial signaling mechanisms and adaptive changes triggered by mechanical stress in cardiac myocytes.

This study was sponsored by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc. 98/11403-7, 99/06088-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Proc. 521098/97-1).

REFERENCES


