ADRIANA SOUZA TORSONI

SINALIZAÇÃO EM MIÓCITOS CARDÍACOS SUBMETIDOS A AUMENTOS DE TENSÃO

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O homem vive de
razões e sobrevive
de sonhos.

La Rochefoucauld

Sumário

RESUMO

Uma variedade de estímulos, como a sobrecarga hemodinâmica, pode levar a um aumento de tensão mecânica em cardiomiócitos, que normalmente é compensado pelo desenvolvimento de um fenótipo hipertrófico. A resposta inicial à sobrecarga é caracterizada por uma rápida e coordenada ativação de vias de sinalização intracelulares que regulam a expressão gênica e culminam com o crescimento hipertrófico de cada cardiomiócito. A importância relativa de cada elemento que participa das vias de sinalização e sua contribuição para as alterações adaptativas observadas nos miócitos cardíacos em resposta à sobrecarga pressora têm sido o foco de diversas pesquisas.

No presente estudo, investigamos a ativação e localização subcelular da FAK em miócitos ventriculares de ratos neonatos (MVRN) submetidos a ciclos de estiramento, além da sua participação na ativação do gene do fator natriurético atrial (ANF). O estiramento pulsátil dos cardiomiócitos, de 5 a 20%, por períodos de 10 a 120 minutos, levou a um aumento da fosforilação da FAK no seu resíduo de tirosina 397, conforme detectado pelo anticorpo fosfoespecífico. Tal ativação foi paralela com uma alteração na localização da Fak em MVRN que, da região perinuclear em miócitos não estirados, passou a distribuir-se ao longo dos miofilamentos, como agregados protéicos, em células estiradas. Além disso, 4 horas de estiramento pulsátil aumentou a atividade do gene repórter da luciferase contendo o promotor do ANF. A interrupção da sinalização pelo complexo endógeno Fak/Src, seja pela expressão de um mutante negativo de Fak, onde a tirosina foi substituída por fenilalanina no resíduo 397, ou pelo tratamento com um inibidor farmacológico da c-Src, diminuiu severamente a ativação da Fak e sua redistribuição ao longo dos miofilamentos, mediados pelo estiramento, além de inibir a ativação do gene do ANF. No entanto, a expressão de um mutante selvagem de Fak potencializou a fosforilação dessa quinase, induzida por estiramento, sem alterar a expressão gênica do ANF, em comparação aos MVRN não transfectados.

Neste trabalho, também realizamos experimentos com o intuito de examinar a importância do complexo RhoA/ROCK na ativação da Fak durante o estiramento pulsátil em MVRN e sua participação na ativação do gene da cadeia pesada de miosina (βMHC). Nessas células, a fosforilação da Fak e de seu efetor Erk1/2, nos

resíduos de Tyr 397 e Thr202/Tyr204, respectivamente, aumentou rapidamente e manteve-se constante por até 2 horas. Experimentos de imunofluorescência mostraram que 2 horas de estiramento pulsátil levaram a uma marcação nuclear da Fak, ao contrário do que se observou em cardiomiócitos não estirados, onde a Fak colocalizou-se com a proteína Rho na região sarcomérica. O bloqueio da sinalização do complexo RhoA/ROCK, através da utilização de inibidores farmacológicos de RhoA e ROCK, reverteu a ativação das proteínas Fak e Erk 1/2 induzida pelo estiramento. Além disso, o tratamento das células com citocalasina D, inibidor da polimerização da actina, impediu a ativação das proteínas Fak/Erk1/2 induzida pelo estiramento. A transfecção dos MVRN com oligonucleotídeos antisense para a RhoA levou a uma diminuição da ativação da Fak, seguida por uma redução significante da expressão do gene da βMHC, fenômeno que também foi observado quando as células foram transfectadas com oligonucleotídeos antisense para a Fak.

Finalmente, também procuramos entender os efeitos da sobrecarga pressora aguda sobre a ativação da p160ROCK em miocárdio de ratos. A sobrecarga pressora, representada pela constrição da aorta transversa, levou a um aumento da associação RhoA/Dbl-3 e RhoA/p160ROCK, sendo que esta última foi máxima aos 30 min, reduzindo após 60 min de sobrecarga pressora. O mesmo perfil de ativação transiente foi observado com relação à atividade do imunoprecipitado de p160ROCK após 10 e 60 min de estímulo. Análises imunocitoquímicas indicaram a formação de agregados de p160ROCK e RhoA, junto ao eixo longitudinal dos miócitos cardíacos, induzidos pela sobrecarga pressora. Experimentos de imunohistoquímica estrutural mostraram que a sobrecarga pressora induziu colocalização de p160ROCK e RhoA, preferencialmente na linha Z, túbulo-T e áreas subsarcolemais.

Todos os dados apresentados, em conjunto, indicam que a Fak desempenha um papel primordial na resposta inicial induzida pelo estiramento em cardiomiócitos, controlando o programa de expressão gênica associado com a hipertrofia do miócito cardíaco, e que sua função está diretamente vinculada à ativação do complexo RhoA/ROCK.

ABSTRACT

A variety of stimuli, such as hemodynamic overload, can lead to an increase in mechanical stress on cardiomyocytes that can be compensed by the development of a hypertrophyc phenotype. The hypertrophyc response is characterized by a rapid and coordinate activation of intracellular signaling pathways that regulate gene expression and results in a hypertrophyc growth of cardiac myocyte. The relative importance of each element that participate of signaling pathway and its contribution for the adaptative changes observed in cardiac myocyte in response to pressure overload, has been the focus of diverse researches.

In the present study we investigated the Fak activation and subcellular localization in neonatal rat ventricular myocytes (NRVMs) submitted to pulsatile stretch, instead its participation in the atrial natriuretic factor (ANF) gene activation. 5% to 20% (10-120 minute) pulsatile stretch of NRVMs lead to an increase of Fak phosphorylation at Tyr-397, as detected by phosphospecific antibody.

This activation was accompanied by a change in Fak localization in NRVMs that, of the perinuclear regions in nonstretched cells change to aggregates regularly distributed along the myofilaments in stretched cells. Furthermore, a 4-hour cyclic stretch enhanced the activity of the ANF promoter-luciferase reporter gene. Disrupting endogenous Fak/Src signaling either by expression of a dominant-negative Fak mutant with phenylalanine substituted for Tyr-397 or by treatment with a c-Src pharmacological inhibitor markedly attenuated stretch-induced Fak activation and its redistribution at myofilaments and inhibited stretch-induced ANF gene activation.

However, the expression of wild-type Fak potentiated the stretch-induced Fak phosphorylation but did not change ANF gene expression compared with the responses of nontransfected NRVMs.

In this study, we performed experiments to examinate the importance of RhoA/ROCK complex in stretch-induced FAK activation in NRVMs and its participation in β-myosin heavy chain (βMHC) gene activation. In these cells, Fak and its effector Erk1/2 phosphorylation at Tyr397 and Thr202/Tyr204, respectively, rapidly increased and sustained up to 2 hours. Immunofluorescence experiments showed that 2-hour period of cyclic stretch lead to Fak staining nuclei in contrast to non-stretched NRVMs, where Fak and RhoA are co-localized in sarcomeric region.

Blockade of RhoA/ROCK signaling by pharmacological inhibitors of RhoA and ROCK reverted the stretch-induced Fak and Erk 1/2 activation.

Moreover, the treatment of cells with the inhibitor of actin polymerization, cytochalasin D, abolished the stretch-induced Fak/Erk1/2 activation. Transfections of NRVM with RhoA antisense oligonucleotide lead to an decreased of stretch-induced Fak activation and reduced the stretch-induced expression of βMHC gene, what was observed when the cells were treated with FAK antisense oligonucleotide.

Finally, we intend to understand the effects of acute pressure overload on activation of p160 ROCK in rat myocardium. The pressure overload, represented by constriction of transverse aorta, induced an increase of RhoA/Dbl-3 and RhoA/p160 ROCK association, where the RhoA/p160 ROCK association had peaking at 30 min but reduced to lower levels by 60 min of pressure overload.

The same profile of transient activation was observed with the p160 ROCK immunoprecipitated activity after 10 min and 60 min of stimulus.

Imunocytochemistry analysis indicated that pressure overload induced the formation of aggregates of p160 ROCK and RhoA along the longitudinal axis of cardiac myocytes. Immunoelectron microscopic experiments showed that pressure overload induced the association of p160 ROCK and RhoA to Z-line, T-tubule, and subsarcolemmal areas.

All these data presented together indicate that Fak play a primordial role in the early responses induced by stretch in cardiac myocytes, controlling gene expression program associated with load-induced cardiac myocyte hypertrophy and that its function is directly bound to RhoA/ROCK complex activation.

1. INTRODUÇÃO

Diversos estímulos patofisiológicos, tais como o infarto do miocárdio, hipertensão, doenças valvulares, miocardites virais e cardiomiopatias podem levar a um aumento na sobrecarga cardíaca e elevar a tensão mecânica sobre os miócitos cardíacos. A tensão mecânica é responsável por ativar um distinto padrão de expressão gênica nos cardiomiócitos, resultando, eventualmente, em alterações qualitativas e quantitativas no conteúdo de proteínas contráteis e na indução de um programa gênico embrionário, que culminam com o aparecimento de um fenótipo hipertrófico.

Quando a sobrecarga persiste, o coração tensionado entra em um estado crítico de transição de hipertrofia compensatória para uma falência cardíaca descompensada. A dilatação das câmaras, o desacoplamento do processo de excitação-contração, a desorganização sarcomérica, o aumento anormal do tecido intersticial, o metabolismo energético alterado e a perda da viabilidade dos miócitos, são características comuns encontradas no estágio final de falência cardíaca.

O grande interesse nessa área de pesquisa está em desvendar como a célula interpreta o estímulo mecânico e conduz à resposta hipertrófica.

1.1 Estrutura do miócito cardíaco

Os miócitos cardíacos são células constituídas de longas fibras que apresentam geralmente um a dois núcleos centrais (SOMMER & JENNINGS, 1992). Essas células se associam através de estruturas denominadas discos intercalares, que fazem com as mesmas estejam arranjadas em uma rede ramificada, formando um sincício funcional.

Os miócitos contêm um grande número de miofilamentos que estão organizados, de modo a delimitar um intercalamento de regiões contendo filamentos espessos e delgados, conferindo à fibra um aspecto estriado e demarcando regiões conhecidas como sarcômeros. O filamento espesso é composto pela miosina, enquanto no filamento delgado a grande proteína contrátil encontrada é a actina (SOMMER & JENNINGS, 1992; revisado por CLARK *et al.*, 2002) (fig 01).

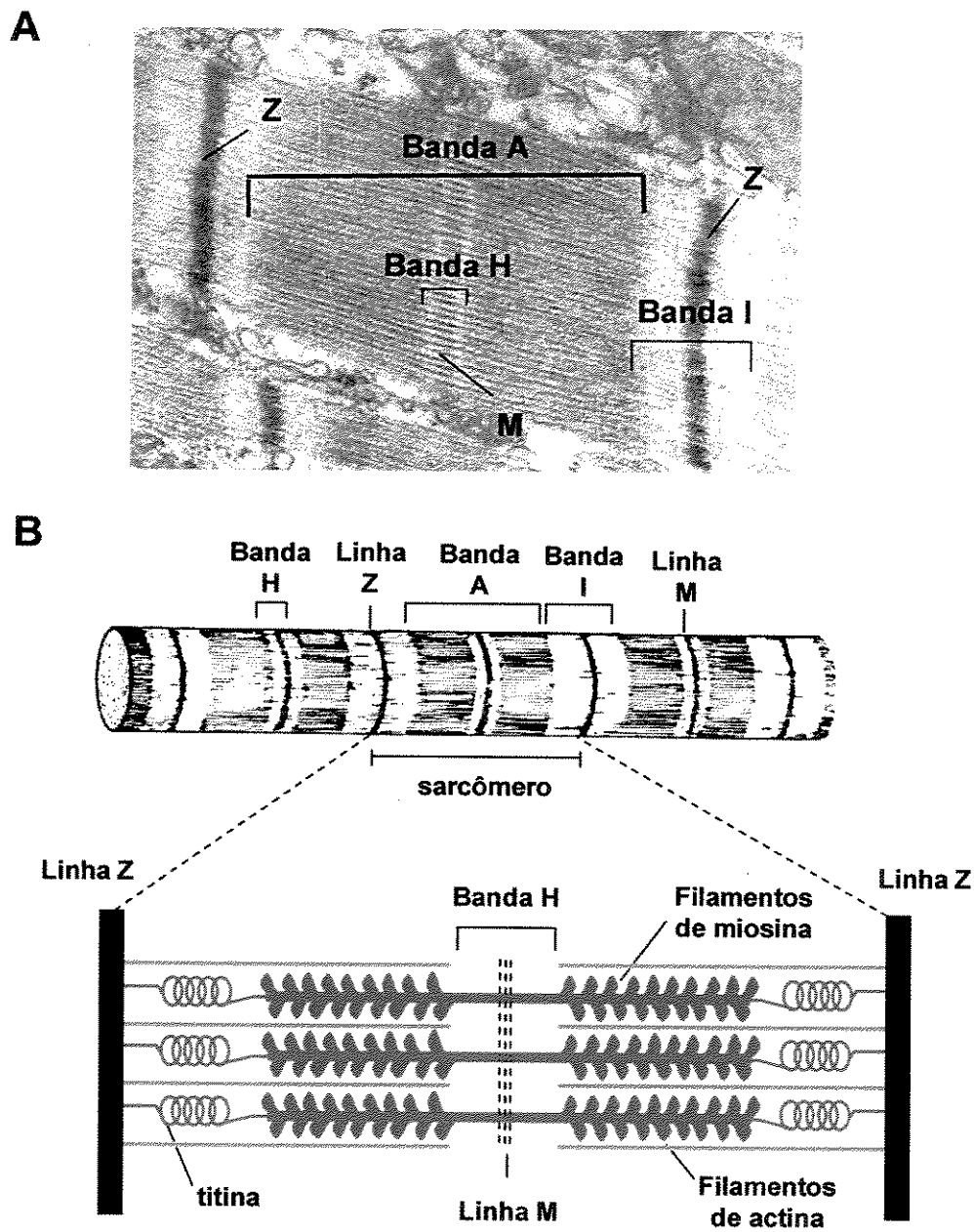

Figura 01: O Sarcômero. A - fotomicrografia de secção de músculo cardíaco de rato adulto (47.260x). Observar o sarcômero e a demarcação das bandas A, I e H e as linhas Z e M. B – Diagrama ilustrando a miofibrila e a posição dos filamentos delgados e espessos do sarcômero, bem como dos filamentos de titina (Modificado por SIESSER, 2004).

A região do sarcômero ocupada pelos filamentos espessos de miosina nos quais os filamentos de actina inserem-se de cada lado, é chamada de banda A. No centro de cada banda A encontra-se a banda H, onde não existe superposição de filamentos delgados. A banda H é dividida em duas partes por uma linha M. A região do sarcômero ocupada somente pelos filamentos delgados de actina é conhecida por banda I; essa banda estende-se da linha Z na direção do centro do sarcômero (revisado por CLARK *et al.*, 2002) (fig 01). A linha Z representa o limite lateral do sarcômero onde, além dos filamentos de actina, ancoram-se proteínas como a titina e a nebulete. O sarcômero é definido, então, como a região entre sucessivas linhas Z, contendo duas meias bandas I e uma banda A (revisado por GRANZIER & LABEIT, 2004; PYLE & SOLARO, 2004) (fig 01). Associadas ao filamento delgado encontram-se proteínas como a tropomiosina e o complexo troponina, que auxiliam na regulação do mecansimo de contração. Próximo à linha Z, os filamentos delgados interagem com proteínas citoesqueléticas, como a α e β actinina, as quais mantêm esses filamentos presos à linha Z (revisado por PYLE & SOLARO, 2004). Entre os miofilamentos, observa-se a presença de mitocôndrias que fornecem a demanda de energia necessária para a contração.

Os cardiomiócitos apresentam-se delimitados por uma complexa estrutura membranosa conhecida como sarcolema, onde estão inseridos receptores transmembrana protéicos que ligam os miócitos à matriz extracelular, além de bombas e canais iônicos que são essenciais para o processo contrátil do miócito (revisado por CLARK *et al.*, 2002). O sarcolema forma duas regiões especializadas dos miócitos, o sistema de túbulos transversos e os discos intercalares, que desempenham papel importante no acoplamento excitação-contração e na comunicação mecânica e elétrica entre as células, respectivamente (SOMMER & JENNINGS, 1992; revisado por CLARK *et al.*, 2002).

A adesão dos cardiomiócitos à matriz extracelular ocorre por meio de regiões especializadas, denominadas costâmeros. Essas regiões ancoram as linhas Z das miofibrilas ao sarcolema, por meio de filamentos intermediários de desmina. Além da adesão, os costâmeros são responsáveis pela transmissão lateral da força

contrátil gerada nos sarcômeros e pela manutenção da integridade do sarcolema durante a contração (DANOWSKI *et al.*, 1992; CLARK *et al.*, 2002).

1.2- Hipertrofia cardíaca

1.2.1- Hipertrofia do miocárdio

Os cardiomiócitos são células que se diferenciam precocemente durante o desenvolvimento do coração, fato que acarreta na perda da capacidade de divisão celular. Desta maneira, quando ocorre um aumento da carga de trabalho, essas células tendem a se adaptar à nova demanda crescendo apenas em tamanho (revisado por AKAZAWA & KOMURO, 2003). Em diversas condições patológicas, as quais impõem uma sobrecarga ao coração, como doenças isquêmicas, hipertensão, doenças vasculares e cardiomiopatias, os cardiomiócitos tornam-se hipertróficos (CHIEN *et al.*, 1993; revisado por FREY *et al.*, 2004).

De um modo geral, o coração responde à sobrecarga de trabalho, melhorando seu desempenho. Esse efeito é decorrente de uma melhora na contratilidade, devido à ativação do mecanismo de Frank-Starling, e do aumento da disponibilidade de cálcio (revisado por LORELL *et al.*, 2000).

Ao nível celular, a hipertrofia cardíaca induz uma série de respostas, incluindo aumento na síntese protéica, aumento do número e organização das subunidades motoras (sarcômeros), liberação de peptídeos vasoativos, além da indução de genes de resposta imediata e reativação da expressão de genes fetais (YUE *et al.*, 2000; revisado por FREY *et al.*, 2004). Adicionalmente, a estimulação hipertrófica parece induzir uma expressão gênica diferencial, incluindo genes que codificam proteínas envolvidas em vias de sinalização e metabolismo energético (revisado por AKAZAWA & KOMURO, 2003).

Aparentemente essa resposta hipertrófica do cardiomiócito é desencadeada por uma combinação de estímulos mecânicos e neurohumorais. Entretanto, independentemente do estímulo, a resposta fenotípica de crescimento hipertrófico

é homogênea, sugerindo a ativação de um mecanismo comum, que culmina com a hipertrofia cardíaca (WAGNER *et al.*, 1999).

Em vários tipos de patologias cardíacas a hipertrofia induzida pela sobrecarga pressora parece ter uma função compensatória que leva à diminuição da tensão na parede e no consumo de oxigênio (revisado por FREY *et al.*, 2004). Este é um mecanismo que, de acordo com a relação de Laplace ($T=PxR/2\varepsilon$), anula o efeito do aumento na tensão provocada pelo aumento da pressão ventricular, seja sistólico ou diastólico (CARO *et al.*, 1978; GUNTHER & GROSSMAN, 1979). Contudo, embora a hipertrofia cardíaca seja inicialmente compensatória, no caso de um aumento da carga de trabalho, a manutenção desse processo por períodos prolongados pode levar a arritmias, falência cardíaca e morte repentina (revisado por AKAZAWA & KOMURO, 2003).

1.2.2- Hipertrofia do miócito cultivado in vitro

Da mesma forma que miocárdios de ratos submetidos à sobrecarga de trabalho desenvolvem o fenótipo hipertrófico, miócitos cardíacos em cultura submetidos a estiramento mecânico apresentam características fenotípicas de crescimento hipertrófico.

Os primeiros estudos utilizando miócitos de ratos neonatos demonstraram que pequenos intervalos de estiramento estático eram suficientes para induzir características de resposta hipertrófica. Experimentos mais recentes, em que miócitos de ratos neonatos foram submetidos a estiramento pulsátil, demonstraram a ativação de inúmeras vias de transdução de sinal que são ativadas em resposta à sobrecarga mecânica *in vivo* (revisado por SAFFITZ & KLÉBER, 2004).

De maneira similar, a liberação de fatores neurohumorais e mediadores celulares, como citocinas, fatores de crescimento, catecolaminas, peptídios vasoativos e hormônios, os quais induzem as reações hipertróficas *in vivo*, têm sido identificados nos cardiomiócitos em cultura (SEKO *et al.*, 1999; revisado por SAFFITZ & KLÉBER, 2004). Isto demonstra que o estímulo hipertrófico induzido

em cultura, leva a uma resposta semelhante àquela observada *in vivo*, regulada durante a hipertrofia compensatória (HEFTI *et al.*, 1997).

Ademais, cardiomiócitos em cultura primária representam um bom modelo *in vitro* para uma análise detalhada de todos os eventos celulares que culminam com o desencadeamento da resposta hipertrófica, uma vez que nos ensaios *in vivo* existe a dificuldade de diferenciar quais os tipos celulares que estão envolvidos com as alterações bioquímicas e morfológicas observadas na hipertrofia cardíaca (STEMMER *et al.*, 1992; HEFTI *et al.*, 1997).

1.2.3- Estímulos Hipertrofiantes

1.2.3.1- Estiramento Mecânico

O estiramento de cardiomiócitos em cultura sobre superfícies deformáveis tem demonstrado induzir características celulares de hipertrofia, aumento na expressão de proteínas contráteis e estimulação de várias vias de sinalização, incluindo vias que envolvem tirosinas quinases, como a quinase de adesão focal, via Ras/MAPK, via JAK/STAT, proteína quinase C e fosfolipases C e D (SEKO *et al.*, 1999; ZHUANG *et al.*, 2000).

Adicionalmente, vários autores relatam que em resposta a um estímulo hipertrófico, MVRN iniciam um rápido e transiente aumento na expressão dos genes de resposta imediata, como c-jun, c-fos, c-myc e ergr-1, seguida pela re-expressão de genes fetais como o gene do fator atrial natriurético (ANF), o gene da cadeia pesada da miosina (βMHC) e o gene da α actina do músculo esquelético (SkM) e, finalmente, a ativação de genes de expressão constitutiva, como o gene da actina cardíaca e o gene da cadeia leve de miosina (MLC) (KOMURO *et al.*, 1988; IZUMO *et al.*, 1988; FULLER *et al.*, 1998; NADRUZ *et al.*, 2003).

Sabe-se que as três MAP quinases ERK, JNK e p38 são mediadoras da sinalização hipertrófica em células miocardiais. ERK e p38 podem ser ativadas pela via de sinalização que envolve integrinas-Fak-Src-Ras, após o estiramento mecânico. Essas proteínas estão envolvidas na fosforilação de substratos no

núcleo (como c-myc, c-jun), o que desencadeia uma reprogramação transcricional, que é igualmente responsável pela expressão gênica alterada associada com a hipertrofia (SUSSMAN *et al.*, 2002).

1.2.3.2- Fatores Humorais e Parácrinos

Vários estudos relatam que a liberação de fatores autócrinos e parácrinos amplifica o estímulo de crescimento inicial, disparado pelo estiramento mecânico. Angiotensina II, endotelina-1, fenilefrina e fator de crescimento endotelial vascular (VEGF) têm sido implicados na hipertrofia celular cardíaca *in vitro* e *in vivo* (SADOSHIMA *et al.*, 1993; SADOSHIMA *et al.*,1995; SADOSHIMA & IZUMO, 1997; SCHEIDEGGER *et al.*, 1999).

Esses fatores humorais são conhecidos por ativar vários sistemas de segundo-mensageiro, através de receptores acoplados à proteína G (SADOSHIMA *et al.*, 1993; SADOSHIMA *et al.*,1995; SADOSHIMA & IZUMO, 1997; SCHEIDEGGER *et al.*, 1999). Tais receptores participam da transdução de sinal induzida por agonistas a efetores intracelulares como enzimas e canais iônicos. A simples ativação desses receptores pode levar à indução de hipertrofia *in vitro* e cardiomiopatia *in vivo* (revisado por FREY *et al.*, 2004). Quando estimulados por agonistas, esses receptores induzem a expressão de c-*fos* e outros genes de resposta imediata, além de promover a reexpressão de genes embrionários, como o ANF (revisado por DOSTAL & BAKER, 1999).

Sabe-se que Angiotensina II, através de seu receptor AT1, induz mobilização de Ca^{+2} intracelular, além de ativação de proteínas tirosinas quinases, como Fak e Shc e de proteínas serina/treonina quinases, como MAP quinases. Vários estudos sugerem que angiotensina II pode ser um fator crítico em mediar hipertrofia cardíaca *in vivo*. SADOSHIMA *et al.* (1993) reportaram que angiotensina II causa resposta hipertrófica em miócitos cardíacos de ratos neonatos e resposta mitogênica em fibroblastos cardíacos *in vitro*. Recentemente esses mesmos autores demonstraram que a estimulação mecânica em miócitos cardíacos de ratos neonatos causa secreção de angiotensina II, e esta age como um mediador inicial de hipertrofia induzida por estiramento, através da ativação de receptores

AT1 (SADOSHIMA & IZUMO, 1997). Nessas células a administração de losartan, antagonista do receptor AT1, inibiu a fosforilação em tirosina de várias proteínas intracelulares e ativação de MAP quinases, induzida pela Angiotensina II (SADOSHIMA *et al.*,1995). BOGOYEVITCH *et al.* (1994) demonstraram que fenilefrina e endotelina-1 também ativam MAP quinases em cultura de cardiomiócitos ventriculares.

No entanto, o estiramento físico de cardiomiócitos adultos ou neonatos é suficiente para induzir expressão gênica hipertrófica e um fenótipo hipertrófico nessas células, mesmo que na ausência de fatores neuronais ou humorais (SADOSHIMA & IZUMO, 1997), o que sugere um mecanismo celular autônomo (revisado por FREY *et al.*, 2004).

1.3- Mecanosensores e a transdução de sinal bioquímico

Os mecanismos moleculares que convertem as forças físicas externas percebidas pelas células em sinais bioquímicos intracelulares e como esses sinais levam a mudanças na programação transcricional que são responsáveis pelas alterações no fenótipo dos cardiomiócitos permanecem ainda pouco claros (revisado por AKAZAWA & KOMURO, 2003). Contudo, vários mediadores mecanosensíveis têm sido propostos, dentre eles algumas proteínas de membrana como canais iônicos ativados por estiramento e algumas classes de receptores, como as integrinas (NARUSE *et al.*, 1998).

1.3.1- Canais Iônicos ativados por estiramento

O aumento na força de contração após o estiramento (mecanismo de Frank-Starling) parece consistir de dois componentes principais: o aumento na afinidade da Troponina C e aumento na concentração de Ca^{+2} transiente (TAVI *et al.*, 1998).

Aumentos na concentração de cálcio podem envolver uma variedade de mecanismos incluindo ativação de canais sensíveis ao estiramento e canais de cálcio tipo L (SUSSMAN *et al.*, 2002).

A presença de canais iônicos sensíveis ao estiramento no sarcolema de miócitos cardíacos sugere o envolvimento dessas estruturas nas alterações da função cardíaca, induzidas pelo estiramento.

É conhecido que a abertura dos canais sensíveis ao estiramento depende do estiramento mecânico da membrana. A maioria dos canais encontrada em cardiomiócitos apresenta permeabilidade tanto a cátions monovalentes, como a divalentes. Dessa maneira, adicionalmente ao aumento na concentração intracelular de Ca^{+2} induzida pela sobrecarga mecânica, ocorre também um aumento na concentração de sódio intracelular. O aumento deste íon leva à ativação da trocadora Na^{+}/Ca^{+2}, o que acarreta um acúmulo adicional de Ca^{+2} no interior da célula. Este Ca^{+2} é, então, bombeado para o retículo sarcoplasmático, por meio da bomba de Ca^{+2}. O aumento na quantidade de cálcio no interior do retículo, leva a uma grande liberação deste íon durante a sístole (TAVI *et al.*, 1998).

O aumento nos níveis de cálcio intracelulares além de desencadear o aumento da contratilidade, também leva à ativação de vias de sinalização molecular que podem resultar em síntese e secreção de peptídeos natriuréticos, reprogramação transcricional e remodelamento estrutural. A sinalização prolongada dependente de cálcio, leva ao estabelecimento do fenótipo hipertrófico, com alterações no tamanho da célula, síntese protéica e desempenho hemodinâmico (SUSSMAN *et al.*, 2002).

Adicionalmente, a alta concentração de Ca^{+2} pode desencadear a ativação de uma tirosina quinase relacionada à Fak, a pyk2/RAFTK. A ativação da pyk2/RAFTK leva à fosforilação da proteína adaptadora paxilina, que é um componente estrutural do complexo de adesão focal (MELENDEZ *et al.*, 2002)

Entretanto, a maior parte dos estudos realizados com canais iônicos sensíveis ao estiramento relacionam sua ativação muito mais à uma adaptação funcional, responsável pelo aumento de força gerada por cada miócito, do que a efeitos anabólicos específicos que determinam a síntese de proteínas contráteis no miócito e que culminam em uma adaptação estrutural da célula.

1.3.2-Receptores Transmembrana

Cerca de 3 classes de receptores são considerados importantes na transmissão da força mecânica entre as células e a matriz extracelular, as integrinas, as caderinas e os receptores com domínio discoidina (DDR). Esses receptores são encontrados tanto em miócitos como em fibroblastos (BOUVARD *et al.*, 2001). Dentre eles, a principal classe é a das integrinas.

As integrinas são receptores heterodiméricos transmembrana, constituídos de subunidades α e β (GIANCOTTI & RUOSLAHTI, 1999). Tais receptores estão localizados nas adesões focais de vários tipos celulares e são responsáveis por realizar a comunicação entre a matriz extracelular e o citoesqueleto de actina. O domínio extracelular da subunidade β é responsável pela ligação com elementos da matriz extracelular, enquanto que seu domínio citoplasmático se associa a proteínas estruturais do citoesqueleto, incluindo a talina, α-actinina e filamina (JULIANO & HASKILL, 1993; SHARMA *et al.*, 1995).

Estudos *in vitro* confirmam que as integrinas localizam-se, a princípio, nas adesões focais, conectando os componentes da matriz extracelular com o citoesqueleto de actina e com componentes de vias de sinalização (ROSS & BORG, 2001). Acredita-se que a ligação desses receptores a elementos de matriz desencadeie a agregação de várias moléculas, as quais sofrem alterações conformacionais, a fim de transmitir o sinal físico recebido a proteínas sinalizadoras intracelulares associadas a estes sítios (revisado por GIANCOTTI & RUOSLAHTI, 1999).

Em cardiomiócitos, as integrinas estão localizadas nos costâmeros (TERRACIO *et al.*, 1989; TERRACIO *et al.*, 1991; IMANAKA-YOSHIDA *et al.*, 1999). Essas estruturas, análogas às adesões focais estão localizadas *in vivo* próximas à linha Z sarcomérica e são responsáveis por conectar a matriz extracelular, o citoesqueleto e complexos sinalizadores. No entanto, apesar da similaridade com as adesões focais, somente recentemente os costâmeros passaram a ser considerados como candidatos a mecanosensores em cardiomiócitos (SUSSMAN

et al., 2002; CLARK *et al.*, 2002; ERVASTI, 2003; PYLE & SOLARO, 2004). Também a presença de disco intercalar, *in vivo*, pode ser importante para a transmissão de força e sua condução entre as células e a matriz extracelular (ROSS & BORG, 2001). Esse fato foi reforçado pela demonstração da localização de proteínas sinalizadoras nessa região após estimulação com endotelina (KOVACIC-MILIVOJEVIC *et al.*, 2001).

Esses receptores, quando mecanicamente estimulados, podem funcionar como sensores que transmitem o sinal mecânico da matriz extracelular para o meio intracelular regulando a expressão gênica, diferenciação e crescimento celulares, cooperativamente com fatores de crescimento (CLARK & BRUGGE, 1995). Seu arranjo e localização na superfície de miócitos e fibroblastos parecem ser cruciais para a geração e transmissão de força (SUSSMAN *et al.*, 2002).

1.4- Importantes proteínas ativadas em resposta a aumentos de tensão

1.4.1-Proteína Quinase de Adesão Focal (Fak)

1.4.1.1- Estrutura da proteína

A Fak é uma tirosina quinase citoplasmática, que se colocaliza com as integrinas nos contatos focais de vários tipos celulares. Esta enzima é constituída de três domínios diferentes em sua estrutura, a saber, um domínio N-terminal, um domínio quinase, responsável por aumentar a atividade enzimática da proteína, e um domínio C-terminal, sendo cada um deles constituído de aproximadamente 400 aminoácidos (CALALB *et al.*, 1995) (fig 02).

Seis resíduos de tirosina na estrutura da Fak foram identificados como sítios de fosforilação *in vivo*. Dois desses sítios de fosforilação (Tyr 397 e 407) estão localizados próximos à porção N-terminal, dois estão próximos ao domínio de ativação de quinase (Tyr 576 e 577) e outros dois próximos ao domínio C-terminal (Tyr 861 e 925) (CALALB *et al.*, 1995) (fig 02).

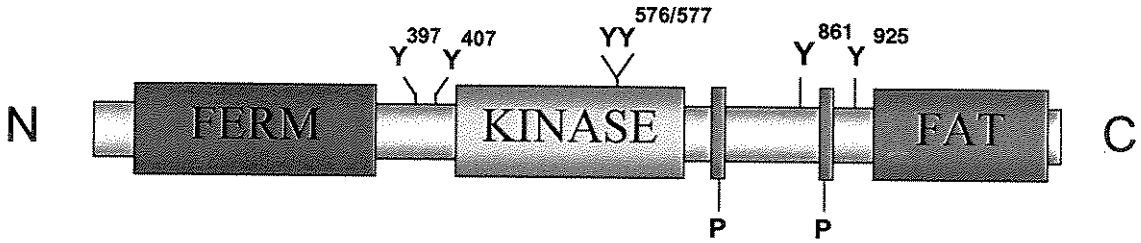

Figura 02: Esquema representativo da estrutura primária da FAK. Detalhe para seus domínios FERM, quinase e FAT, sítios de fosforilação em tirosina (Y) e seqüências ricas em prolina (P).

O domínio N-terminal da Fak liga-se ao domínio citoplasmático da β integrina, estabilizando a associação Fak-integrina no contato focal (SCHALLER *et al.*, 1995). Este domínio é formado, em sua maior parte, pelo domínio FERM, análogo àquele encontrado em proteínas da família das JAK (Janus quinase) e proteínas ERM, que está vinculado a um mecanismo de autoinibição da Fak (GIRAULT *et al.*, 1999; COOPER *et al.*, 2003).

Próximo ao domínio N-terminal localiza-se o maior sítio de autofosforilação da molécula, a tirosina 397 (CALALB *et al.*, 1995; ZHANG *et al.*, 1999; REISKE *et al.*, 1999; HAN & GUAN, 1999). A autofosforilação deste resíduo pode ser desencadeada em resposta a estímulos como fatores de crescimento, fatores humorais, adesão celular e estiramento mecânico, e seu bloqueio pode levar à desestruturação do sítio de adesão focal e à morte dos cardiomiócitos (HEIDKAMP *et al.*, 2002).

O domínio C-terminal contém sítios de ligação para uma série de proteínas associadas ao complexo de adesão focal, tais como talina e paxilina (TUNER & MILLER, 1994; CHEN *et al.*, 1995), além de outras proteínas sinalizadoras, como GRAF (GTPase associada à Fak) e pCas130 (SHEN & SCHALLER, 1999). Esta região é conhecida como FAT (*focal adhesion targeting*) e é responsável por direcionar a Fak à malha de actina que ancora o complexo integrina/Fak ao

citoesqueleto, estabilizando-a nos contatos focais (HILDEBRAND *et al.*, 1993; COOLEY *et al.*, 2000).

1.4.1.2- Ativação da proteína

Diversos estímulos podem levar à ativação da Fak, sejam eles diretos, como a presença de fatores de crescimento e humorais, ou indiretos, como a ativação via integrinas (SCHLAEPFER *et al.*, 1994; HEIDKAMP *et al.*, 2002). Em ambos os casos, a ativação se processa de maneira análoga. A agregação de integrinas, por exemplo, pode promover a ligação do domínio N-terminal da Fak ao domínio citoplasmático da própria integrina, levando à exposição e autofosforilação do resíduo de Tyr 397 (SCHLAEPFER *et al.*, 1994). Especificamente, a autofosforilação da Fak no resíduo de tirosina 397, resulta na criação de um sítio de alta afinidade para a Src (quinase relacionada ao oncogene do sarcoma de Roux) (HANKS *et al.*, 1992; SCHALLER *et al.*,1994; YAMADA & MIYAMOTO, 1995; CALALB *et al.*,1996; SCHLAEPFER & HUNTER, 1996). Este aumento da afinidade da Fak pela Src resulta na fosforilação das Tyr 576 e 577, localizadas no domínio catalítico (de ativação) da Fak, levando à máxima atividade de quinase desta proteína (SCHAEPFER & HUNTER, 1996). Paralelamente à ativação da Fak pela Src, a própria Src se autofosforila em seu resíduo de tirosina 418, após associação com o sítio de fosforilação 397 da Fak, havendo, portanto, uma ativação mútua do complexo Fak/Src (SCHAEPFER & HUNTER, 1996).

Adicionalmente, a ativação do complexo Fak/Src leva à ligação da Fak à proteínas sinalizadoras, como a subunidade regulatória da PI3K (CHEN *et al.*, 1996), Shc (SCHLAEPFER *et al.*, 1994) e Nck (GOICOECHEA *et al*, 2002). A associação da Fak com a PI3-quinase desencadeia a ativação da proteína quinase Akt, influenciando vias de sinalização celular, como a anti-apoptótica, por meio da fosforilação e regulação de componentes da maquinaria que controla a morte celular, incluindo BAD e caspase-9 (revisado por VIVANCO & SAWYERS, 2002).

Outros resíduos de tirosina da Fak não menos importantes, como a tirosina 925, são sítios que sofrem fosforilação pela Src (SCHAEPFER *et al.*, 1994; CALALB *et*

al., 1995; CALALB *et al.*, 1996; SCHAEPFER & HUNTER, 1996). Especificamente, a fosforilação da tirosina 925 regula as interações proteína-proteína por meio da criação de um sítio de ligação para proteínas com domínios SH2, tal como a proteína adapatadora Grb2 (SCHLAEPFER *et al.*, 1994; SCHLAEPFER & HUNTER, 1996). Quando ligada à Fak, esta proteína ativa a via de sinalização para crescimento celular, a qual envolve a ativação da via ERK1/2/MAP quinases (CHARDIN *et al.*, 1993; SCHLAEPFER *et al.*,1994; SCHLAEPFER & HUNTER, 1996).

Evidências da ativação precoce do complexo Fak/Src, em resposta a sobrecarga pressora aguda, em miocárdio de ratos foram demonstradas em estudos do nosso laboratório (FRANCHINI *et al.*, 2000), paralelamente à associação da Fak com Grb2 e PI3-quinase, além da ativação de ERK1/2 e Akt. Adicionalmente, foi demonstrado em nosso laboratório que o estiramento, mais do que a contratilidade, é o estímulo mecânico mais importante para ativar a Fak em corações isolados de ratos (DOMINGOS *et al.*, 2002).

O complexo Fak/Src tem como principal substrato a proteína CAS (revisado por HANKS *et al.*, 2003), que, quando fosforilada, leva à ativação da via de sinalização associada à JNK, contribuindo para a regulação do mecanismo de sobrevivência celular (ALMEIDA *et al.*, 2000).

A fosforilação da Fak também parece ser controlada por alterações no citoesqueleto de actina, mediadas pela proteína Rho, uma vez que a ativação desta proteína reguladora do citoesqueleto é essencial para a ativação da Fak em resposta a vários estímulos (WANG *et al.*, 1979; REN *et al.*, 1999).

1.4.2- Proteína Homóloga à Ras (Rho)

1.4.2.1- Estrutura da proteína

A Rho é uma pequena proteína G, membro da superfamília de proteínas Ras, que, no início da década de 90, foi descrita em fibroblastos 3T3 de camundongos *Swiss*, como sendo uma proteína que age controlando vias de transdução de sinal

que ligam receptores de membrana ao citoesqueleto. Dentre várias funções, essa proteína tem demonstrado participar das vias de sinalização que regulam o citoesqueleto de actina, tais como, a via que regula a formação das fibras de estresse e a via de adesão celular. A Rho também está envolvida na regulação da morfologia, agregação e motilidade celular, na citocinese, na contração do músculo liso, no processo de endocitose, na expressão gênica e na síntese protéica (CLERCK & SUGDEN, 2000).

As proteínas da superfamília Ras (Ras, Rho (RhoA, B, C e G Rac 1 e 2, Cdc42 e TC10), Raf, Rab, Ran, Arf,) são constituídas de cadeias polipeptídicas simples, apresentando 189 resíduos de aminoácidos no total, ligadas à membrana plasmática da célula por uma cauda lipídica presente no domínio C-terminal da proteína. Todos os membros dessa família de proteínas apresentam-se ligados a um nucleotídeo de guanina (GTP ou GDP, dependendo do estado de ativação da proteína) e todos têm função GTPásica (GOMPERTS *et al.*, 2002).

A homologia dentro do grupo de proteínas Ras é muito grande, sendo que a distinção entre elas ocorre apenas pela divergência na sequência do domínio C-terminal, por volta do resíduo 165 até o 189 (fig. 03). É exatamente nesse domínio que ocorre uma modificação pós-traducional, onde há a adição de um ácido graxo farnesil que garante a associação dessas proteínas à superfície interna da membrana plasmática. Ao longo da cadeia polipeptídica, vários resíduos (10-17, 32-36, 58-61, 116-119, 143-147) são responsáveis pela ligação da proteína a um nucleotídeo de guanina. Entre os resíduos 26 e 45 encontra-se a região efetora, que comunica essas proteínas com outras mais à frente na via de sinalização. Dentro dessa região, há uma porção extremamente conservada, entre os resíduos 30 a 40 que, quando mutada, gera uma proteína inativa. A sequência de aminoácidos entre os resíduos 97 e 108 é responsável pela interação com fatores trocadores de nucleotídeo, chamados GEFs (fig 03) (GOMPERTS *et al.*, 2002).

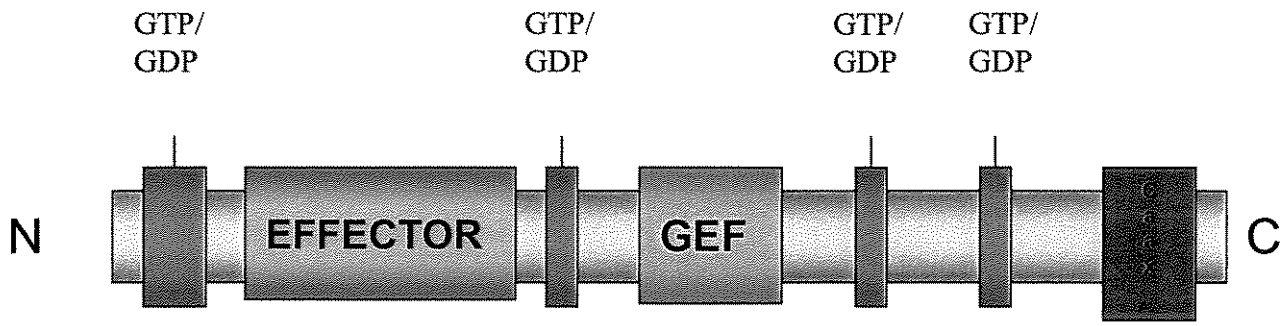

Figura 03: Esquema representativo da estrutura primária das proteínas pertencentes à superfamília Ras. Detalhe dos domínios envolvidos na ligação de nucleotídeos de guanina (GTP ou GDP), domínio efetor, domínio de interação com GEFs e sequência Caax Box, substrato para a modificação pós-traducional por isoprenilação.

1.4.2.2- Ativação da proteína

A proteína Rho é ativada, e pode exercer seus efeitos biológicos quando ligada ao GTP. De maneira contrária, sua ligação com o GDP torna-a inativa. A conversão entre as formas ligadas a GTP ou a GDP é regulada por diversas proteínas (MACKAY & HALL, 1998; KAIBUCHI *et al.*, 1999).

A ativação da GTPase, através da troca de GDP por GTP é promovida por fatores trocadores de nucleotídeo guanina (GEFs). Esses fatores facilitam a liberação de GDP da proteína, promovendo a ligação de GTP, uma vez que a concentração citosólica deste nucleotídeo é cinco vezes maior que a de GDP (KAIBUCHI *et al.*, 1999). Os RhoGEF são proteínas complexas, com vários sítios diferentes em sua estrutura, que incluem SH2, SH3, além dos domínios de homologia à Dbl (DH) e de homologia à pleckstrina (PH), os quais são essenciais para a atividade dos GEFs e identificam os membros desta classe de proteínas (KAIBUCHI *et al.*, 1999).

A inativação da proteína Rho, devido a um aumento na hidrólise do GTP, é estimulada pelas proteínas ativadoras de GTPase (GAPs). Tais moléculas agem como reguladores negativos, aumentando a atividade intrínseca da Rho GTPase, levando à sua conversão ao estado inativo ligado ao GDP.

Adicionalmente, proteínas inibidoras da dissociação de nucletídeos (GDIs), são moléculas que estabilizam a forma inativa da proteína Rho, uma vez que apresentam potencial para diminuir a taxa de dissociação de GDP (MACKAY & HALL, 1998; KAIBUCHI *et al.*, 1999). Essa proteína age como uma "chaperone", regulando a translocação da Rho GTPase entre a embrana e o citosol (KAIBUCHI, 1999).

A primeira indicação de que a Rho estaria envolvida na organização do citoesqueleto de actina, foi obtida por um experimento usando C3 exoenzima em células Vero, o qual resultou no arredondamento das células e dissolução das fibras de actina das mesmas (NARUMIYA, 1996). Em fibroblastos 3T3 a ativação da proteína Rho levou à reunião de filamentos contráteis de actina e miosina (fibras de stress) e à associação de complexos de adesão focal (HALL, 1998; MACKAY & HALL, 1998) (fig 04).

A atividade da Rho, realmente, parece ser necessária para manter a adesão focal em células dependentes de ancoragem, tanto que, com apenas 15 minutos de inativação celular da Rho, a reunião de integrinas não pode mais ser vista na superfície celular.

Alguns autores sugerem que as GTPases da família da Rho são todas responsáveis pela organização do citoesqueleto de actina, mas cada uma age em diferentes locais na célula (MACKAY & HALL, 1998; KAIBUCHI *et al.*, 1999). As RhoGTPases apresentam duas funções básicas: uma é induzir a polimerização da actina, e a outra é organizar o complexo focal em certos locais na célula, função esta desempenhada por RhoGTPases diferentes (NARUMIYA, 1996). O que permanence inexplicável é se a RhoGTPase promove a reunião do complexo de adesão diretamente, pela modificação de um ou mais de seus componentes, ou indiretamente, através da ligação cruzada dos filamentos de actina, estrutura onde

muitos dos constituintes do complexo de adesão permanecem ligados (HALL, 1998).

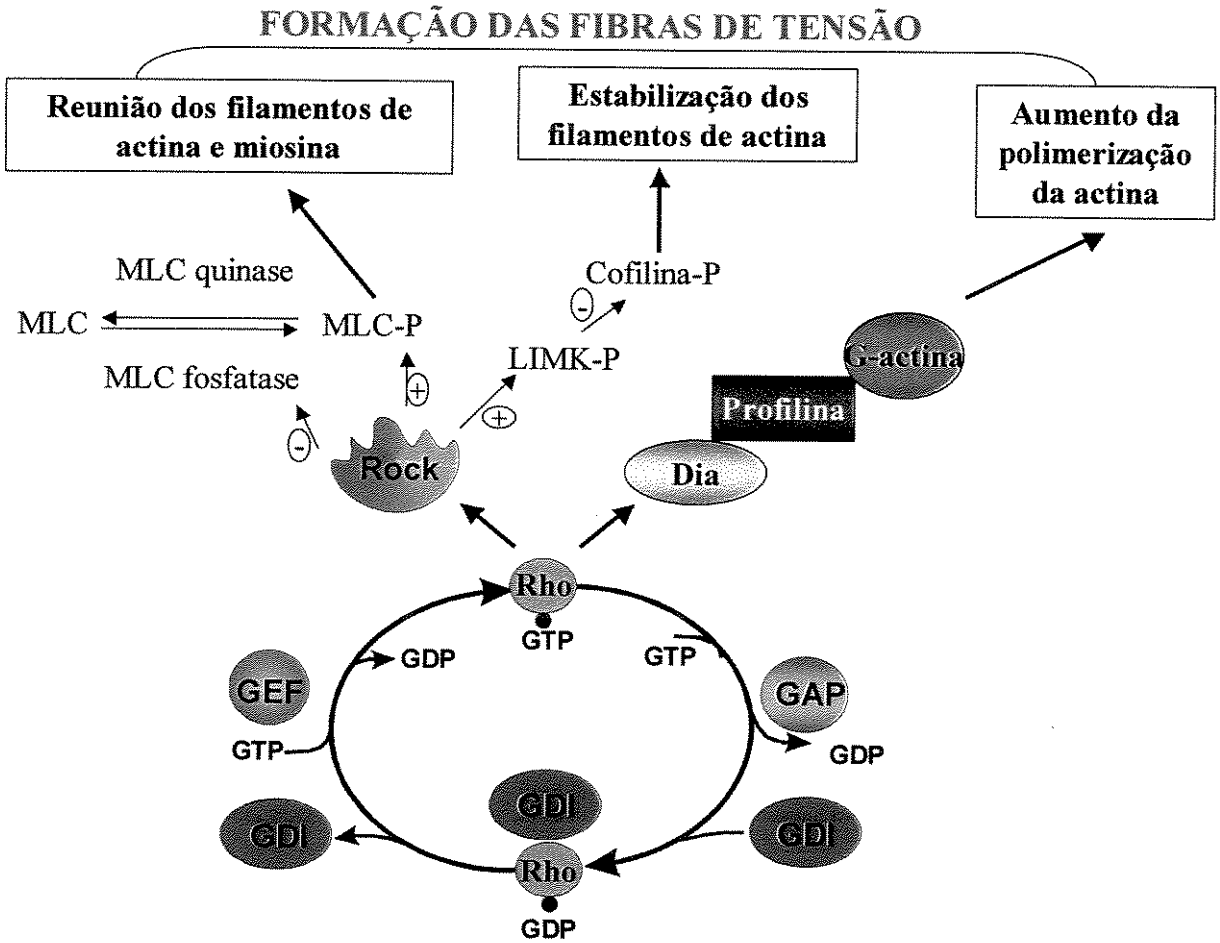

Figura 04: Esquema das vias de transdução de sinal envolvidas na formação das fibras de estresse induzida pela Rho (BISHOP & HALL, 2000).

A Rho também participa da regulação da contração do músculo liso vascular e da morfologia, agregação e motilidade celulares. Estudos recentes têm demonstrado que a Rho está envolvida, também, na ativação da ERK, uma quinase que, reconhecidamente, participa da regulação de eventos iniciais da resposta hipertrófica, através de mecanismos dependentes de integrina em

miócitos cardíacos submetidos a *stress* mecânico (NUMAGUCHI *et al.*, 1999; AIKAWA *et al.*, 1999; YUE *et al.*, 2000).

As proteínas que são reguladas pela Rho incluem a proteína quinase N, a Rho quinase e a subunidade ligada à miosina da miosina fosfatase (HALL, 1998; KAIBUCHI *et al.*, 1999). Outros alvos da Rho incluem a rhofilina, rhotekina, citron e a PIP5-quinase (fosfatidilinositol-4-fosfato 5-quinase) (KAIBUCHI *et al.*, 1999).

De maneira similar à Ras, que é responsável por ativar a via das MAP Kinases, também é esperado que a Rho inicie uma cascata de ativação de Kinases. Estudos anteriores mostraram que membros da família da Rho ativam vias de quinases, paralelamente à via Ras/MAP kinases (NAKAGAWA, 1996).

Algumas proteínas serina/treonina quinases que interagem com Rho, foram identificadas como alvo preferencial dessa pequena GTPase. Uma dessas proteínas é a p160ROCK, uma quinase isolada de plaquetas humanas, que apresenta grande homologia com a Rho-quinase. Essa família de quinases apresenta homologia em seu domínio quinase com a quinase da distrofia miotônica, a qual está especificamente localizada na junção miotendinosa do músculo esquelético e disco intercalar do músculo cardíaco. Neste local, as fibras musculares fundem-se à membrana e muitas proteínas de adesão focal acumulam-se. Experimentos com Rho-kinase indicaram que ela se associa à miosina fosfatase, inibindo sua atividade através da fosforilação da porção que se liga à miosina II, o que sugere uma importante ligação entre a via de ativação da Rho e o processo de contratilidade (BURRIDGE & CHRZANOWSKA-WODNICKA, 1996). KIMURA *et al.* (1996) mostraram que a Rho-kinase também fosforila a MLC e, portanto, ativa a miosina ATPase, estimulando a contratilidade (fig 04). Por sua vez, esse processo leva à organização dos filamentos de actina em fibras de estresse, além da reunião de integrinas, fenômeno visto também para p160ROCK em fibroblastos (NUMAGUCHI *et al.*, 1999) e músculo liso (AOKI *et al.*, 2000). As fibras de tensão aderem-se a estruturas celulares chamadas contatos focais, originando, portanto, os complexos de adesão focais (NARUMIYA, 1996; KAIBUCHI *et al.*, 1999). Certas moléculas como vinculina, α-actinina e talina,

juntamente com a integrina, fazem parte da adesão focal e servem como "âncoras" para as fibras de estresse (BURRIDGE *et al.*, 1996, KAIBUCHI *et al.*, 1999).

Um outro alvo da proteína ROCK é a proteína quinase LIM, que, quando fosforilada, é capaz de inibir, também por fosforilação, a proteína cofilina, levando à estabilização dos filamentos de actina (revisado por BISHOP & HALL, 2000). Entretanto o domínio catalítico da ROCK, por si só, não é capaz de induzir a formação de fibras de estresse organizadas corretamente. Estudos recentes mostram que, quando a ativação da ROCK é combinada com a ativação da proteína Dia, um efetor de Rho, a formação das fibras de estresse é induzida. A proteína Dia liga-se a uma segunda proteína, conhecida como profilina, capaz de se ligar à G-actina. Essa interação leva à contribuição na polimerização de actina e à organização da F-actina em fibras de estresse (revisado por BISHOP & HALL, 2000) (fig 04).

Recentemente NUMAGUSHI *et al.* (1999) mostraram que o estiramento mecânico ativa Rho em células de músculo liso de vasos, translocando parcialmente esta proteína do citosol, onde ela provavelmente estaria ligada a um GDI e, conseqüentemente, inativa, para a fração de membrana, onde ela poderia interagir com um GEF, tornando-a ativa e desencadeando os mecanismos bioquímicos de organização das proteínas do citoesqueleto.

Como foi abordado até o momento, os dados presentes na literatura sustentam a idéia de que estímulos de naturezas variadas podem ser convertidos em sinais bioquímicos que, em última instância, determinam a resposta hipertrófica em cardiomiócitos. Adicionalmente, crescem as evidências da importância da sinalização da FAK, bem como a participação da RhoGTPase e seus efetores na organização do citoesqueleto e na transdução de sinais, vinculada à resposta hipertrófica em cardiomiócitos. Nesse contexto, o entendimento da ativação da Fak e da Rho, bem como dos mecanismos intracelulares ativados por essas enzimas, são fundamentais para o conhecimento da mecanotransdução em miócitos cardíacos.

Assim sendo, o presente estudo pretendeu avaliar a sinalização intracelular em miócitos cardíacos em cultura, em resposta a aumento de tensão. Para tanto, foram levados em consideração os eventos relacionados à sinalização da FAK que resultam no desencadeamento da resposta hipertrófica, bem como a descrição do papel da RhoGTPase na ativação da Fak e no processo de formação do complexo de adesão focal. Propusemo-nos também, a inferir sobre à ativação e localização da Rho e de seu efetor, a proteína ROCK, em cardiomiócitos de ratos adultos submetidos à sobrecarga pressora aguda.

2. OBJETIVOS

Objetivos Específicos- Capítulo 1

- Padronizar a técnica de isolamento de cardiomiócitos ventriculares, obtidos de ratos neonatos. para utilização em estudos envolvendo sinalização intracelular;

- Padronizar a duração e amplitude de estiramento dos cardiomiócitos ventriculares obtidos de ratos neonatos, para estudos de ativação da via de sinalização envolvida com a Fak;

- Investigar a ativação do complexo Fak/Src e sua influência na ativação do fator atrial natriurético (ANF), em resposta ao estiramento pulsátil, em cultura de miócitos ventriculares de ratos neonatos;

- Avaliar a influência da Angiotensina II na ativação da Fak pelo estímulo mecânico.

Objetivos Específicos- Capítulo 2

- Analisar a contribuição da ativação da Rho GTPase no processo de formação do complexo de adesão focal, via ativação precoce da Fak em cardiomiócitos ventriculares obtidos de ratos neonatos;

- Avaliar a importância dos mecanismos de sinalização mediados pela Fak e RhoA, no efeito de crescimento de cardiomiócitos e alterações na expressão de marcadores moleculares de hipertrofia, induzido pelo estiramento mecânico em cardiomiócitos ventriculares obtidos de ratos neonatos;

- Avaliar a importância da ativação da Rho-GTPase na polimerização do citoesqueleto de actina e sua relação com a ativação da via Fak/MAPK, induzida

pelo estiramento pulsátil de cardiomiócitos ventriculares obtidos de ratos neonatos.

Objetivos Específicos- Capítulo 3

- Analisar o envolvimento da Rho GTPase e do fator trocador de nucleotídeo Dbl-3 na ativação precoce da p160ROCK, em miocárdio de ratos submetidos a sobrecarga pressora aguda;

- Examinar a atividade, expressão e localização celular da proteína citoplasmática p160ROCK em miocárdio de ratos submetidos a sobrecarga pressora aguda.

3. CAPÍTULO 1

"Focal adhesion kinase is activated and mediates the early hypertrophic response to stretch in cardiac myocytes."

Circulation Research, 93(2):140-147, 2003

Focal Adhesion Kinase Is Activated and Mediates the Early Hypertrophic Response to Stretch in Cardiac Myocytes

Adriana S. Torsoni,* Sabata S. Constancio,* Wilson Nadruz, Jr, Steven K. Hanks, Kleber G. Franchini

Abstract—Previously we reported that the rapid activation of the Fak/Src multicomponent signaling complex mediates load-induced activation of growth and survival signaling pathways in adult rat heart. In this study, we report that 5% to 20% (10-minute) cyclic stretch (1 Hz) of neonatal rat ventricular myocytes (NRVMs) was paralleled by increases of Fak phosphorylation at Tyr-397 (from 1.5- to 2.8-fold), as detected by anti-Fak-pY^{397} phosphospecific antibody. Moreover, 15% cyclic stretch lasting from 10 to 120 minutes increased Fak phosphorylation at Tyr-397 by 2.5- to 3.5-fold. This activation was accompanied by a dramatic change in Fak localization in NRVMs from densely concentrated in the perinuclear regions in nonstretched cells to aggregates regularly distributed along the myofilaments in stretched cells. Furthermore, a 4-hour cyclic stretch enhanced the activity of an atrial natriuretic factor (ANF) promoter-luciferase reporter gene by 2.7-fold. Disrupting endogenous Fak/Src signaling either by expression of a dominant-negative Fak mutant with phenylalanine substituted for Tyr-397 or by treatment with a c-Src pharmacological inhibitor (PP-2) markedly attenuated stretch-induced Fak activation and clustering at myofilaments and inhibited stretch-induced ANF gene activation. On the other hand, overexpression of wild-type Fak potentiated the stretch-induced Fak phosphorylation but did not enhance either baseline or stretch-induced ANF promoter-luciferase reporter gene activity compared with the responses of nontransfected NRVMs. These findings identify Fak as an important element in the early responses induced by stretch in cardiac myocytes, indicating that it may coordinate the cellular signaling machinery that controls gene expression program associated with load-induced cardiac myocyte hypertrophy. **(*Circ Res*. 2003;93:140-147.)**

Key Words: focal adhesion kinase ■ mechanotransduction ■ cell signaling ■ hypertrophy

Mechanical overload is both cause and consequence of most heart diseases.[1] Cardiac myocytes respond to increased mechanical load by hypertrophic growth, but mechanical stress is also an important stimulus for triggering the initial steps toward cardiac myocyte degeneration and death, which play a critical role in the maladaptive myocardial remodeling and heart failure.[1,2] A major goal in this field is to decipher the mechanisms that link biomechanical forces to the activation of signaling pathways that mediate the hypertrophic as well as maladaptive responses of cardiac myocytes to mechanical stress.

The mechanistic pathways that link mechanical stimuli to biochemical signals in cardiac myocytes are presently unclear, but a growing body of evidence indicates that costameres (complex structures constituted by integrins and cytoskeletal proteins at the junction of sarcolemma and Z-discs) have a critical role in sensing and transducing mechanical stress into biochemical signals that coordinate growth responses to hypertrophic stimuli in both cardiac and skeletal muscle.[3–7] The prominent location of integrins at the junction of extracellular matrix to Z discs makes them candidates for acting as biomechanical sensors in cardiac myocytes. Accordingly, overexpression of β_1-integrins in cardiac myocytes induces hypertrophic gene expression, whereas disruption of integrin function by conditional Cre-loxP gene targeting in adult mice results in intolerance to hemodynamic overload and abnormal cardiac function.[8,9] In addition, Fak, a primary integrin effector that is known to play a key role in the responses of cells to mechanical stimuli through focal adhesion sites,[10–12] is rapidly activated by mechanical stimuli in cultured neonatal rat ventricular myocytes (NRVMs)[13,14] and in overloaded myocardium of adult animals.[15–18] Data obtained in NRVMs have shown that Fak is also involved in the regulation of early gene transcription in response to hypertrophic agonists,[19–22] indicating that this kinase may function as a node for the convergence of multiple signaling pathways involved in the hypertrophic growth of cardiac myocytes. However, a clear demonstration that Fak plays a role in the control of early gene expression in response to mechanical stimuli in cardiac myocytes is still lacking.

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Thus, in this study we investigated the activation of Fak/Src complex by cyclic stretch and its influence on the early activation of atrial natriuretic factor (ANF) promoter reporter gene (ANF-LUC) in response to cyclic stretch in cultured neonatal rat ventricular myocytes. By disrupting Fak/Src signaling, either through expression of a dominant-negative Fak mutant (Phe-397) or through the inhibition of Src activity with the pharmacological inhibitor PP-2, we showed that Fak plays an important role in the stretch-induced ANF transcriptional activation in cardiac myocytes.

Materials and Methods

Detailed methods are described in the online data supplement and in published studies, as referenced.

Cell Culture

Primary cultures of NRVMs (1- to 2-day-old Wistar rats) were prepared as previously reported.[8] Briefly, the myocytes were purified on a discontinuous Percoll gradient, suspended in a plating media containing 10% horse serum, 5% fetal serum, and 0.5% penicillin/streptomycin, and plated in type I collagen Bioflex plates (Flexcell International Corp) coated with gelatin at 500 000/well. After 24 hours, the medium was replaced with serum-free DMEM and incubated for 24 to 48 hours under 95% air plus 5% CO_2 before being used for study.

Cell Stretching

NRVMs cultured in Bioflex plates were stretched in a Flexercell FX-3000 strain unit to 115% of resting length at a frequency of 1 Hz (0.5-s stretch/0.5-s relaxation) for variable periods, depending on the protocol. Control nonstretched NRVMs were also cultured in Bioflex plates and incubated in serum-free medium. At the conclusion of the experimental protocol, cells were either scraped from membranes and lysed for immunoblot analysis or fixed for confocal immunofluorescence analysis.

Plasmid Transfection and Dual Reporter Gene Assays

Constructions of murine Fak pRc/CMV-FAKwt (wild-type [WT-Fak]) and pRc/CMV-FAKF397 (mutant [MT-Fak]) were described previously.[23] WT-Fak and MT-Fak constructs were subcloned into pRc/CMV cytomegalovirus promoter-driven eukaryotic expression vector containing c-myc epitope tag (Invitrogen). Rat ANF promoter luciferase reporter gene (NP328, 700 bp of ANF flanking sequences containing luciferase reporter gene into pXP2) was obtained from Dr Mona Nemer (Institut de Recherches Cliniques de Montréal, Canada). NRVMs were cotransfected with 2 μg of ANF-LUC, 0.1 μg of the internal control SV40-renilla luciferase, and 2 μg of WT-Fak, MT-Fak, or empty plasmid, and 48 hours after transfection they were stretched for 4 hours. All *firefly* luciferase values were normalized to *renilla* activities.

Subcellular Fractionation

This procedure was performed as previously reported.[28] Briefly, NRVMs were homogenized in lysis buffer and centrifuged (100 000*g*, 1 hour). The supernatant (S) fraction was concentrated to 10% of original volume. The particulate (P) fraction was resuspended in a buffer with 1% Triton X-100 and 0.1% SDS and recentrifuged (10 000*g*, 20 minutes). S and P fractions were separated by SDS-PAGE.

Immunoblotting

NRVMs homogenated in lysis buffer were resolved on SDS-PAGE and transferred to nitrocellulose membranes. For immunoprecipitation, normalized samples were incubated with anti-c-myc monoclonal antibody and collected after addition of 25 μL of protein G-Sepharose beads. The membranes were incubated with primary antibodies (anti-Fak, anti-Fak-pY^{397}, anti-Fak-pY^{577}, anti-c-Src, or anti-c-Src-pY^{418}). Band intensities were quantified by optical densitometry of the developed autoradiographs.

Laser Confocal Analysis

NRVMs were fixed with 4% paraformaldehyde/sucrose and incubated with anti-Fak primary antibody. This was followed by incubation with biotin-conjugated secondary anti-rabbit antibody and then with streptavidin-Cy2 and rhodamine-conjugated phalloidin. Images were obtained with laser confocal microscope (Zeiss LSM510).

Statistical Analysis

Data are presented as mean±SEM. Differences between the mean values of the densitometric or luciferase readings were tested by ANOVA and Bonferroni multiple-range test. $P<0.05$ indicated statistical significance.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Stretch-Induced Fak Activation in NRVMs

The tyrosine residue Tyr-397 has been shown to be phosphorylated via an autophosphorylation process, being critical for FAK activation.[23–25] Phosphorylated Tyr-397 then recruits Src family kinases, which lead to an increase in Fak enzymatic activity. We examined the effects of the amplitude and duration of cyclic stretch (1 Hz) on Fak activity by Western blotting NRVM extracts with phosphospecific antibody directed against the autophosphorylation site of Fak [anti-Fak-Tyr(P)-397 antibody (anti-Fak-pY^{397})]. As shown in Figure 1A, 5% cyclic stretch enhanced the phosphorylation of Fak at Tyr-397 to ≈150%. Additional increases were seen in NRVMs subjected to 10%, 15%, and 20% stretch (to ≈210%, 230%, and 280%, respectively). The time course of Fak phosphorylation at Tyr 397 was examined in NRVMs subjected to 15% cyclic stretch up to 120 minutes (Figure 1B). The amount of Fak detected with the anti-Fak-pY^{397} antibody increased 2.5-fold within 10 minutes to up to 3.5-fold within 120 minutes of cyclic stretch compared with nonstretched cells. No change was observed in the amount of protein detected with anti-Fak antibody in both stimulus intensity and time course experiments.

The subcellular distribution of Fak in nonstretched and stretched NRVMs was first examined in soluble (S) and particulate (P) fractions obtained with differential centrifugation of NRVMs extracts. As shown in Figure 2A, in nonstretched cells, most of Fak was present in the S fraction, whereas stretch resulted in a marked reduction of Fak in S fraction and an increase in P fraction.

Fak distribution in NRVMs was also evaluated by confocal microscopy in cells doubled-stained with anti-Fak/streptavidin-Cy2 and rhodamine-conjugated phalloidin. In nonstretched NRVMs, Fak was densely concentrated in the perinuclear regions, but less markedly at the cellular periphery, where it was diffusely distributed (Figures 2B and 2C). We used rhodamine-conjugated phalloidin, which labels sarcomeric actin, to define the precise localization of Fak. NRVMs staining with this procedure revealed the typical sarcomeric pattern of repetitive striations, with the labeled structure representing the actin

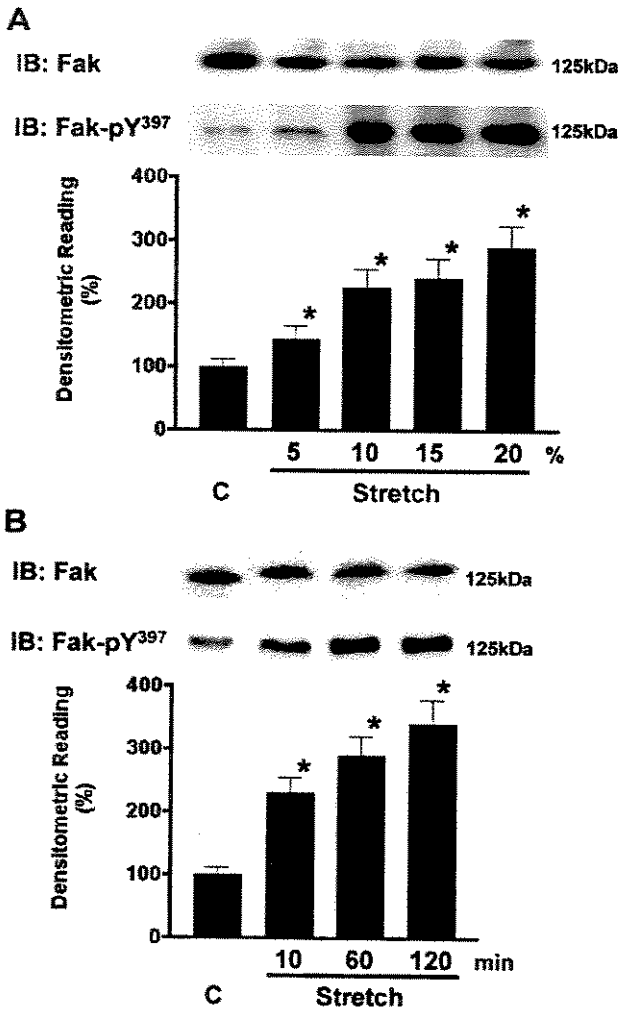

Figure 1. Stretch-induced Fak activation. A, NRVMs were stretched (5% to 20%) for 10 minutes and cell extracts blotted with antibodies against Fak (anti-Fak) or phosphospecific antibody directed against the autophosphorylation site of Fak (anti-Fak^{p397}). Graphic shows average values (6 experiments) of the percent changes in the amount of Fak detected with anti-Fak^{p397} in stretched cells compared with control (C, nonstretched) values, quantified with scanning densitometry. B, NRVMs were stretched at 15% for periods ranging from 10 to 120 minutes. Graphic shows average values of 5 experiments. *$P<0.05$ compared with nonstretched cells.

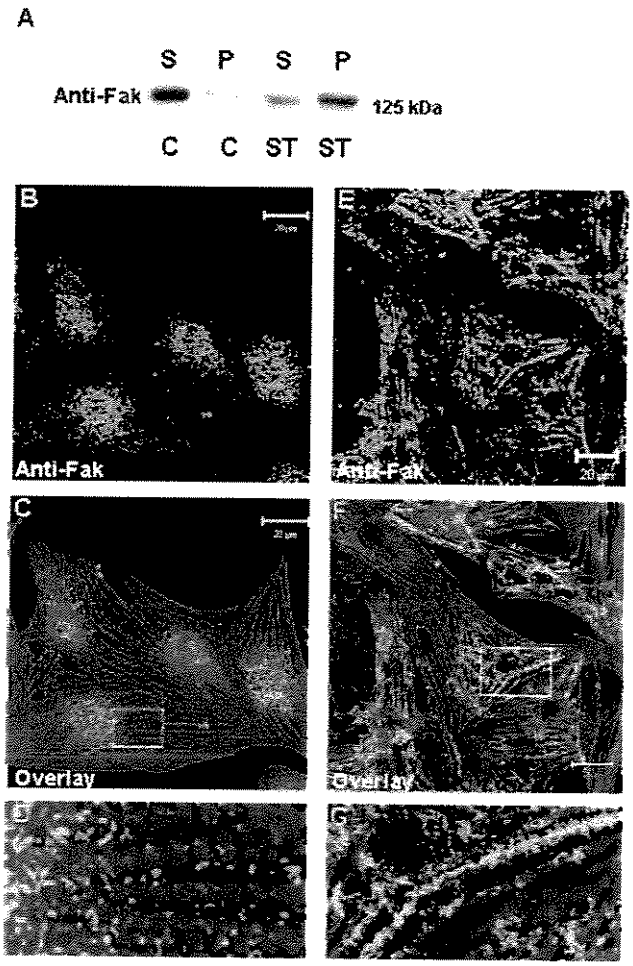

Figure 2. Stretch-induced Fak redistribution in NRVMs. A, Representative blot of soluble (S) and particulate (P) fractions of nonstretched (c) and stretched (ST) NRVMs probed with anti-Fak antibody. B through G, Nonstretched (B through D) and stretched (E through G) NRVMs were fixed, double-labeled with TRITC-conjugated anti-Fak antibody and rhodamine-conjugated phalloidin (actin filaments labeling), and viewed under a laser confocal microscope. In nonstretched cells, Fak (A, green) was concentrated at the perinuclear region and much less at cell periphery as seen in detail at higher magnification (D). After 30 minutes of cyclic stretch (15%), Fak aggregates were seen decorating myofilaments (F and G). Areas of Fak/phalloidin colocalization appear as yellow.

array of two adjacent sarcomeres, where presumably Z discs and costameres are located. At higher magnification (Figure 2D), it was possible to see that at cell periphery, although Fak was detected close to myofilaments, it was not possible to define any distribution pattern or a consistent superimposition to myofilaments. In 30-minute stretched cells, anti-Fak staining was clearly reduced at perinuclear regions and increased at the cell periphery, where it distributed regularly along the myofilaments (Figures 2E and 2F). At higher magnification (Figure 2G), Fak-specific staining was seen as clusters overlapping the regions stained with phalloidin, consistent with the localization of costameres.

Angiotensin II–Induced Fak Activation in NRVMs

FAK has been shown to be activated in response to GPCR agonists, including angiotensin II (Ang II).[27] Furthermore, Ang II may act as autocrine/paracrine mediator of stretch-induced cardiomyocyte hypertrophy.[5] Thus, we tested the possibility that cyclic stretch induces Fak activation via an Ang II–mediated mechanism. As shown in Figure 3A, Ang II induced a concentration-dependent increase of FAK phosphorylation at Tyr-397 in NRVMs. This effect was completely inhibited by addition to the medium of the AT1-specific receptor antagonist (DUP-753; 10 μmol/L, 1-hour preincubation), indicating that Ang II–induced FAK phosphorylation occurred via AT1 receptor–dependent signaling (Figure 3B). As shown in Figure 3B, cyclic stretch still activated Fak in NRVMs treated with the AT1-antagonist

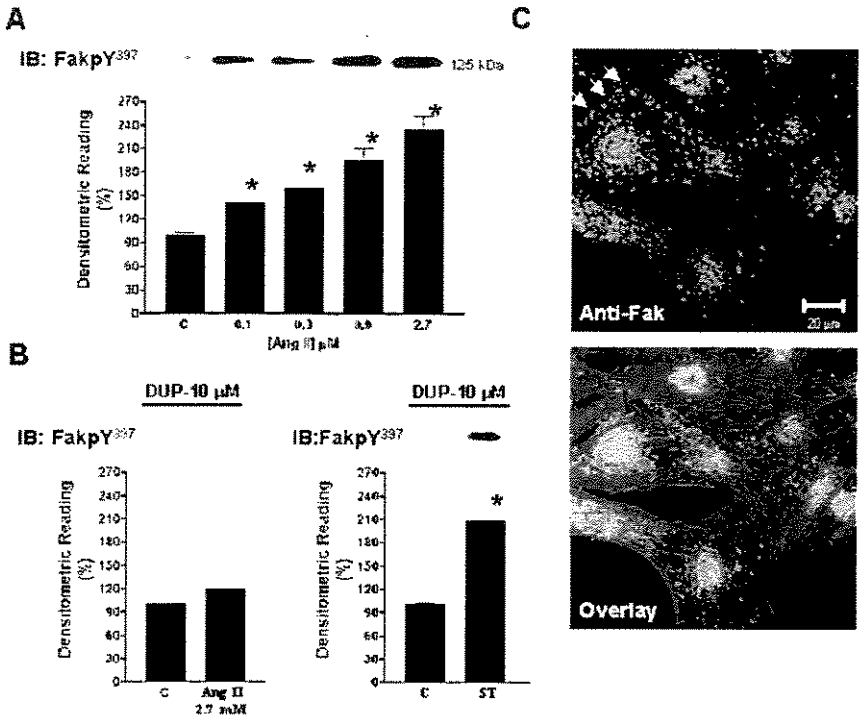

Figure 3. Effects of Ang II treatment on Fak activity in NRVMs. A, Representative blot and graphic showing the average values (4 experiments) of the percent changes in the amount of Fak detected with anti-Fak^{p397} in Ang II–treated cells compared with control (c) values, quantified with scanning densitometry. B, Representative blots probed with Fak anti-Fak^{p397} and average values of densitometric readings of Ang II+DUP-753–treated (2.7 μmol/L and 10 μmol/L, respectively) and stretched (15%, 30 minutes) NRVMs. C, NRVMs treated with Ang II, double-labeled with TRITC-conjugated anti-Fak antibody (arrows) and rhodamine-conjugated phalloidin (actin filaments labeling), and viewed under a laser confocal microscope. *$P<0.05$ compared with untreated cells.

DUP-753, indicating that the stretch and Ang II activate Fak by distinct mechanisms.

The effect of Ang II on Fak localization in NRVMs was then evaluated by confocal microscopy of cells double-stained with anti-Fak/streptavidin-Cy2 and rhodamine-conjugated phalloidin. Distinct from cyclic stretch that induced Fak to aggregate at myofilaments, treatment with Ang II did not change the distribution of Fak in NRVMs, except for the augmentation of spot areas around the edge of the cells, consistent with the localization of focal adhesions (Figure 3C, arrows).

Effects of WT-Fak and MT-Fak on Stretch-Induced Expression of ANF-LUC Reporter Gene

To test the role of Fak on the stretch-mediated NRVM hypertrophic response in vitro, we transiently cotransfected NRVMs with Myc-tagged WT-Fak or MT-Fak constructs driven by cytomegalovirus promoter and ANF-LUC/SV40-*Renilla* reporter genes. Because Fak constructions were Myc-tagged, we initially tested the ability of NRVMs to express WT-Fak or MT-Fak by immunoprecipitation using anti-c-Myc monoclonal and anti-Fak antibodies. As shown in Figure 4A, a considerable amount of Myc-tagged Fak was detected in the immunoprecipitates of both WT-Fak–transfected and MT-Fak–transfected NRVMs but not in nontransfected cells. Furthermore, Fak expression was estimated in whole-cell lysates by immunoblotting with anti-Fak antibody. Figure 4B shows that transfection with 2 μg total DNA of WT-Fak or MT-Fak plasmids (48 hours) enhanced the amount of Fak detected by anti-Fak antibody by ≈14-fold compared with the amount of Fak detected in control nontransfected NRVMs. Transfection with WT-Fak and MT-Fak did not change the amount of Fak detected with anti-Fak-pY^{397} antibody in nonstretched NRVMs compared with nontransfected cells (Figure 4C). Nevertheless, transfection with WT-Fak potentiated stretch-induced Fak activation in NRVM, as demonstrated by a marked increase (to 14-fold) in the amount of Fak detected by anti-Fak-pY^{397} after 30 minutes of cyclic stretch. In contrast, NRVM transfection with MT-Fak abolished the stretch-induced Fak activation.

To address the effects of transient transfection with WT-Fak and MT-Fak on cell viability as well as in Fak protein distribution, NRVMs were double-stained with anti-c-Myc antibody/streptavidin-Cy2 and rhodamine-conjugated phalloidin and analyzed by confocal microscopy. In nontransfected and nonstretched NRVMs (Figures 5A and 5B), the anti-c-Myc monoclonal antibody detected only a small amount of c-Myc restricted to perinuclear areas. Cyclic stretch slightly increased the amount of c-Myc in these cells restricted to perinuclear areas (Figures 5C and 5D). Cells transfected with WT-Fak (Figures 5E through 5H) or MT-Fak (Figures 5I through 5L) constructs (48 hours) showed morphological characteristics comparable to those of nontransfected cells. Marked increases were seen in the anti-c-Myc staining of these compared with nontransfected cells. In nonstretched cells, anti-c-Myc staining was observed throughout the cells, although it was densely concentrated in the perinuclear areas. In stretched WT-Fak–transfected cells, anti-c-Myc staining was associate with myofilaments, as was the staining with anti-Fak antibody of stretched nontransfected NRVMs (Figures 5G and 5H), but the perinuclear regions still showed a highly intense anti-c-Myc staining. However, in stretched MT-Fak–transfected cells, the distribution of anti-c-Myc staining remained similar to that of nonstretched cells (Figures 5K and 5L) without a clear association with the myofilaments.

We next assessed the effect of cyclic stretch on the activity of transiently transfected ANF-LUC. As shown in Figure 6, a

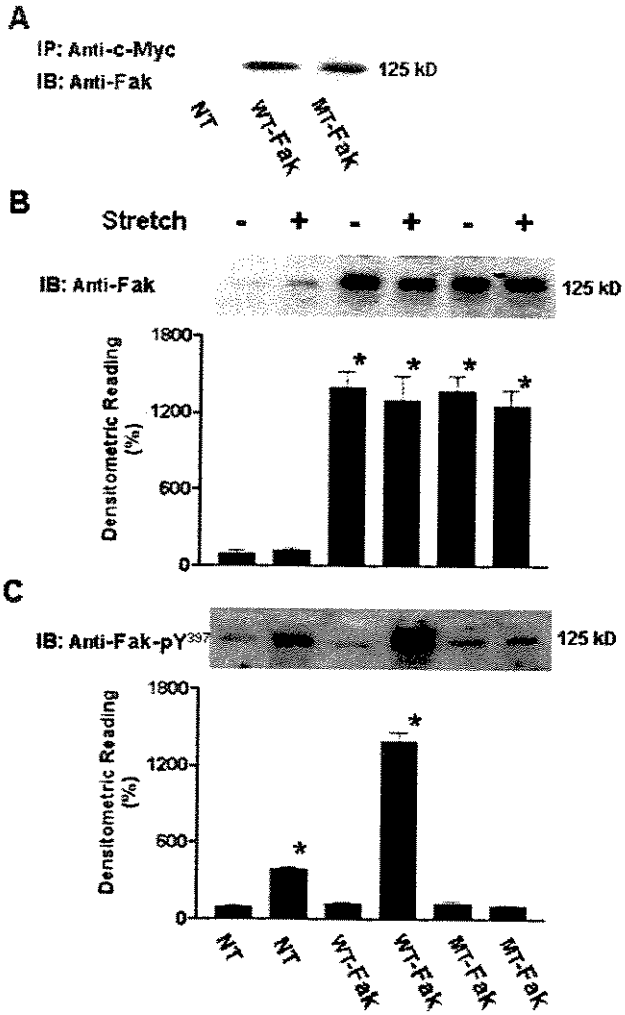

Figure 4. Effects of WT-Fak or MT-Fak overexpression on stretch-induced Fak activation. NRVMs were transfected with WT-Fak or MT-Fak (2 μg DNA, 48 hours) and then stretched (15%). The ability of NRVMs to express WT-Fak and MT-Fak was initially tested by immunoprecipitation performed with anti-c-Myc antibody and blotted with anti-Fak antibody (A, representative example of 3 experiments). To estimate the amount of WT-Fak and MT-Fak expressed in transfected NRVMs, cell extracts were blotted with anti-Fak antibody and the values compared with blots of nontransfected cell extracts. B, Representative Western blots of nontransfected, WT-Fak–transfected, and MT-Fak–transfected cells probed with anti-Fak antibody (average values from 6 experiments). C, Representative examples and the average values (6 experiments) of anti-Fak-pY^{397} Western blotting experiments performed with extracts obtained from nontransfected, WT-Fak–transfected, and MT-Fak–transfected NRVMs stretched or nonstretched. Data are mean±SEM. *P<0.05 vs its corresponding nonstretched control.

4-hour stretching protocol increased the normalized luciferase activity of cotransfected NRVMs by 2.7-fold. Overexpression of WT-Fak did not significantly increase the baseline ANF-LUC expression compared with values of nonstretched cells. Likewise, WT-Fak overexpression did not enhance the ANF-LUC activity induced by cyclic stretch compared with nontransfected cells. MT-Fak overexpression did not change the baseline but reduced significantly the

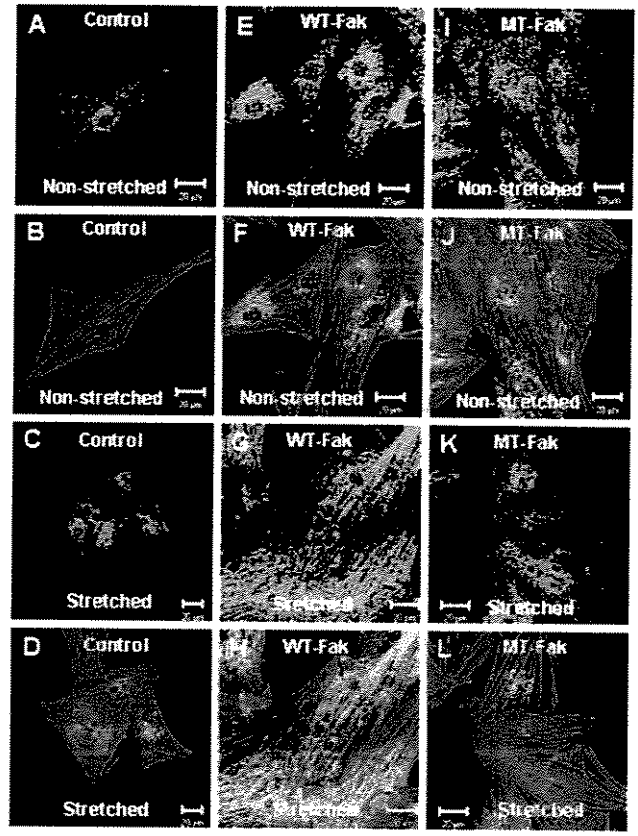

Figure 5. Effects of WT-Fak and MT-Fak overexpression on Fak distribution in NRVMs. Nonstretched and stretched NRVMs nontransfected and transfected with WT-Fak or MT-Fak were fixed, double-labeled with TRITC-conjugated anti-Myc antibody and rhodamine-conjugated phalloidin, and viewed under a laser confocal microscope. In nontransfected and nonstretched NRVMs (A and B), the anti-c-Myc monoclonal antibody detected a small amount of c-Myc (green) restricted to perinuclear areas. Cyclic stretch increased the anti-c-Myc staining, which remained most restricted to perinuclear areas (C and D). In nonstretched WT-Fak (E and F) and MT-Fak (I and J), transfected cell anti-c-Myc staining was intense and distributed over the cells. In WT-Fak–transfected cells, cyclic stretch (G and H) caused an increase in anti-c-Myc staining associated with myofilaments, whereas no clear aggregation of Fak was seen at myofilaments in stretched MT-Fak–transfected cells (K and L).

stretch-induced increase in ANF expression in NRVMs compared with the responses of nontransfected cells.

Effects of PP2 on Stretch-Induced Fak and ANF-LUC Activation

Fak activation in response to cell adhesion is dependent on a reciprocal catalytic activation of Fak and Src family kinases.[23–25,29] Autophosphorylation of Fak Tyr-397 creates a high-affinity binding site for the SH2 domain of Src, and Src associated with Fak promotes maximal Fak catalytic activity by phosphorylating Fak Tyr-576 and Tyr-577 located in the kinase catalytic domain. Accordingly, treatment with the selective Src family kinase inhibitor PP-2 has been shown to inhibit the phosphorylation of Tyr-397 and Fak activation induced by integrin engagement.[26,27] To additionally evaluate the role of stretch-induced activation of the Fak/Src complex on the early regulation of ANF-LUC activity in NRVMs, we

pretreated cells with PP-2. NRVM stretch was accompanied by c-Src activation, as indicated by the increase in the amount of c-Src detected with the phosphospecific antibody directed against c-Src Tyr-418 (Figure 7A). Cyclic stretch also increased Fak phosphorylation at Tyr-576 and Tyr-577 in addition to Fak phosphorylation at Tyr-397. Treatment with PP-2 (1 μmol/L) inhibited stretch-induced c-Src and Fak activation, as indicated by the reduction of c-Src-pY^{418}, Fak-pY^{397}, and Fak-pY^{577}, respectively, additionally suggesting that stretch-induced Fak activation is dependent on integrin engagement and c-Src activation.

As shown in the representative examples of Figure 7B, despite the marked inhibition of stretch-induced Fak, treatment with PP-2 for 4 hours did not alter the morphology or viability of NRVM. In addition, image analysis indicated that PP-2 treatment did not change the distribution of Fak in nonstretched cells, because it remained densely concentrated in the perinuclear regions and diffusely distributed at the cell periphery. However, in contrast to what was seen in untreated stretched NRVMs, treatment with PP-2 was not accompanied by a consistent aggregation of Fak at myofilaments.

Experiments performed with ANF-LUC–transfected NRVMs showed that PP-2 treatment markedly attenuated the stretch-induced ANF-LUC activation (Figure 7C). In untreated cells, cyclic stretch increased ANF-LUC expression by 2.7-fold, whereas in PP-2–treated cells, stretch increased ANF-LUC activity by only 1.3-fold.

Discussion

The present study demonstrates that Fak activation plays an important role in the early upregulation of ANF transcription induced by mechanical stress in cardiac myocytes, indicating that its activation by mechanical stress is not simply involved in the initiation of biochemical signals but also that it coordinates cellular signaling machinery that controls gene expression associated with load-induced cardiac myocyte hypertrophy.

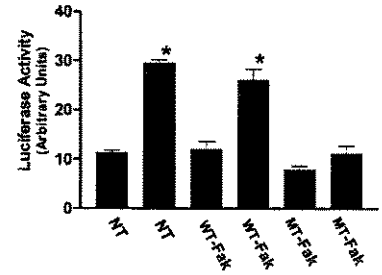

Figure 6. Overexpression of WT-Fak or MT-Fak and stretch-induced ANF regulation in NRVMs. Graphic representing the average values of *firefly* luciferase activity normalized to *renilla* luciferase activity present in samples of nonstretched and stretched NRVMs nontransfected or transfected with WT-Fak or MT-Fak. *$P<0.05$ compared with values of nontransfected and nonstretched NRVMs.

Stretch-Induced Fak Activation in NRVMs

Fak was found to be rapidly activated in NRVMs by cyclic stretch, as reported previously.[13,14] This activation was shown to be roughly parallel to the amplitude and duration of the stretch. In addition, we have shown here that stretch induced Fak to migrate from soluble to particulate fraction of NRVMs extracts. Additional analysis with confocal microscopy demonstrated that stretch induced Fak to aggregate at myofilaments in a distribution pattern that follows that of costamere sites. Simultaneous with the aggregation at myofilaments, there was a marked reduction of specific Fak staining in the perinuclear regions with no change in the amount of Fak detected by Western blot, supporting the conclusion that cyclic stretch induces Fak to translocate and cluster at costameres sites of NRVMs. These results indicate that activation of Fak by stretch is dependent on its proper

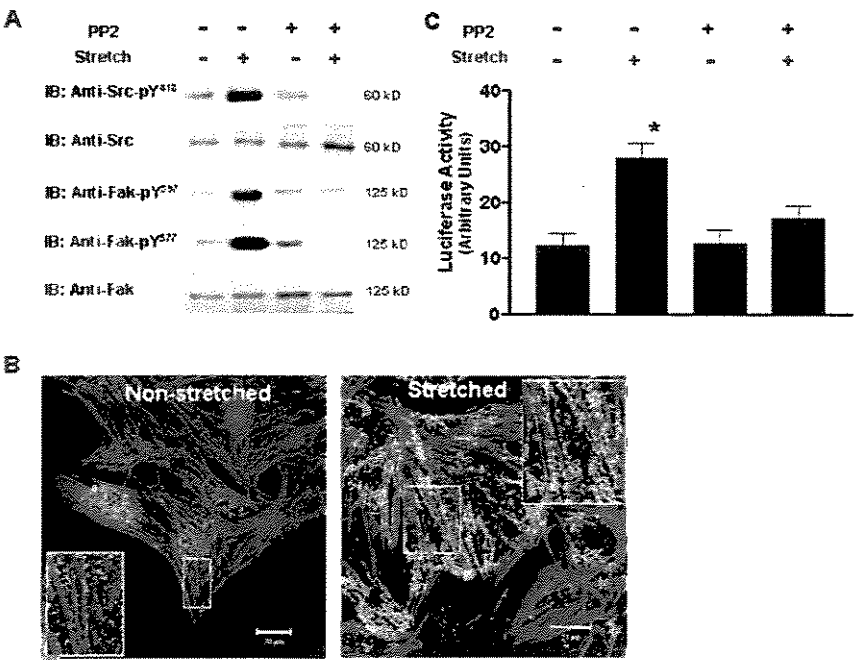

Figure 7. Effect of treatment with PP-2 on Fak activity and stretch-induced ANF-LUC activation in NRVMs. A, Effect of PP-2 treatment (1 μmol/L) on stretch-induced c-Src (phosphorylation of Tyr-418) and Fak activation (phosphorylation of Tyr-397 and Tyr-577). B, Nonstretched and stretched NRVMs treated with PP-2 double-labeled with TRITC-conjugated anti-Fak antibody and rhodamine-conjugated phalloidin. C, Graphic representing the average values of *firefly* luciferase activity normalized to *renilla* luciferase activity present in samples of nonstretched and stretched NRVMs treated or not with PP-2. $P<0.05$ compared with values of nonstretched and untreated cells.

location at the costameres, where local mechanical forces are transduced into biochemical signals. This is consistent with our previous demonstration that mechanical stress induces a translocation of Fak to the actin cytoskeletal compartment in rat myocardium.[15] Moreover, this generally agrees with data from recent studies that demonstrated that displacement of Fak from focal adhesion sites by overexpressed FRNK (FAK-related non-kinase) in cultured NRVMs impairs Fak activation.[19,20,28] However, in contrast to our results, in those studies Fak was found most consistently in classical focal adhesion sites at the edges of cultured myocytes, where it increased in response to agonist treatment. This suggests that displacement of Fak by FRNK might occur preferentially at these sites instead of costameres. The reason for such differences might be related to differences in the nature of stimulus (ie, mechanical versus chemical). This idea is supported by the results of our experiments performed with Ang II. Although Ang II activates Fak in NRVMs, it does not induce the migration of Fak to myofilaments. Instead, Ang II enhanced Fak aggregation at classical focal adhesion sites, indicating that Ang II and stretch activate Fak by distinct mechanisms and sites of NRVMs. This is additionally supported by our demonstration here that AT1 receptor antagonist did not impair the stretch-induced Fak activation although it is able to blockade the Ang II–induced Fak activation. Moreover, this generally agrees with the previous demonstration[27] that signaling events leading to Fak activation by GPCR agonists are Src independent whereas those stimulated by integrin receptors require Src activation.

Experiments designed to disrupt stretch-induced activation of FAK/Src signaling, either by the dominant-negative Fak overexpression or pharmacological inhibition of Src, abolished the stretch-induced Fak aggregation at NRVM myofilaments. Because both strategies disrupted Fak signaling by affecting Fak autophosphorylation at Tyr-397, our results indicate that stretch-induced Fak clustering at myofilaments is dependent on autophosphorylation of Tyr-397. Similarly, a critical role of Tyr-397 autophosphorylation to Fak activation and clustering also has been shown at focal adhesion sites of noncardiac cells.[29,30] The importance of Tyr-397 to Fak translocation and clustering probably relies on the fact that autophosphorylation of Tyr-397 is responsible for recruiting and activating Src family kinases, which in turn additionally enhance Fak activity by transphosphorylating and recruiting additional Fak molecules to specific sites.[23–25] Thus, the impairment of Tyr-397 autophosphorylation would prevent Fak/Src clustering at costameres in cardiac myocytes.

In this regard, one might expect that Fak clustering at costameres optimizes the stretch-induced Fak signaling in cardiac myocytes, not only because of the location at strategic sites that convey mechanical stimuli but also because the molecular proximity in clusters may serve to enhance and sustain Fak signaling. Accordingly, recent studies by Katz et al[31] in fibroblasts have shown that Fak clustering enhances and sustains Fak activation, allowing the recruitment and activation of additional cellular signaling pathways such as those involved in the activation of growth and survival pathways. Additional studies are required to dissect the potential role of stretch-induced Fak clustering at costameres of cardiac myocytes to the long-term effect of mechanical stress in cardiac myocytes.

Control of Gene Expression by Stretch-Induced Fak Activation

The impairment of Fak activation markedly attenuated ANF promoter activity induced by stretch in NRVMs, indicating that Fak plays a central role to signaling events involved in the early regulation of gene expression in response to mechanical stress in cardiac myocytes. These results extend to mechanical stimuli previous demonstrations that Fak is required for early gene upregulation in response to hypertrophic agonists in cultured NRVMs.[19,20,22] Moreover, the demonstration here that stretch-induced Fak activation in cultured NRVMs occurs at costameres indicates that this is a specific phenomenon directly related to the mechanical forces imposed by stretch on the costameres and also that this phenomenon might be present in cardiac myocytes in intact myocardium.

A role for Fak in cell growth mediated by integrin signaling has been demonstrated in many cell systems.[32,33] Early work[34] showed that Fak/Src complex activates putative downstream signaling pathways, leading to activation of phosphoinositide 3-kinase, protein kinase C, ERK, Jun N-terminal kinase, and p38 mitogen-activated protein kinase pathways. Although the downstream mediators of the regulation of ANF expression induced by Fak were not explored in the present study, previous evidence indicates that the Fak/Src complex may activate extracellular signal-regulated kinase (ERK) 1 and ERK2 in NRVMs and in rat myocardium,[15–17,20] which potentially may mediate the early regulation of ANF expression. Accordingly, the early activation of ERK1/2 has been suggested to contribute to the reexpression of fetal ventricle genes.[35] Transfection of constitutively active MEK1 (immediate upstream activator of ERK1/2) has been shown to augment ANF promoter activity in cultured cardiomyocytes, whereas a dominant-negative MEK1 construct attenuated its activity.[36] However, the present results do not exclude the possibility that multiple downstream effectors are involved in the influence of Fak on early gene regulation in response to mechanical stress. Thus, additional studies are necessary to clarify the relative importance of the various candidate signaling molecules to Fak influence on early gene regulation in response to mechanical stress.

Interestingly, WT-FAK overexpression in NRVMs did not significantly activate ANF gene expression in nonstretched cells, nor did it enhance stretch-induced ANF-LUC activity despite the fact that it substantially increased the amount of activated Fak in response to stretch in NRVMs. These results might indicate that WT-Fak overexpression alone was not sufficient to activate baseline nor to potentiate the stretch-induced ANF transcription. Alternatively, these results might indicate that Fak-independent mechanisms are responsible for the stretch-induced ANF transcriptional activation. Similarly, previous studies have shown that overexpression of WT-Fak constructs in low-density cultured NRVMs did not enhance Fak activation by endothelin.[19] However, transfection of WT-Fak in high-density cultured NRVMs has been shown to stimulate the transcription of fetal genes associated with the

hypertrophic phenotype.[21,37] The reasons for such discrepancies are not apparent from our results. One may still speculate that under the culture conditions used here, the basal ANF expression was already quite high; thus, despite the potentiation in stretch-induced Fak activation caused by transfection with WT-Fak, ANF transcriptional activity could not increase substantially.

In conclusion, our present results corroborate and extend to isolated cardiac myocytes previous findings in diverse experimental systems that Fak plays a central role as a biomechanical sensor that responds to changes in load in cardiac myocytes. Our data also indicate that signaling by the Fak/Src complex initiated in response to continuous stimulation by mechanical stress coordinates the cellular signaling machinery that controls gene expression program associated with load-induced cardiac myocyte hypertrophy.

Acknowledgments

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4. CAPÍTULO 2

"RhoA/ROCK signaling is critical to Fak activation by cyclic stretch in cardiac myocytes."

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RHOA/ROCK SIGNALING IS CRITICAL TO FAK ACTIVATION BY CYCLIC STRETCH IN CARDIAC MYOCYTES

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Running head: RhoA/ROCK signaling in cardiac myocyte

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ABSTRACT

The activation of FAK signaling has been demonstrated to play a critical role to the coordination of early gene expression in cardiac myocytes in response to mechanical stress. Here, we investigated the regulation of stretch-induced FAK activation in neonatal rat ventricular myocytes (NRVMs) by RhoA/ROCK signaling. Immunoblotting analysis of NRVMs extracts showed a rapid and sustained (up to 2 hours) activation of Fak and Erk½ indicated by increased phosphorylation of Fak at Tyr397 and Erk½ at Thr202/Tyr204. Blockade of RhoA/ROCK signaling by pharmacological inhibitors of RhoA (*C. botulinum* C3-Exoenzyme) and ROCK (Y-27632; 10 μmol/l, 1 h) abolished the stretch-induced Fak and Erk ½ activation. Moreover, stretch-induced Fak/Erk1/2 activation were abolished by treatment of cells with the inhibitor of actin polymerization, cytochalasin D. Transfections of cells with RhoA antisense oligonucleotide not only attenuated the stretch-induced Fak activation but also abolished the stretch-induced expression of β-myosin heavy chain mRNA. Similar inhibition of the stretch-induced expression of β-myosin heavy chain mRNA was observed in cells treated with FAK antisense oligonucleotide. Thus, these findings demonstrate that RhoA/ROCK signaling plays a crucial role in stretch-induced Fak activation, indicating that upstream events operationally linked to actin cytoskeleton may play an important role in the regulation of Fak activity by mechanical stress.

Key Words: Mechanical stress, hypertrophy, cell signaling

INTRODUCTION

Mechanical stress induces functional and structural changes in cardiac myocytes that are implicated both in myocardial hypertrophy and failure (6, 16). Although a number of diverse signaling molecules have been shown to be activated by mechanical stress, the mechanisms by which mechanical forces are sensed and activate such signaling molecules in cardiac myocytes are still largely unknown. One important development in this field is the recognition in recent years that the integrity of structures such as Z-disc, costameres and intercalated discs play an important role in the ability of cardiac cells to appropriately respond to mechanical stress (3, 10, 18, 29, 33). A current hypothesis imply that such structures monitor mechanical forces and communicate strain through molecules which are then able to activate signal transduction pathways that ultimately coordinate the functional and structural changes in response to mechanical stress.

Fak, a tyrosine kinase linked to integrin signaling (13, 21, 34), has been shown to be rapidly activated by mechanical stimuli in cardiac myocytes (2, 4, 8, 11, 20, 26, 31). Several lines of evidence also support a role for Fak in the regulation of early gene transcription in response to hypertrophic agonists and mechanical stress (9, 19, 23, 30, 31), indicating that this kinase may coordinate the convergence of multiple signaling pathways involved in the hypertrophic growth of cardiac myocytes. Although the molecular mechanism responsible for Fak activation by mechanical stress in cardiac myocytes is still elusive, our previous studies have demonstrated that Fak activation by mechanical stress is associated with its clustering and association with myofilaments (31). In addition, in cardiac myocytes as well as in other cell types Fak activation either by agonists or mechanical stress have been shown to be influenced by a cooperative interaction with F-

actin cytoskeleton (14, 32, 35). On the other hand, it has been shown that Fak activation by agonists, such as endothelin, is dependent on RhoA/ROCK signaling and the integrity of F-actin cytoskeleton (14). More recently, studies performed in isolated cardiac myocytes (17) and in left ventricle of adult rats (32) have shown that mechanical stress induce a rapid but transient activation of both RhoA and ROCK, respectively, indicating that RhoA/ROCK signaling may have a function in the early activation of signaling mechanisms induced by mechanical stress in cardiac myocytes. Whether stretch-induced Fak activation in cardiac myocytes is also dependent on the integrity of RhoA/ROCK and F-actin integrity remains unknown.

Thus, to gain insight into the activation of Fak by mechanical stress in cardiac myocytes we tested the hypothesis that RhoA and ROCK signaling pathway plays a role in stretch-induced Fak activation in cardiac myocytes. By disrupting RhoA signaling, either through pharmacological inhibition with C3-Exoenzyme or transfection with RhoA-antisense oligonucleotide or ROCK specific inhibitor Y27632 we showed that RhoA/ROCK signaling is critical to the stretch-induce Fak activation in cardiac myocytes as well as to the regulation of the stretch-induced expression of β-myosin heavy chain in cardiac myocytes.

Material and Methods

Reagents. Bioflex culture plates (Flex I® Collagen Culture Plate # Cat. No 35-P-1001C - Type-I) were from Flexcell International Corp. Protein A conjugated with ^{125}I was purchased from Du Pont –New England Nuclear Co. Dulbecco's modified Eagle's medium (DMEM), horse serum (HS) and fetal bovin serum (FBS) were from Gibco BRL Co.

Pancreatin was from Sigma, colagenase type II from Worthington. Rabbit polyclonal anti-Fak, anti- Erk½ and mouse monoclonal anti-RhoA antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti-pFak-Tyr397 and anti-pErk½ Thr202/Tyr204, were from Biosource International. Cytochalasin D, *C. botulinum* C3-Exoenzyme and Y-27632 ROCK inhibitor and was from Calbiochem. Oligonucleotides were synthesized by Life Technologies.

Neonatal rat ventricular myocytes (NRVMs) culture. Primary cultures of NRVMs (1- to 2-day-old Wistar rats) were prepared as previously reported (31). Briefly, the myocytes were purified on a discontinuous Percoll gradient, suspended in plating medium containing 10% horse serum, 5% fetal serum, and 0.5% penicillin/streptomycin, and plated in type I collagen Bioflex plates (Flexcell International Corp) coated with gelatin at 500000/well. After 24 hours, the medium was replaced with serum-free DMEM and incubated for 24 to 48 hours under 95% air plus 5% CO_2 before being used for study. NRVMs cultured in Bioflex plates were stretched in a Flexercell FX-3000 strain unit to 115% of resting length at a frequency of 1 Hz (0.5-s stretch/0.5-s relaxation) for variable periods, depending on the protocol. Control nonstretched NRVMs were also cultured in Bioflex plates and incubated in serum-free medium. At the conclusion of the experimental protocol, cells were either scraped from membranes and lysed for immunoblot analysis or fixed for confocal immunofluorescence analysis.

Immunoblotting. NRVMs were lysed in assay lysis buffer containing freshly added protease and phosphatase inhibitors (1% Triton, 10mM sodium pyrophosphate, 100mM NaF, 10 μg/mL aprotinin, 1mM phenylmethylsulfonyl fluoride, 0.25mM sodium orthovanadate). The samples were centrifuged for 20 min at 11,000g and the soluble fraction was ressuspended in 50μL of Laemmli loading buffer (2% SDS, 20%glycerol, 0.04mg/mL bromophenol blue, 0.12M Tris-HCl, pH 6.8, 0.28M b-mercaptoethanol) before

separation on 8% SDS-PAGE gels. Proteins were transferred from the gels to a nitrocellulose membrane. Membranes were blocked for 2 hours at room temperature with 5% skim milk/TBST buffer (10mM Tris-HCl, pH 8; 150mM NaCl, 0.05% Tween 20). Membranes were exposed to primary antibodies overnight at 4°C, washed in TBST and exposed to [^{125}I]Protein A.

Oligodeoxynucleotides (ODNs). Fak-antisense ODN was a 16-mer (5'-GATAAGCAGCTGCCAT -3') directed against the initiation of translation site of rat Fak mRNA sequence. Fak sense sequence (5'- CGGCTAACCGAAGTGA -3') was used as control. RhoA-antisense ODN was a 16-mer (5'- TCCTGATGGCAGCCAT -3') directed against the initiation of translation site of rat RhoA mRNA sequence. RhoA sense sequence (5'- GTTAATCTTGCAGGTA -3') was used as control. All bases were phosphorothioate-protected and obtained from Life Technologies. The sequences were confirmed in the GeneBank database for uniqueness. Transfections ODNs in NRVMs were performed as previously described (22). Cells were serum-starved for 6 hours and transfected with 1μmol/L antisense or sense ODN and 12μg lipofectin in serum-starved DMEM without antibiotics (final volume – 1mL) for 6 hours. NRVMs were washed with DMEM and maintained in DMEM containing 10% serum for 18 hours before used in experiments.

RT-PCR analysis. NRVMs were homogenized in Trizol reagent, and total RNA was isolated by precipitation with isopropyl as previously described (22). A 5-μg aliquot of total RNA was used for cDNA synthesis with the Superscript preamplification system (Life Technologies) according to the manufacturer's instructions. cDNA was amplified by PCR using *Taq* DNA polymerase with oligonucleotides derived from the β-MHC gene (5'-

CCAACACCAACCTGTCCAAGTTC-3' and 5'-TGCAAAGGCTCCAGGTCTGAGGGC-3') or β-actin gene (5'-TTCTACAATGAGCTGCGTGTGGCT-3' and 5'-GCTTCTCCTTAATGTCACGCACGA-3'). Oligonucleotides were synthesized by Life Technologies. The amplification conditions consisted of denaturing at 94°C for 2 min, annealing at 45°C (β-actin) and 54°C (β-MHC) for 1 min, and extension at 72°C for 2 min. The number of cycles was 25. PCR products were size fractionated with agarose gel electrophoresis. After being stained with ethidium bromide, the DNA bands were visualized with a UV transilluminator.

Immunohistochemistry and Laser confocal analysis. NRVMs were fixed with 4% paraformaldehyde and 4% sucrose in 0.1 mol/L phosphate-saline buffer, pH 7.4, for 15 min at room temperature. The slides were preincubated in blocking buffer (3% nonfat dry milk on 0.1 mol/L PBS) containing 0.6% Triton X-100 for 45 min at room temperature, followed by overnight incubation with the primary antibody (1:75 in PBS) anti-FAK at 4°C. The slides were extensively rinsed in 0.1 mol/L PBS and incubated with goat anti-rabbit biotin-conjugated secondary antibody (1:250 in 1% skim milk in PBS) for 2 hours at 25°C followed by incubation with streptavidin-Cy2 (1:500 in PBS) and Rhodamine-conjugated Phalloidin (1:500 in PBS). For RhoA staining cells were incubated with monoclonal anti-RhoA antibody followed by incubation with rabbit anti-mouse biotin-conjugated secondary antibody and streptavidin-Cy2 (1:500 in PBS) and Rhodamine-conjugated Phalloidin. Positive immunoreactivity was visualized by laser confocal scanning (Zeiss LM510). In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining.

Statistical Analysis

Data are presented as mean±SEM. Differences between the mean values of the densitometric readings were tested by ANOVA and Bonferroni multiple-range test. $P<0.05$ indicated statistical significance.

RESULTS

Cyclic stretch induces Fak and Erk1/2 activation in NRVM

Cardiac myocytes were extracted from 1-2 day neonatal rats and cultured in silicon plates for 72 hours before they were subjected to cyclic stretch (to 115% at 1Hz) for periods ranging from 10 up to 120 min. As shown in Figure 1 A, B, cyclic stretch in NRVMs induced a rapid (10 min) and sustained (up to 120 min) Fak and Erk ½ activation, as indicated by the increases in the amount of Fak and Erk ½ detected by phosphospecific antibodies against Fak Tyr-397 and Erk ½ (Thr202/Tyr204). No change was found in the amount of Fak, Erk ½ expressed by NRVMs along the experimental period.

Fak and RhoA are co-localized in non-stretched NRVMs

We have previously shown that Fak activation by cyclic stretch is accompanied by its aggregation at cardiac myocytes myofilaments (31). In the present study we extended the observation of Fak localization by studying NRVMs subjected to cyclic stretch up to 120 min. Figure 2 A-F shows the immunolocalization of Fak in non-stretched and stretched (10 and 120 min) NRVMs. In non-stretched cells, Fak was found most concentrated at perinuclear areas and along the myofilaments. NRVMs stained with rhodamine-conjugated

phalloidin revealed the typical sarcomeric pattern of repetitive striations, with the labeled structure representing the actin array of two adjacent sarcomeres. In 30 min-stretched cells, anti-Fak staining was found regularly distributed along the myofilaments (Fig 2 D, E). After 1-2 hours of cyclic stretch anti-Fak antibody staining was most found at cardiac myocytes nuclei (Fig 2 E-F) with weak staining at cytoplasm. Interestingly, in this period, most of Fak staining observed at the cytoplasm was seen as spot areas around the edge of the cells, consistent with the localization of focal adhesions (Fig. 2 E-F).

Figure 3A-C, shows a respresentative example of NRVMs double stained with anti-RhoA antibody and with rhodamine-conjugated phalloidin. The striated pattern of anti-RhoA staining and the alternate localization of the RhoA and actin labeling in the sarcomere indicate a potential localization of RhoA in the A-band. Cyclic stretch did not change the localization of RhoA in NRVMs (Fig 3D-F).

Fak activation by stretch is dependent on RhoA activity

We next examined the effect of C3 exoenzyme on Fak activation induced by mechanical stress in NRVMs. C3 exoenzyme selectively ADP-ribosylates small G proteins of the Rho subfamily at Asn-41, thereby blocking their action. As shown in Figure 4A, although pretreatment with the C3 exoenzyme had no effect on basal Fak activity, it markedly inhibited the stretch-induced Fak activation. Notably, the blockade of Fak activation by cyclic stretch in NRVMs was also accompanied by a reduction in the stretch-induced activation of Erk ½.

Next, we investigated whether ROCK signaling, a RhoA downstream serine kinase involved in the actin cytoskeletal organization, mediates stretch-induced activation of Fak

in NRVMs. The cells were pretreated with the selective ROCK inhibitor Y-27632 (10 μmol/l, 1 h) and then subjected to cyclic stretch for 30 min. Figure 4 B shows that stretch-induced Fak phosphorylation at Tyr-397 was markedly reduced after Y-27632 treatment, but no change was observed in basal Fak phosphorylation or expression. Erk ½ activation was also markedly reduced in stretched myocytes treated with Y-27632.

Because in non-myocyte cells RhoA/ROCK signaling has been implicated in the organization of stress fibers (34), we next examined the influence of cytochalasin D, which induces actin filament disassembly, in the stretch-induced Fak phosphorylation at Tyr-397. As shown in Figure 5, NRVMs pretreated with cytochalasin D exhibited a marked reduction in stretch-induced Fak and Erk ½ activation.

We further examined the role of RhoA proteins in stretch-induced Fak activation by transfecting NRVMs with RhoA antisense. The effectiveness of RhoA oligonucleotide antisense was demonstrated by its ability to specifically reduce RhoA expression in NRVMs. As shown in Figure 6A, immunofluorescence analysis showed that although RhoA oligonucleotide markedly reduced the expression of RhoA in NRVMs it did not interfere with cell viability and morphology. Moreover, as shown in the blots of Figure 6B, RhoA antisense oligonucleotide markedly reduced the expression of RhoA in NRVMs, but it did not affect the expression of Fak or Erk ½ in these cells indicating its specificity. The reduction of RhoA expression by RhoA antisense oligonucleotide markedly reduced the stretch-induced Fak and Erk ½ activation.

RhoA activity influences stretch induced hypertrophic gene activation

To test the role of RhoA and Fak on the stretch-mediated NRVMs hypertrophic response *in vitro*, cells transfected with RhoA or Fak antisense oligonucleotides were analyzed to the expression of β-MHC. As shown in the representative examples of Figure 7, cyclic stretch lasting from 2- to 4-hour period markedly increased (2.7-fold) the amount of β-MHC expressed by NRVMs. Transfection with RhoA or Fak antisense oligonucleotides significantly reduced the stretch-induced expression of β-MHC in NRVMs, while transfection with sense oligonucleotides did not change the stretch-induced expression of β-MHC in NRVMs.

DISCUSSION

We have previously (31) provided data showing that Fak activation by cyclic stretch in NRVMs is critically dependent on Tyr397 phosphorylation and clustering at myofilaments. This implies that re-location at subcellular structures might be important to the role of this kinase in the mechanotransduction and coordination of hypertrophic gene expression in response to mechanical stress. Our present data extended these previous observations to show that the stretch-induced Fak activation in NRVMs is influenced by the small GTPase RhoA signaling. This is supported by our demonstration here that 1) reductions of RhoA signaling in NRVMs, either by the selective Rho inhibitor, C. *botulinum* C3 Exoenzyme, or by the reduction of RhoA expression by oligonucleotide antisense, markedly attenuated stretch-induced Fak phosphorylation at Tyr397; 2) treatment of NRVMs with pharmacological inhibitor of ROCK, a downstream effector of RhoA, attenuated the stretch-induced Fak phosphorylation at Tyr397; and 3) Cytochalasin D, an inhibitor of actin filament polymerization, also reduced the stretch-induced Fak

phosphorylation. In addition, the demonstration here that attenuation of either Fak or RhoA signaling, by specific antisense oligonucleotides, considerably reduced the stretch-induced expression of the β-myosin heavy chain in NRVMs support the idea that RhoA/ROCK and Fak signaling cooperate to regulate the expression of hypertrophic genes in response to mechanical stress in cardiac myocytes.

RhoA/ROCK signaling influences Fak activation by cyclic stretch

Like Fak, RhoA has been demonstrated to be rapidly activated and involved in the up-regulation of hypertrophic genes by mechanical stress in cardiac myocytes (1, 17, 31). Our present data indicate that RhoA/ROCK signaling is upstream to stretch-induced Fak activation in NRVMs. This is in agreement with data from non-myocyte cells indicating that RhoA activation increases, while its inhibition attenuates Fak phosphorylation (7, 37). This also agrees with previous data from cardiac myocytes indicating that Fak mediates the effects of RhoA signaling triggered by constitutively active RhoA or by the activation of G-protein coupled receptors (14, 35). Furthermore, our demonstration here that Cytochalasin D treatment attenuated the stretch-induced Fak phosphorylation at Tyr397, indicate the requirement of an intact actin cytoskeleton for Fak activation. Incidentally, it has been advocated that the assembly of actin stress fibers (7), is necessary for proper activation of Fak, most probably by allowing the recruitment of Fak and other signaling molecules, to specific subcellular regions. Indeed, our present as well as previous (31) data indicate that Fak activation is accompanied by its aggregation at myofilaments, suggesting that sub-celullar re-location is important to Fak signaling by mechanical stress. The concept that Fak re-location is associated with F-actin was advanced by our previous demonstration in rat

myocardium that activated Fak is precipitated by an anti-F-actin antibody (11). Given that RhoA/ROCK signaling pathways are believed to regulate the formation of actin stress fibers (15), it is conceivable that the dramatic reduction in stretch-induced Fak phosphorylation after treatment of NRVMs with RhoA/ROCK signaling inhibitors or Cytochalasin D are commonly determined by an impairment of actin stress fibers assembly. A major problem with this hypothesis is that there is no clear demonstration that cardiac myocytes have bona fide actin stress fibers. However, it has been reported that cardiac myocytes from neonatal rats have a highly developed actin cytoskeletal meshwork which is closely related to sarcomeres (24, 25). Thus, it is reasonable to assume that upon mechanical stress some form of actin microfilaments remodeling might induce the recruitment of Fak to sub-cellular sites of NRVMs, including the myofilaments. However, further studies are necessary to dissect out how mechanical stress and RhoA/ROCK signaling promote the remodeling of actin microfilaments in cardiac myocytes.

The analysis by immunohistochemisty and confocal microscopy also revealed a distinct pattern of nuclear staining in two-hour stretched NRVMs, indicating that mechanical stress induces Fak to translocate to nuclei of cardiac myocytes. Similar translocation to nucleus was recently reported in cardiac myocytes of spontaneously hypertensive heart failure rats, suggesting that Fak might play a role in the regulation of nuclear processes in response to mechanical stress (36). These findings imply that besides being the trigger of early signaling events Fak may be important to nuclear processes that lead to the regulation of gene expression. However, further investigation is necessary to clarify this issue.

Immunohistochemical analysis were extended here to examine the distribution of RhoA in NRVMs. Anti-RhoA antibody showed a distinct pattern of sarcomeric staining

which was intercalated with that of phalloidin, suggesting that RhoA is localized in the sarcomeric A-bands or even possibly at sarcolemal or sub-sarcolemmal sites close to sarcomeric A-bands. Interestingly, in contrast to Fak, there was no change in RhoA sub-cellular distribution in stretched as compared to non-stretched NRVMs, indicating that RhoA activation by mechanical stress is not accompanied by its translocation to other subcellular sites. In this context, it has been previously shown that upon mechanical stress RhoA dissociate from the membrane caveolas, where it is localized in non-stretched cells, by a mechanism which appears to require the organization of the actin cytoskeleton (17). Thus, the lack of a significant change in subcellular localization of RhoA in stretched NRVMs, as demonstrated here, might indicate that upon mechanical stress RhoA associates with sub-sarcolemmal or sarcomeric A-band sites.

Regulation of stretch-induced hypertrophic genes by RhoA/Fak signaling

Previous studies have provided evidence that RhoA plays a crucial role in the control of gene transcription in response to agonists and stretch-induced hypertrophy of cardiac myocytes (1, 17, 35). Our demonstration here that the attenuation of RhoA signaling by antisense oligonucleotide markedly reduced the stretch-induced expression of β-MHC confirmed the importance of this signaling protein to the regulation of gene expression by mechanical stress in NRVMs. In addition, our demonstration here that inhibition of RhoA/ROCK signaling markedly attenuated the stretch-induced Fak activation and that Fak antisense oligonucleotide produced a comparable reduction of stretch-induced β-MHC expression, support the idea that Fak mediates the influence of RhoA on stretch-induced gene expression in NRVMs. This agrees with previous demonstration (35) that the

impairment of Fak signaling abrogated the stimulation of α-actin promoter induced by overexpression of a constitutively active RhoA in NRVMs.

Although the downstream mechanisms that mediate the influence of RhoA/Fak signaling on the hypertrophic gene expression was not explored in the present study, our demonstration here that Fak activation is paralleled by the activation of Erk1/2 and that the impairment of RhoA/ROCK signaling also block the stretch-induced Erk1/2 activation, suggest that these MAP kinases might play a role in the regulation of hypertrophic genetic program by RhoA/ROCK/Fak multicomplex signaling. The early activation of Erk1/2 has been suggested to contribute to the re-expression of fetal ventricle genes (28). Transfection of constitutively active MEK1 (immediate upstream activator of ERK1/2) has been shown to augment atrial natriuretic factor (ANF) promoter activity in cultured cardiomyocytes, whereas a dominant-negative MEK1 construct attenuated its activity (12). However, the present results do not exclude the possibility that multiple downstream effectors are involved in the influence of Fak on early gene regulation in response to mechanical stress. Thus further studies are necessary to clarify the relative importance of the various candidate signaling molecules to Fak influence on early gene regulation in response to mechanical stress.

In conclusion, the present report demonstrates that RhoA/ROCK signaling plays a critical role on Fak activation by mechanical stress in cardiac myocytes. Furthermore, the demonstration here that both, RhoA and Fak signaling, are critically important to the stretch-induced expression of β-MHC in NRVMs indicate that both RhoA and Fak are key elements to the regulation of hypertrophic genetic program in cardiac myocytes induced by

mechanical stress. The relative importance of these mechanisms to phenotypic myocardial changes, such as hypertrophy and heart failure, needs further investigation.

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LEGENDS

FIGURE 1. *Cyclic stretch-induced focal adhesion kinase (FAK) and Erk1/2 phosphorylation.* Neonatal rat ventricular myocytes (NRVMs) were stretched for 10, 30 or 60 min (1Hz; 15%). A. Western blots performed with whole cell extracts were probed with antibodies that recognize RhoA and both the phosphorylated (Tyr397) and unphosphorylated forms of FAK and phosphorylated (Thr202/Tyr204) and unphosphorylated forms of ERK1/2. *B.* Quantitative analysis of densitometric readings of 4 western blot experiments performed with anti-Fak-Tyr397 and anti-Erk1/2 Thr202/Tyr204. $P < 0.05$ vs. *C.

FIGURE 2. *Stretch-induced Fak redistribution in NRVMs.* Non-stretched (A, B) and 30 (C, D) and 60 min (E, F) stretched NRVMs were fixed, double-labeled with anti-Fak and rhodamine-conjugated phalloidin (actin filaments labelling), and viewed under a laser confocal microscope. In non-stretched cells, Fak was distributed at the perinuclear region and in cell periphery. After 30 minutes of cyclic stretch (15%) Fak aggregates were seen decorating myofilaments. Areas of Fak/phalloidin colocalization appear as yellow. After 60 minutes of cyclic stretch anti-Fak staining was seen in cardiac myocyte nuclei

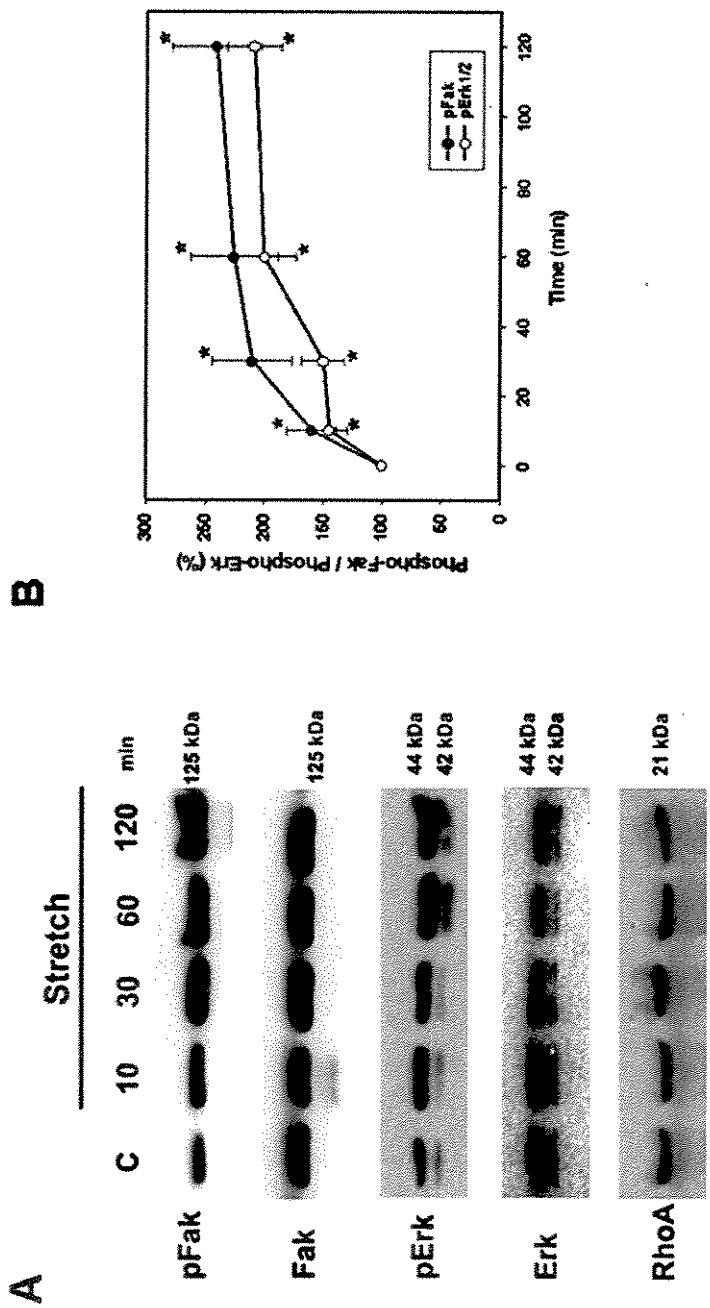
FIGURE 3. *RhoA localization in NRVMs.* A-C. Non-stretched, D-F stretched NRVMs double-labeled with anti-RhoA and rhodamine-conjugated phalloidin (actin filaments labelling), and viewed under a laser confocal microscope.

FIGURE 4. *Inhibition of RhoA/ROCK signaling reduced stretch-induced Fak activation in NRVMs*. A, Representative blot showing the results of western blots performed with anti-Fak, -pFak, -Erk1/2 and –pErk1/2 in cells treated with the RhoA inhibitor *C. botulinum* C3 Exoenzyme. Graphic shows the average values of densitometric analysis of western blots (5 experiments) performed with anti-pFak antibody. B, Representative blot showing the results of western blots performed with anti-Fak, -pFak, -Erk1/2 and –pErk1/2 in cells treated with ROCK inhibitor Y-27632. Graphic shows the average values of densitometric analysis of western blots (4 experiments) performed with anti-pFak antibody. $*P<0.05$ versus C.

FIGURE 5. *Cytochalasin D reduced stretch-induced Fak activation in NRVMs.* Representative blot showing the results of western blots performed with anti-Fak, -pFak, -Erk1/2 and –pErk1/2 in cells treated with the inhibitor of actin organization Cytochalasin D. Graphic shows the average values of densitometric analysis of western blots (4 experiments) performed with anti-pFak antibody.

FIGURE 6. *RhoA antisense ODN attenuates stretch-induced Fak and Erk1/2 activation in NRVMs*. A-D, Laser confocal analysis of control (A-B) and antisense-transfected (C-D) NRVMs double-labeled with phalloidin and anti-RhoA antibody. Cells were collected after maintenance in medium containing serum for 18 hours. E. Representative immunoblotting (from 3 experiments) of extracts from non-stretched and stretched NRVMs transfected with antisense and sense RhoA ODNs and performed with anti-RhoA, -Fak, pFak, Erk1/2 and pErk1/2 antibodies. S- sense; AS – antisense.

FIGURE 7. *RhoA and FAK antisense ODNs block stretch-induced β-myosin heavy chain (β-MHC) mRNA expression in NRVMs.* RT-PCR of β-MHC and β-actin mRNA performed in NRVMs transfected with RhoA or Fak antisense ODNs, as indicated. Gels were obtained total mRNA extracted from 3 experiments with NRVMs.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Phospho-Fak / Phospho-Erk (%)
Time (min)
pFak
pErk1/2

Figure 1

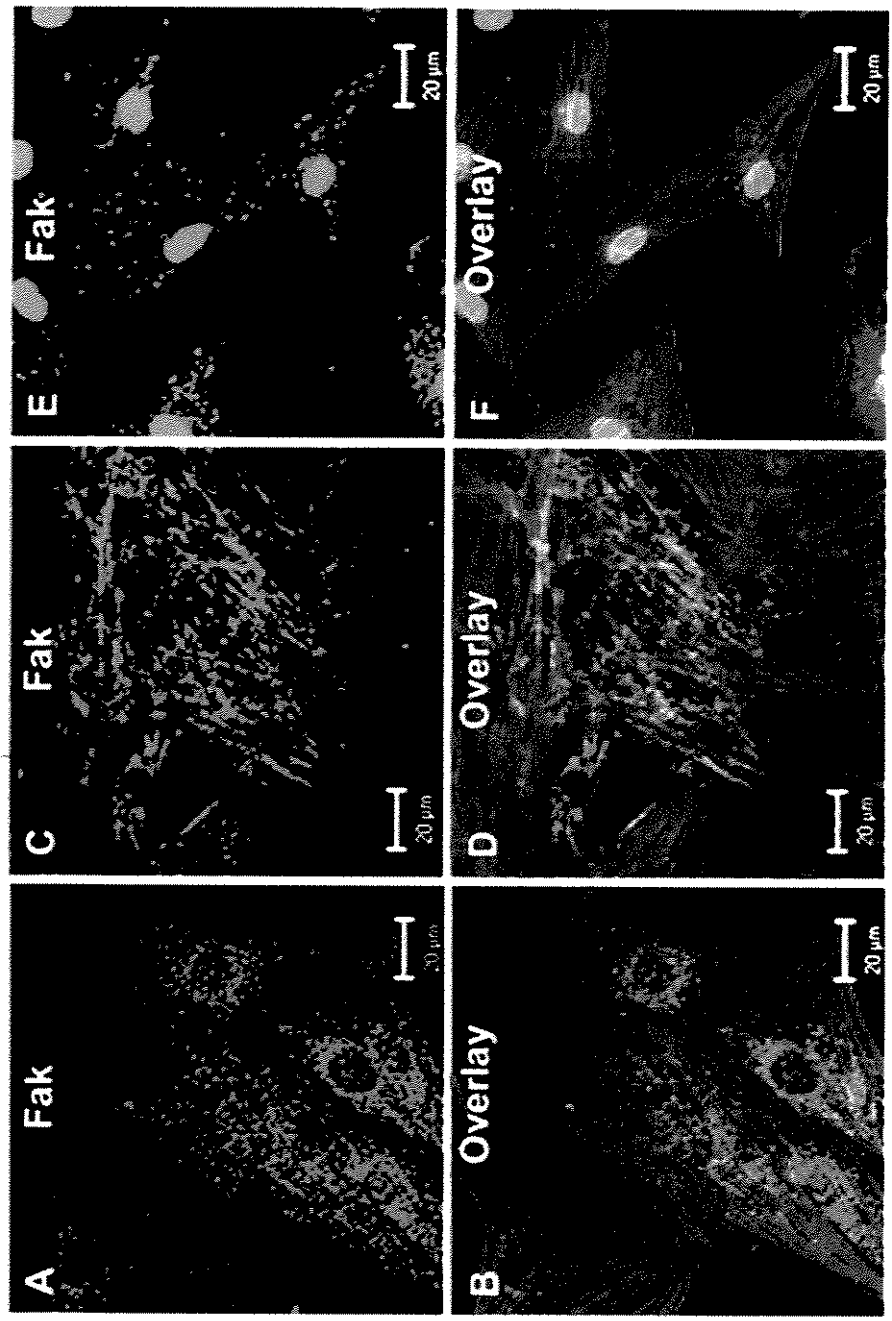

Figure 2

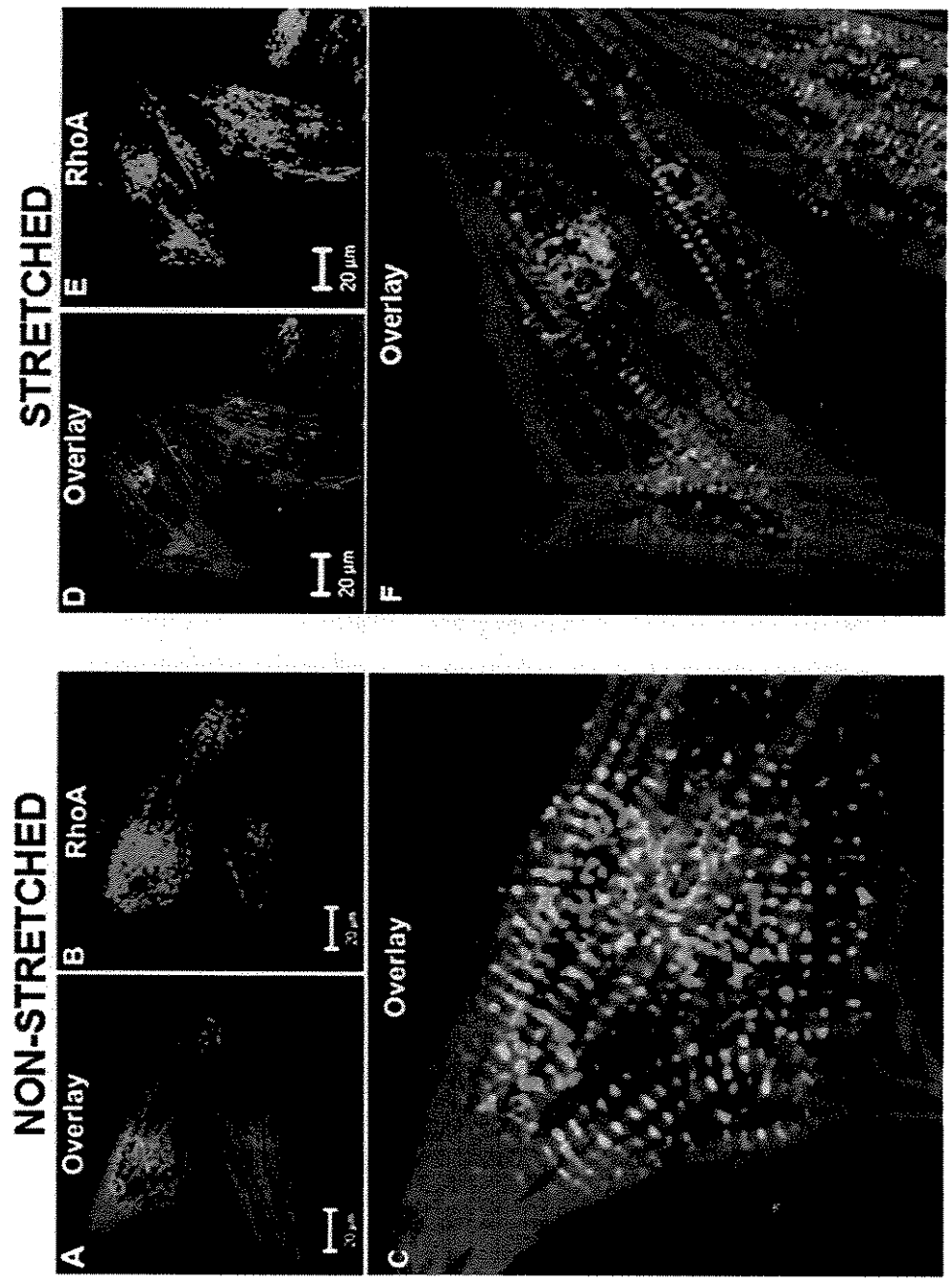
NON-STRETCHED
STRETCHED
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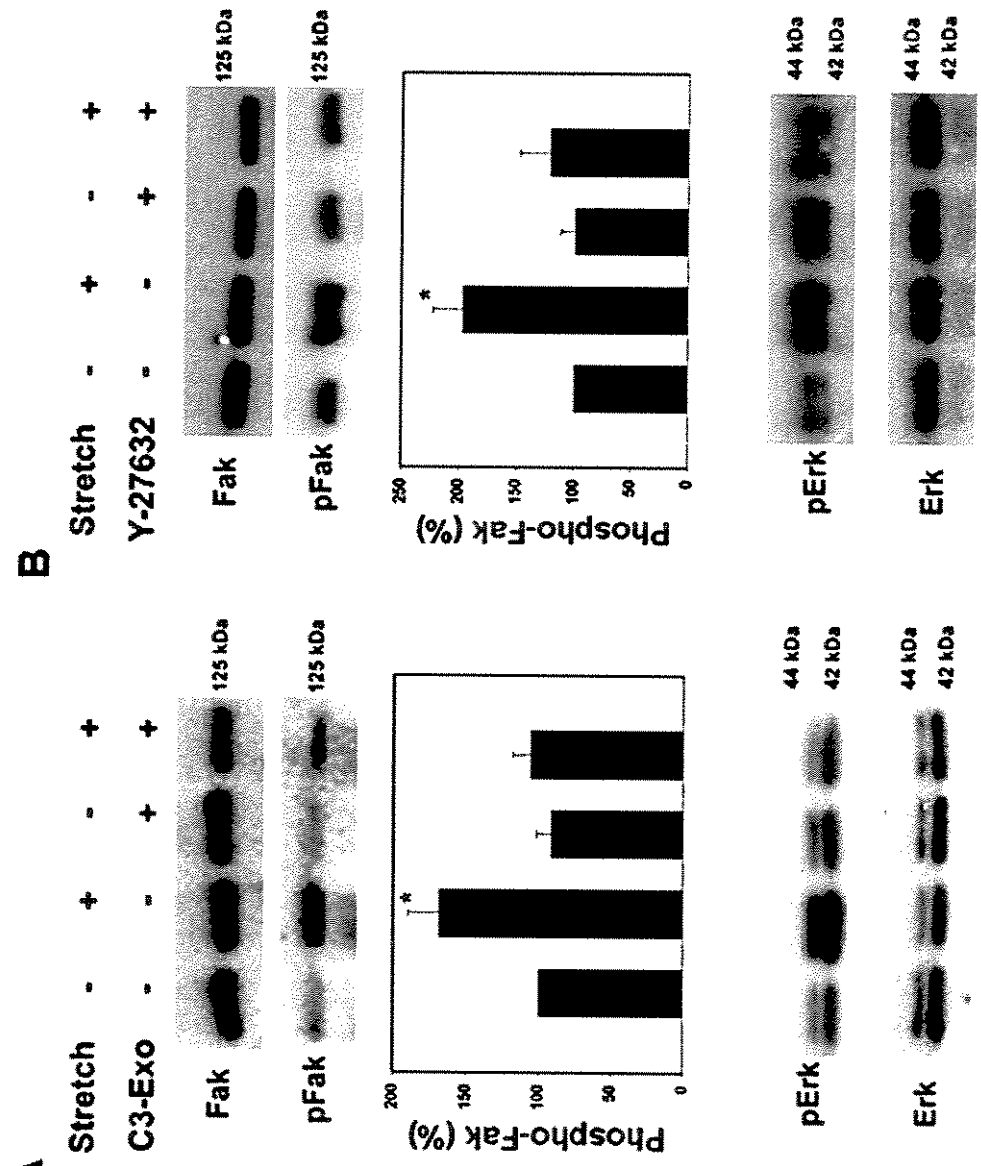
Figure 3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Phospho-Fak (%)
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FIGURE 4

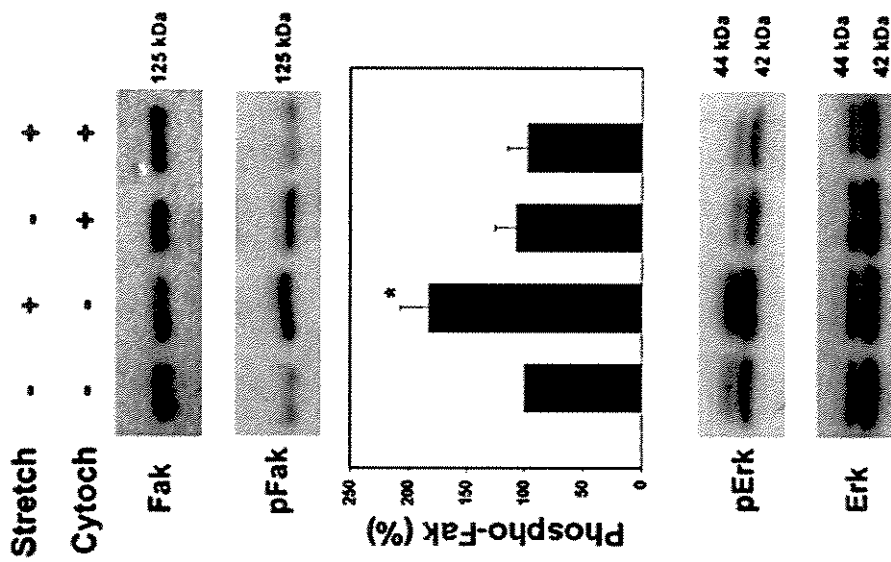
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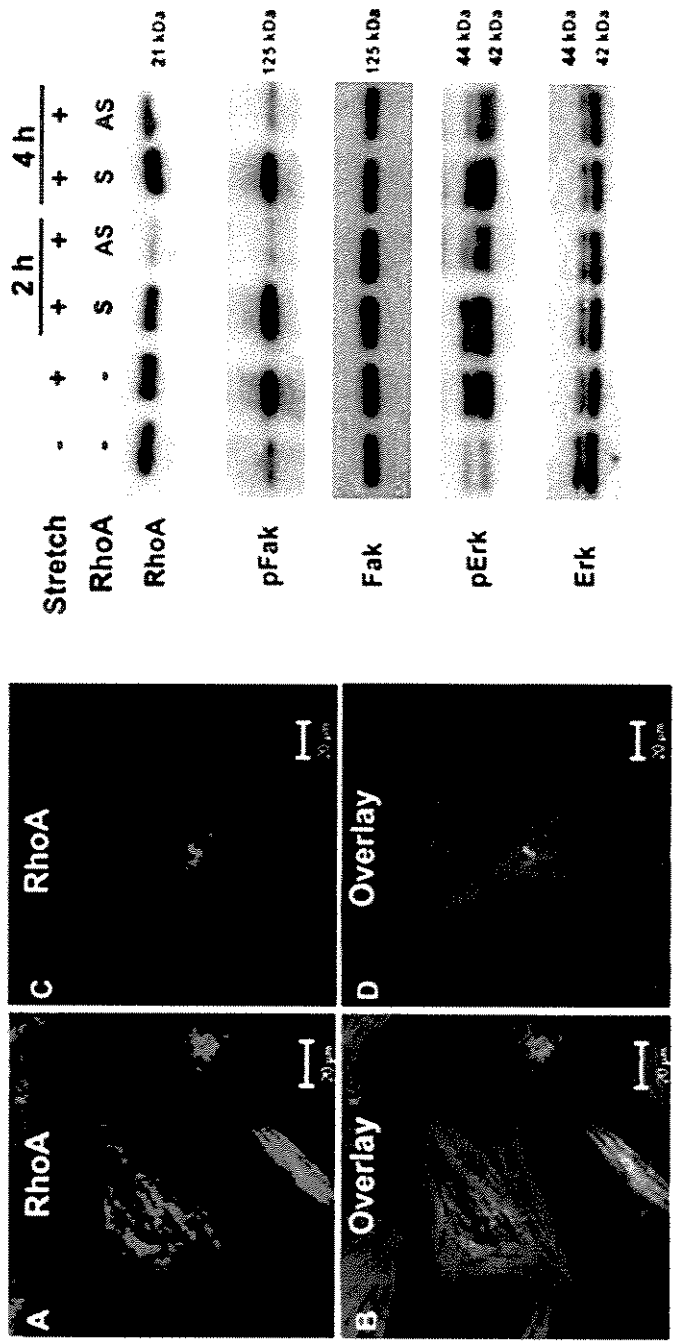
FIGURE 5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RhoA
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D
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42 kDa

FIGURE 6

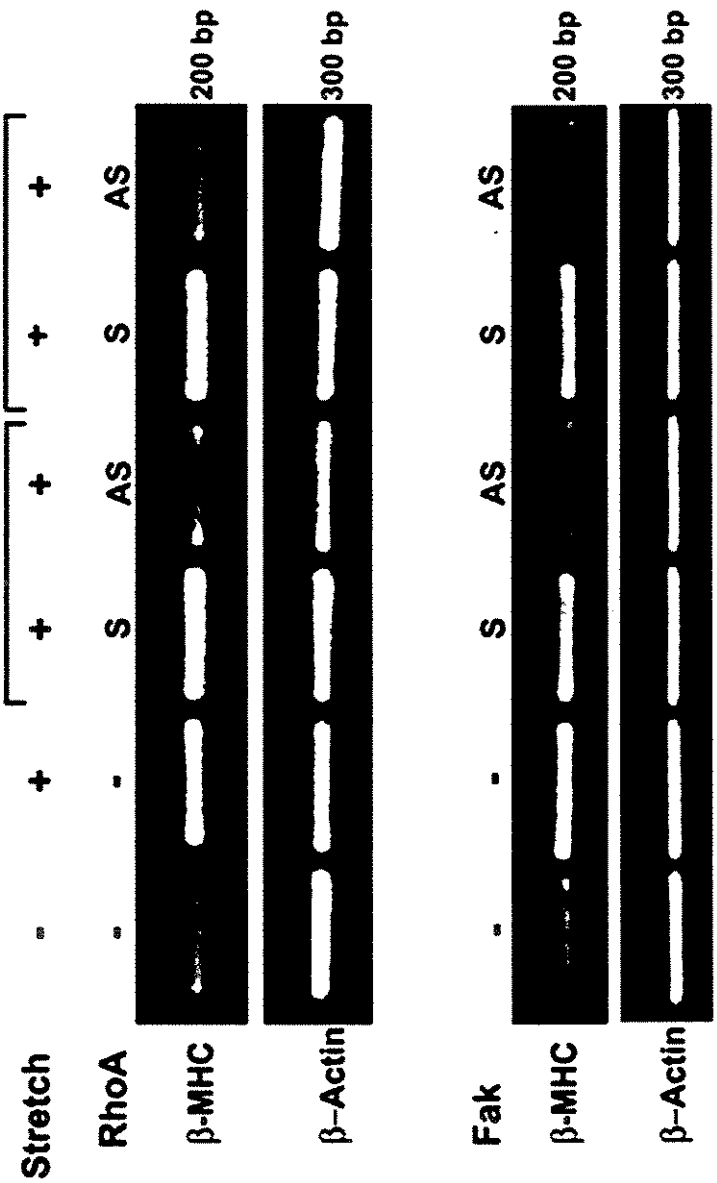
Stretch
RhoA
β-MHC
β-Actin
Fak
β-MHC
β-Actin
2 h
4 h
S
AS
200 bp
300 bp

FIGURE 7

5. CAPÍTULO 3

"Early activation of $p160^{ROCK}$ by pressure overload in rat heart."

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Early activation of p160^{ROCK} by pressure overload in rat heart

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Torsoni, Adriana S., Priscila M. Fonseca, Daniela P Crosara-Alberto, and Kleber G. Franchini. Early activation of p160^{ROCK} by pressure overload in rat heart. *Am J Physiol Cell Physiol* 284: C1411–C1419, 2003. First published February 5, 2003; 10.1152/ajpcell.00098.2002.—We investigated the effects of acute pressure overload on activation of p160^{ROCK} 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 p160^{ROCK}. The binding of p160^{ROCK} 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 p160^{ROCK} 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 p160^{ROCK} and RhoA along the longitudinal axis of cardiac myocytes. Immunoelectron microscopic analysis showed that pressure overload induced the association of p160^{ROCK} and RhoA to Z-line, T-tubule, and subsarcolemmal areas. The rapid activation of p160^{ROCK} 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-

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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 $p160^{ROCK}$ 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 $p160^{ROCK}$ in the overloaded myocardium.

METHODS

Antibodies and chemicals. Polyclonal goat antibody against $p160^{ROCK}$, 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), ^{125}I-protein A, [γ-^{32}P]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 (50 mg/kg rat wt) administered via intraperitoneal injections and maintained on a temperature-controlled surgical table for periods ranging from 5 to 60 min. Supplemental doses of pentobarbital sodium were administered along the experimental period, as necessary. Pulsatile arterial pressure was continuously monitored from catheters placed in the carotid and femoral arteries and processed with WINDAQ-PRO data acquisition software (DATAQ Instruments, Akron, OH). The transverse aorta was reached by surgical incision at the second left intercostal space. The micro-Blalock clamp was adjusted around the transverse aorta between the right and left common carotid arteries, and the constriction was adjusted to produce stable peak systolic pressure gradients of ~40 mmHg between the ascending and abdominal aorta. Control rats were sham operated. At the end of the arterial pressure monitoring period, the animals were euthanized with a supplemental dose of pentobarbital sodium administered via an endovenous catheter placed in the right jugular vein. The thoracic cavity was then opened, and the ventricles were rapidly removed for Western blot, immunohistochemical, and immunoelectron microscopy studies. All procedures and the care of the rats were in accordance with the university's *Guide for the Care and Use of Laboratory Animals.*

Adult rat ventricular myocytes. Cardiac myocytes were isolated from the left ventricle of adult Wistar rats at 160–180 g by collagenase (type IA; Sigma) digestion using a modified Langendorff perfusion according to methods previously described (37). Briefly, rats were anesthetized, and the heart was excised, taking care to remove the pericardium. The aorta was tied onto the cannula, and the heart was perfused with O_2-saturated HEPES-buffered solution (10 mM HEPES, 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 6 mM glucose, 1 ml of heparin, and 1 ml of Xylocaine, pH 7.4). After 2 min, the solution was switched to HEPES-buffered solution plus EGTA (4 mM) solution for 10 min, and the heart was then perfused with enzyme solution for about 20 min (HEPES-buffered solution with 0.1% BSA and 300 μg/ml collagenase). At the end of the digestion, the heart was cut off the cannula and the atria and aorta were dissected away. On a sterile petri dish the ventricular tissue was chopped with small scissors, and the suspension was then filtered through cotton gauze. The myocytes were placed in 40 ml of O_2-saturated HEPES-buffered solution plus 0.1% BSA plus a crescent concentration of Ca^{2+} solution at 37°C. At the end of this period, the Ca^{2+} concentration was 1.2 mM. The myocytes were double-centrifuged at 12 *g* for 10 min each time. The viability of the isolated cardiac myocytes was 40% as determined by rod-shaped morphology and lack of granulation 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 NaH_2PO_4, 100 mM NaF, 10 mM EDTA, 10 mM Na_3VO_4, 2 mM PMSF, 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 ^{125}I-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-$p160^{ROCK}$ antibody was measured by using MLC as substrate. The immune complex of anti-$p160^{ROCK}$ 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 $MnCl_2$, 5 mM DTT, and 500 μM Na_3VO_4) containing 2 μCi [γ-^{32}P]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 ^{32}P-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 anti-$p160^{ROCK}$ or anti-RhoA antibodies in 1% nonfat milk in PBS overnight at 4°C, followed by incubation with FITC-conjugated secondary antibody 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 parafilm. 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 Scheffé'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. 2*A*, 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. 2*B*). 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,

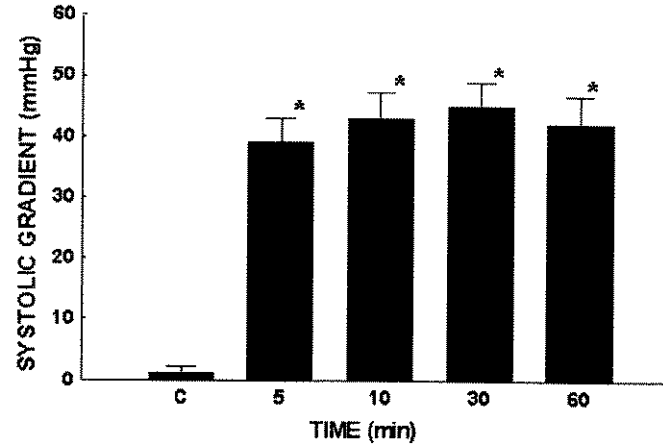

Fig. 1. Systolic gradient across the aortic constriction. Rats (n = 12) were subjected to transverse aortic constriction for periods ranging from 5 to 60 min, and blood pressure was measured in the ascending and abdominal aorta simultaneously. *$P < 0.05$ compared with values of control (C) rats.

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. 3*A*, 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. 3*B*, 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. 4*A*). 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 sarcoplasma (Fig. 4). Pressure overload enhanced the spot-pattern staining along the myocytes (Fig. 4*B*). 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*).

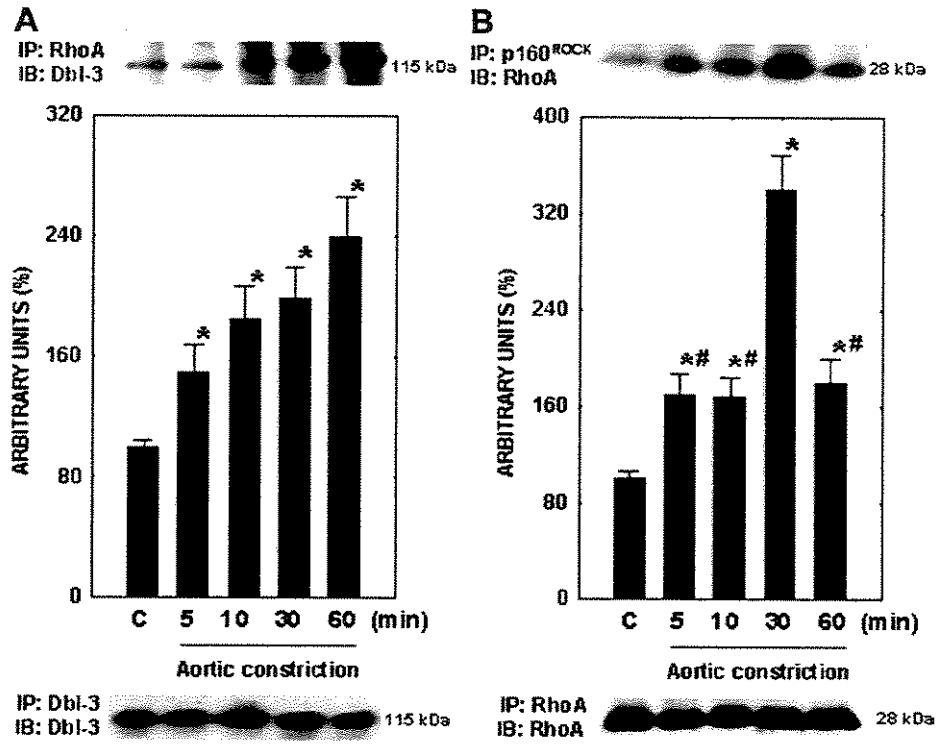

Fig. 2. *A*: representative example (*top*) and average values (*middle*) of Dbl detected in immunoblots (IB) of immunoprecipitated (IP) RhoA. *Bottom*: amount of Dbl-3 recovered from heart homogenates. *B*: representative example (*top*) and average values (*middle*) of RhoA detected in immunoblots of immunoprecipitated p160ROCK. *Bottom*: amount of RhoA recovered from heart homogenates. $*P < 0.05$ compared with control hearts. $\#P < 0.05$ compared with values at 30 min of pressure overload.

Isolated myocytes also showed consistent staining with anti-p160ROCK antibody, and the distribution patterns of p160ROCK generally reproduced those seen in left ventricular sections of control (Fig. 5, *A–C*) and overloaded hearts (Fig. 5, *D–F*), including the enhanced aggregation and the number spots of p160ROCK staining induced by pressure overload.

Experiments were performed with immunogold electron microscopy of myocardial sections to further explore the specific location of p160ROCK in cardiac myocytes. In sections obtained from control hearts, specific labeling with anti-p160ROCK antibody was found to be evenly distributed in the sarcolemma and close to the region of Z lines (Fig. 6*A*). In the sections obtained from

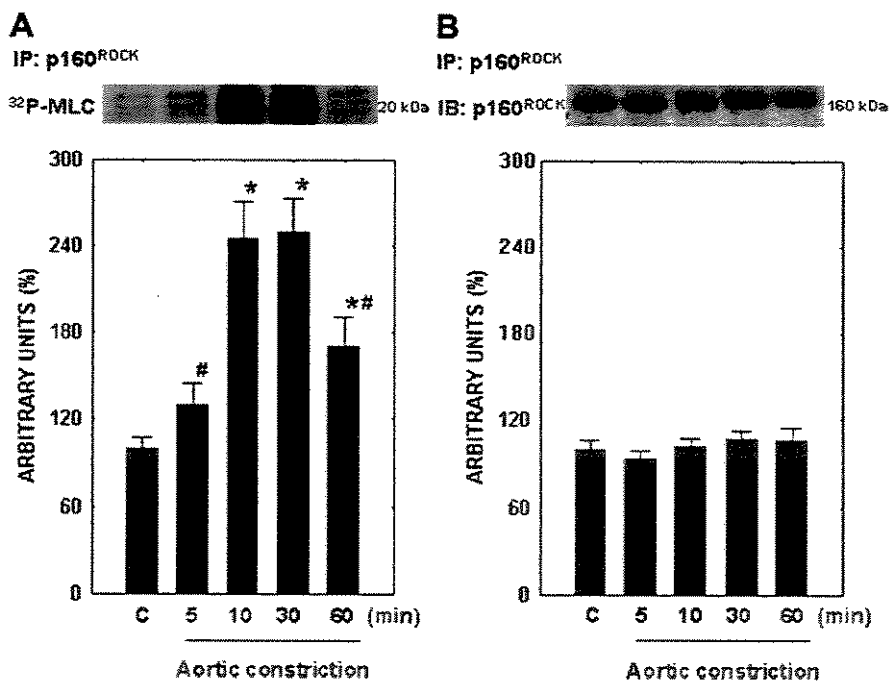

Fig. 3. *A*: kinase activity of the p160ROCK immune complex toward myosin light chain (MLC). p160ROCK was immunoprecipitated from myocardial homogenates of control and overloaded (5–60 min) hearts. A representative blot of ^{32}P-labeled MLC (*top*) and average values (*middle*) of p160ROCK detected from myocardial homogenates are shown. *B*: representative example (*top*) and average values (*middle*) of p160ROCK immunoprecipitated from heart homogenates. $*P < 0.05$ compared with unloaded hearts (C). $\#P < 0.05$ compared with values at 30 min of pressure overload.

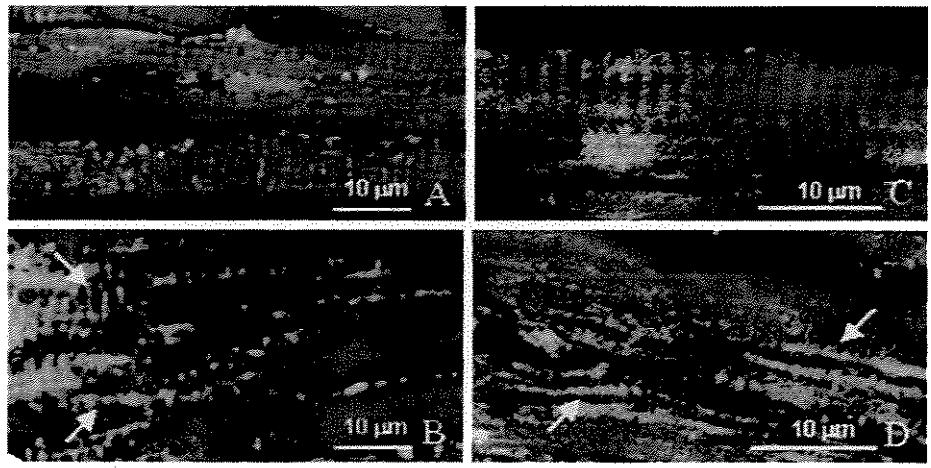

Fig. 4. Confocal laser scanning microscopy (CLSM) studies showing the immunolocalization of p160ROCK (green) and sarcomeric actin (red; phalloidin) in sections of rat left ventricle. *A*: left ventricular section from a control heart, showing the distribution of p160ROCK throughout the sarcoplasma of 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 sarcoplasma 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.

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. 6*B*). 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. 4*D*. 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*).

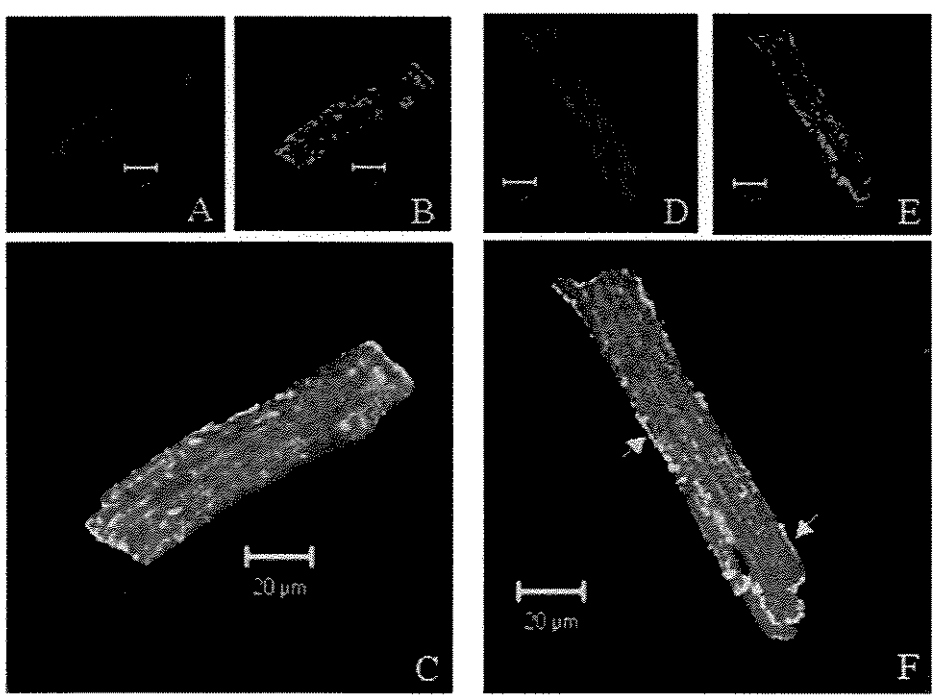

Fig. 5. CLSM studies showing the immunolocalization of p160ROCK (green) and sarcomeric actin (red; phalloidin) in isolated adult rat ventricular myocytes. *A*: phalloidin staining in isolated myocytes from control rats. *B*: representative example of anti-p160ROCK staining of isolated myocytes from control 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.

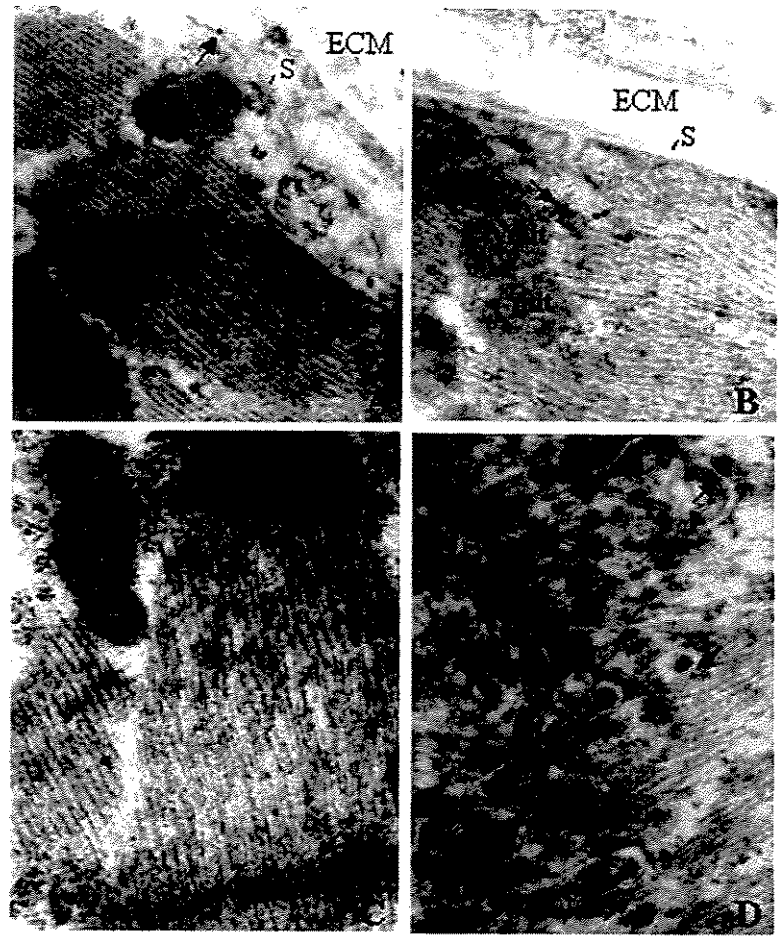

Fig. 6. Immunoelectron micrographs of anti-p160ROCK staining in rat left ventricle. *A*: representative example of p160ROCK staining in the sarcomere and sarcolemma (arrows) in a myocardial section from a control rat. ECM, extracellular matrix; mit, mitochondrion; S, sarcolemma. Magnification, ×50,600. *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*).

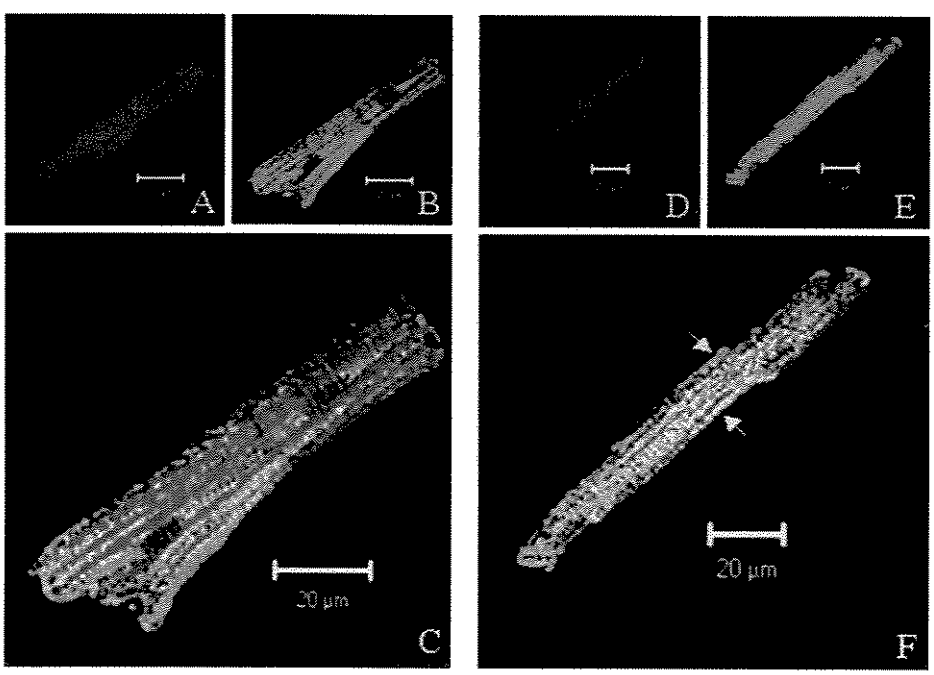

Fig. 7. CLSM studies showing the immunolocalization of RhoA (green) and sarcomeric actin (red; phalloidin) in isolated adult rat ventricular myocytes. *A*: phalloidin staining in isolated myocytes from control rats. *B*: anti-RhoA staining of isolated myocytes from control rats. *C*: RhoA/phalloidin 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. 8*A*). 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 $p160^{ROCK}$ in the adult rat myocardium. Because this activation was paralleled by increases in $p160^{ROCK}$/RhoA and RhoA/Dbl-3 association, our data also indicate that the rapid activation of $p160^{ROCK}$ 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 $p160^{ROCK}$ 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 $p160^{ROCK}$ as well as of RhoA, regularly arranged on similar regions along the longitudinal axis of cardiac myocytes. The aggregation of $p160^{ROCK}$ and RhoA staining in cardiac myocytes of overloaded hearts were confirmed by the immunogold electron microscopy, which showed the appearance of clusters of $p160^{ROCK}$ 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/$p160^{ROCK}$ signaling complex at structures compromised with force transmission in adult rat cardiac myocytes.

The finding here that pressure overload induces $p160^{ROCK}$ as well as RhoA to localize and cluster at specific subcellular structures simultaneously to $p160^{ROCK}$/RhoA association detected by coimmunoprecipitation 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. $p160^{ROCK}$ contains multiple domains, including a kinase domain in the NH_2 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 $p160^{ROCK}$ to cytoskeletal proteins (9, 23). The location at specific subcellular structures might confer to $p160^{ROCK}$ the ability to contribute to signaling mechanisms involving cell membrane and cytoskeleton. Thus one could argue that the load-induced activation and target of $p160^{ROCK}$ 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/$p160^{ROCK}$ 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).

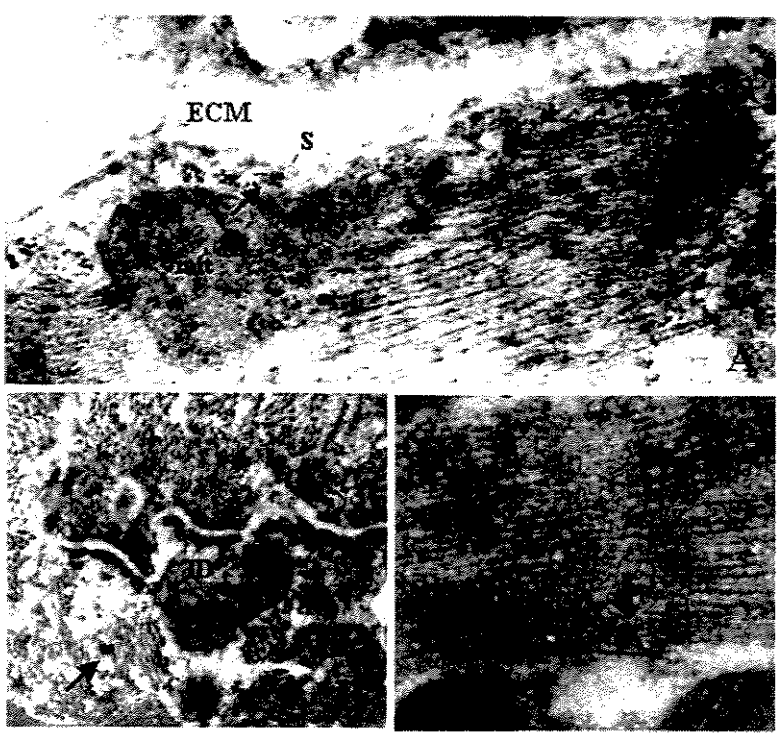

Fig. 8. Immunoelectron micrographs of anti-Rho-A staining in rat left ventricle. *A*: representative example showing sparse RhoA staining in the sarcomere and sarcolemma (arrows) in a myocardial section from a control rat. Magnification, ×41,217. *B*: after 10 min of pressure overload, note clusters (arrow) of colloidal gold particles at intercalated disk. Magnification, ×40,109. *C*: staining of RhoA in 30-min overloaded hearts shows localization of RhoA at Z disk (arrow). Magnification, ×85,319.

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 Dbl/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 role 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 α_1-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 Dbl/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.

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6. DISCUSSÃO

Nos diferentes capítulos apresentados no presente trabalho, foram realizados experimentos com o intuito de 1) demonstrar a ativação e localização da Fak, além de seu papel na regulação da transcrição do ANF, induzida pelo estiramento pulsátil em MVRN; 2) mostrar que a ativação da Fak pelo estiramento pulsátil de MVRN, é dependente de mecanismos de sinalização mediados pela proteína RhoGTPase, além de reforçar a idéia de uma cooperação entre as vias de sinalização de RhoA/ROCK e Fak, como mecanismos críticos na regulação gênica e celular precoce em resposta ao estiramento mecânico em miócitos cardíacos; 3) avaliar a ativação e distribuição da proteína ROCK em miocárdio de ratos submetidos à sobrecarga pressora, ativação esta mediada pelo complexo Dbl3-RhoA.

Esses estudos só foram possíveis, graças à padronização da técnica de extração de cardiomiócitos ventriculares de ratos neonatos, para a manutenção em cultura primária. Inúmeras tentativas foram feitas a fim de se estabelecer cultura primária de miócitos cardíacos adultos em nosso laboratório. No entanto, devido à grande dificuldade de manutenção dessas células em cultura, optamos pela padronização de cardiomiócitos obtidos de ratos neonatos. Essas células são mais facilmente destacadas do miocárdio e têm servido como um excelente modelo para o estudo de vias de sinalização celular em miócitos cardíacos, como demonstrado pela diversidade de trabalhos na literatura nessa área (WEISENSEE *et al.*, 1995; SADOSHIMA & IZUMO, 1997; FULLER *et al.*, 1998; HOSHIJIMA *et al.*, 1998;). Além disso, essas células, submetidas a forças de estiramento, representam um modelo *in vitro* adequado para análise mais detalhada da reação hipertrófica em nível celular com respeito a estímulos individuais, uma vez que experimentos *in vivo* são incapazes de distinguir a contribuição relativa de diferentes fatores que conduzem a mudanças bioquímicas e morfológicas específicas observadas na hipertrofia cardíaca (SIMPSON *et al.*, 1989; STEMMER *et al.*, 1992; HEFTI *et al.*, 1997).

Ademais, os ensaios *in vitro* são vantajosos em relação aos testes *in vivo*, uma vez que fornecem melhor controle sobre as condições experimentais, permitindo

boa reprodutibilidade dos ensaios e dos resultados, diminuição dos custos efetivos e redução do número de animais de experimentação (CHU, 1995).

Capítulos 1 e 2

Recentes estudos mostraram que a Fak é rapidamente ativada pelo estiramento pulsátil em MVRN (SEKO *et al.*, 1999; AIKAWA *et al.*, 2002). No presente trabalho demonstramos que essa ativação é paralela à amplitude e duração do estiramento. Além disso, também observamos que o estiramento pulsátil induziu a Fak a migrar da fração solúvel citosólica para a fração de citoesqueleto nos homogenatos de MVRN. Utilizando microscopia confocal, demonstramos que o estiramento induz a agregação da Fak nos miofilamentos, em um padrão de distribuição parecido àquele das estruturas conhecidas como costâmeros no tecido muscular cardíaco. Simultaneamente a essa agregação em miofilamentos, houve uma importante redução na marcação específica da Fak na região perinuclear, sem que fosse observada qualquer alteração na quantidade de Fak detectada por "Western Blot", sugerindo que o estiramento pulsátil induz a translocação e agregação da Fak em regiões semelhantes aos costâmeros nos MVRN.

Esses resultados indicam que a ativação da Fak pelo estiramento é, de alguma forma, dependente de sua localização nos possíveis costâmeros, estrutura considerada como um sensor mecânico, onde um sinal físico mecânico pode ser transformado em sinal bioquímico, por meio da ativação de proteínas sinalizadoras intracelulares. Estas, por sua vez, culminariam com o desencadeamento da resposta hipertrófica, necessária à adaptação cardíaca à sobrecarga de trabalho. Esses dados corroboram nossa prévia demonstração que o estiramento mecânico induz uma translocação da Fak para o compartimento citoesquelético de actina em miocárdio de ratos (FRANCHINI *et al.*, 2000).

Adicionalmente, dados de estudos recentes demonstraram que a remoção da Fak de sitos de adesão focal pela superexpressão do fragmento C-terminal da Fak (FRNK), em cultura de MVRN, diminuiu a ativação da Fak (EBLE *et al.*, 2000; TAYLOR *et al.*, 2000; HEIDKAMP *et al.*, 2002). Entretanto, em contraste com

nossos resultados, nesses estudos a Fak foi encontrada principalmente em sítios clássicos de adesão focal na região periférica da célula, onde ela aumentou em resposta ao tratamento com agonistas. Isso sugere que a remoção da Fak pelo fragmento FRNK poderia ocorrer preferencialmente nesses sítios de adesão focal ao invés dos costâmeros. Uma possível explicação para essas diferenças pode estar relacionada com a natureza do estímulo. Sendo assim, se o estímulo for mecânico, haveria uma tendência de se encontrar a Fak nos sítios costaméricos, enquanto que se o estímulo for químico, a tendência seria da Fak se localizar nos sítios de adesão focal.

Essa hipótese é sustentada por resultados de experimentos realizados com Angiotensina II (AII) em MVRN. Vários autores relataram que o estímulo mecânico causa secreção de AII em miócitos cardíacos, agindo como um mediador inicial de hipertrofia induzida por estiramento, através da ativação de receptores AT1 em MVRN, causando fosforilação em tirosina de várias proteínas intracelulares e ativação de MAP quinases (SADOSHIMA *et al.*, 1993; SADOSHIMA *et al.*, 1995). Embora o tratamento com AII leve à ativação da Fak em MVRN, a presença deste hormônio não induz a migração da Fak para os miofilamentos. Ao invés disso, a AII aumenta a agregação da Fak em sítios de adesão focal clássicos, indicando que a AII e o estiramento ativam a Fak por diferentes mecanismos e em sítios distintos do MVRN.

A demonstração, no presente trabalho, que o antagonista do receptor AT1, losartan, não diminuiu a ativação da Fak induzida pelo estiramento, suporta essa idéia de que há uma ativação diferencial da Fak, dependente do estímulo transmitido. Ademais, de maneira geral, isso concorda com a demonstração prévia (SALAZAR *et al.*, 2001) de que os eventos de sinalização que levam à ativação da Fak, por agonistas que se ligam a receptores acoplados a proteínas G, são independentes da Src, enquanto que aqueles estimulados por receptores de integrina, requerem a ativação dessa quinase.

Experimentos realizados com o intuito de impedir a ativação da sinalização Fak-Src induzida pelo estiramento, seja pela superexpressão de um dominante

negativo de Fak, ou pela inibição farmacológica da Src, através do tratamento com PP2, aboliram a agregação da Fak nos miofilamentos de MVRN induzida pelo estiramento pulsátil. Uma vez que as duas abordagens eliminam a sinalização da Fak, por afetar sua autofosforilação na tirosina 397, nossos resultados indicam que a agregação da Fak nos miofilamentos, induzida pelo estiramento, deva ser dependente da autofosforilação da Fak na tirosina 397. De maneira similar, a importância da autofosforilação na tirosina 397 para a ativação e agregação da Fak, também tem sido mostrada em sítios de adesão focal de células não cardíacas (OWEN *et al.*, 1999; SCHALLER *et al.*, 1999).

A importância do resíduo de tirosina 397 para a translocação e agregação da Fak nos diferentes tipos celulares, reforça o fato que a autofosforilação da tirosina 397 é responsável pelo recrutamento e ativação de quinases da família da Src, as quais por sua vez, aumentam a atividade da Fak devido à fosforilação de outros resíduos de tirosina e recrutamento de moléculas adicionais de Fak para sítios específicos (CALALB *et al.*, 1995; SCHLAEFER *et al.*, 1994; POLTE & HANKS, 1997). Assim, a diminuição da autofosforilação da tirosina 397, poderia prevenir a agregação das moléculas de Fak/Src nos costâmeros de miócitos cardíacos. Dessa maneira, pode-se esperar que a agregação da Fak nos costâmeros, melhore a sinalização dessa enzima induzida pelo estiramento pulsátil em miócitos cardíacos, não apenas pela localização dessa proteína em pontos estratégicos de mecanotransdução, mas também, porque a proximidade molecular nos agregados pode servir para aumentar e sustentar a sinalização de Fak.

Adicionalmente, a demonstração que a ativação da Fak, induzida pelo estiramento, em cultura de MVRN ocorre em sítios semelhantes aos costâmeros, indica que esse é um fenômeno específico, diretamente relacionado à imposição de forças mecânicas, representadas pelo estiramento pulsátil, sobre possíveis sítios de mecanotransdução e, também, que esse fenômeno pode estar presente em miócitos cardíacos de miocardios intactos.

KATZ *et al.* (2002), desenvolvendo estudos em fibroblastos, demonstraram que a agregação da Fak aumenta e sustenta sua ativação, permitindo o recrutamento

e a ativação de vias de sinalização celular adicionais, tais como aquelas envolvidas na ativação de vias de crescimento e sobrevivência.

A ativação de vias de sinalização, que culminam no crescimento celular do miócito cardíaco, interfere com a indução da re-expressão de genes fetais, como o gene do ANF e da βMHC.

Nesse contexto, estudos recentes desenvolvidos por diferentes grupos mostraram que a ativação da Fak é importante na regulação gênica precoce, em resposta a estímulos provenientes de agonistas hipertróficos, em cultura de MVRN (EBLE *et al.*, 2000; TAYLOR *et al.*, 2000; KOVACIC-MILIVOJEVIC *et al.*, 2001). No presente trabalho, demonstramos que a diminuição da ativação da Fak atenuou significativamente a atividade do promotor do ANF, induzida pelo estiramento pulsátil em MVRN, indicando que a Fak desempenha papel fundamental nos eventos de sinalização envolvidos com a regulação da expressão gênica, em resposta ao estiramento mecânico em miócitos cardíacos.

O papel da Fak no crescimento celular, mediado pela sinalização de integrina, vem sendo demonstrado em muitos sistemas celulares (ZHAO *et al.*, 1998; ZHAO *et al.*, 2000). Um trabalho recente, desenvolvido por SCHLAEPFER *et al.* (1998), mostrou que o complexo Fak/Src ativa vias de sinalização posteriores, levando à ativação de fosfoinositídio 3-quinase, proteína quinase C, ERK, JUNK e vias de proteínas quinases ativadas pelo mitógeno p38.

Embora o efetor da regulação da expressão do ANF, induzida pela Fak, não tenha sido explorado no presente estudo, evidências prévias indicam que o complexo Fak/Src pode ativar as isoformas da ERK1/2 em MVRN e em miocárdio de ratos (FRANCHINI *et al.*, 2000; LASER *et al.*, 2000; DOMINGOS *et al.*, 2002; TAYLOR *et al.*, 2000), a qual pode potencialmente mediar a regulação precoce da expressão do ANF. A ativação precoce da ERK1/2 parece contribuir para a re-expressão de genes ventriculares fetais (SUGDEN & CLERCK, 1998). A transfecção de uma MEK1 (proteína ativadora de ERK1/2), constitutivamente ativa em cardiomiócitos em cultura, mostrou um aumento na atividade do promotor de ANF, enquanto que a transfecção com um dominante negativo de MEK1 atenuou

sua atividade (GILLESPIE-BROWN *et al.*, 1995). Entretanto, os presentes resultados não excluem a possibilidade do envolvimento de múltiplos efetores participando da regulação gênica precoce em resposta ao estiramento mecânico, mediado pela Fak.

Em nossos estudos, a superexpressão do mutante selvagem de Fak (WT-FAK) em MVRN não ativou significativamente a expressão do gene ANF em células não-estiradas, apesar de ter aumentado a quantidade de Fak ativada em resposta ao estiramento em MVRN. Esses resultados podem indicar que a superexpressão apenas do mutante WT-Fak foi insuficiente, tanto para a ativação basal do ANF, como para potencializar a transcrição gênica induzida pelo estiramento pulsátil. Esses resultados podem indicar que mecanismos não somente dependentes da ativação da Fak poderiam ser responsáveis pela ativação transcricional do ANF induzida pelo estiramento pulsátil.

Estudos similares corroboram essa hipótese, mostrando que a superexpressão de construções WT-Fak em MVRN, cultivados em baixa densidade, não aumentou a ativação da Fak induzida pela endotelina (EBLE *et al.*, 2000). No entanto, a transfecção de construções WT-Fak em MVRN, cultivados em alta densidade, tem mostrado estimular a transcrição de genes fetais associados com o fenótipo hipertrófico (PHAM *et al.*, 2000; EBLE *et al.*, 2000a). As razões para essas diferenças observadas ainda não são conhecidas. Podemos especular que, sob as condições de cultivo utilizadas em nosso trabalho, a expressão basal de ANF deva ter sido alta e que, portanto, apesar de ter ocorrido uma potencialização na ativação da Fak induzida pelo estiramento pulsátil, decorrente da transfecção com o mutante WT-Fak, a atividade transcricional do ANF não poderia aumentar visivelmente.

Um fato de extrema relevância que deve ser considerado é que a ativação da Fak e sua participação na regulação da expressão gênica diferencial em MVRN, induzida pelo estiramento, é criticamente dependente da ativação da proteína Rho.

Esse fato pode ser explicado por nossas observações de que i- a redução na sinalização de RhoA em MVRN, seja pela administração de um inibidor farmacológico seletivo, a Exoenzima C3, específico para proteínas G de baixo peso molecular da subfamília das RhoGTPases, ou pela redução da expressão de RhoA através da transfecção dos MVRN com uma sequência de oligodeoxinucleotídeo antisense para RhoA, atenuou de modo significativo a fosforilação da Fak no resíduo de tirosina 397, induzida pelo estiramento; ii- houve uma drástica redução na fosforilação da Fak no resíduo de tirosina 397, induzida pelo estiramento, em MVRN tratados com o inibidor farmacológico específico para a ROCK, a proteína efetora da RhoA; e iii- a abolição da fosforilação da Fak no resíduo de tirosina 397 pela Citocalasina D, um inibidor da polimerização de filamentos de actina, mostrou ser um evento mediado pela via de sinalização que envolve as proteínas RhoA/ROCK. Adicionalmente, a redução na sinalização de Fak ou RhoA, induzida pelo estiramento, reduziu consideravelmente a expressão de β-MHC em MVRNs, um conhecido marcador hipertrófico. Paralelamente, dados de imunocitoquímica utilizando a microscopia confocal, mostraram a co-localização de RhoA e Fak, sugerindo uma estreita relação entre as duas proteínas.

Assim como a Fak, a RhoA mostrou ser uma proteína rapidamente ativada e essencial para a regulação inicial do programa genético hipertrófico desencadeado nos miócitos cardíacos em resposta ao estiramento mecânico.

Evidências mostradas no presente estudo indicam que a sinalização RhoA/ROCK precede a ativação da Fak pelo estiramento mecânico. Esses dados corroboram demonstrações prévias da literatura, em que a diminuição na sinalização da Fak em miócitos cardíacos, decorrente da superexpressão do FRNK, aboliu completamente a indução do promotor da α-actina esquelética, provocada pela transfecção de uma RhoA constitutivamente ativa em MVRN (SCHWARTZ & SHATTIL, 2000). Outros dados mostram que a sinalização RhoA/ROCK medeia a ativação da Fak e a expressão do gene do ANF induzida por endotelina (HEIDKAMP *et al.*, 2002).

Apesar de não se conhecer o mecanismo molecular que determina o controle da atividade da Fak pela interação entre RhoA/ROCK, nossos resultados indicam que a integridade do citoesqueleto de actina é fundamental nesse processo.

Trabalhos recentes sugerem que a ativação da Fak possa ser iniciada pela reunião e contração das fibras de estresse de actina, fenômenos regulados especialmente pela sinalização das proteínas RhoA/ROCK. A expressão de dominantes ativos de ROCK induziu a formação das fibras de estresse em fibroblastos e células MDCK. De maneira contrária, a expressão de um dominante negativo inibiu a formação das fibras de estresse induzida pela Rho (KAIBUCHII *et al.*, 1999). A hipótese de que a ativação da Fak é dependente desse processo, vem sendo mantida principalmente por experimentos realizados com inibidores farmacológicos específicos da polimerização de actina, incluindo a citocalasina D, em miócitos cardíacos e em outros tipos celulares, os quais, invariavelmente, resultam na atenuação da ativação da Fak induzida pela adesão celular e por fatores de crescimento (CLARK *et al.*, 1998). Sugere-se que a contração das fibras de estresse esteja envolvida na ativação da Fak, ou porque ela ativaria diretamente esta enzima, ou porque ela poderia agrupar outras moléculas sinalizadoras, como a Src, por exemplo, que poderiam ser importantes para a ativação da Fak.

A nossa demonstração no presente trabalho que o tratamento com Citocalasina D aboliu a atividade da Fak induzida pelo estiramento mecânico, corrobora resultados anteriores da literatura e reforça a idéia de que a ativação da Fak pelo estiramento mecânico em MVRN é dependente da integridade do citoesqueleto de actina, sugerindo que a especificidade da transdução do sinal não está apenas relacionada à interação proteína-proteína, mas também ao arranjo espacial dos componentes da cascata de sinalização celular.

O grande problema desta hipótese é que não existe uma demonstração clara que miócitos cardíacos tenham fibras de estresse de actina (CLARK *et al.*, 1998). Entretanto, em MVRN, tão bem como em miócitos cardíacos adultos, microfilamentos de actina extra-sarcoméricos podem ser alvo para a sinalização

de RhoA/ROCK (SADOSHIMA *et al.*, 1992; ROTHEN-RUTISHAUSER *et al.*, 1998). Embora não se possa afirmar que tais microfilamentos organizem-se como fibras de estresse, em resposta a estímulos celulares, demonstramos previamente, em outro estudo, que o estiramento mecânico leva a Fak a associar-se com a fração citoesquelética de actina de miocárdio de ratos (FRANCHINI *et al.*, 2000), indicando que algum tipo de reorganização dos microfilamentos de actina deva ocorrer em miócitos cardíacos e explique a ativação da Fak, induzida pelo estiramento pulsátil, e o efeito da Citocalasina D sobre a sinalização da Fak em MVRN. Entretanto, esta hipótese necessita ser confirmada pela demonstração de uma interação direta da Fak com microfilamentos de actina.

Nossa demonstração, no presente estudo, que a RhoA está co-localizada com a Fak na banda A sarcomérica de MVRN não estirados, sugere que a ativação de Fak e RhoA, induzida pelo estiramento, está relacionada com a localização de ambas neste sítio sarcomérico. Entretanto, em nosso modelo de cultura em monocamada, o sarcolema pode estar muito próximo ao aparato de miofilamentos. Assim, a co-localização das proteínas Fak/RhoA ao nível da banda A, deve representar uma localização subsarcolêmica de uma ou ambas proteínas. Vale ressaltar, que estudos recentes demonstraram (KAWAMURA *et al.*, 2003) que a localização da RhoA em cavéolas é necessária para a ativação dessa proteína, induzida pelo estiramento em miócitos cardíacos. Ademais, mostrou-se que, após o estiramento de MVRNs, a RhoA dissocia-se do compartimento caveolar por um mecanismo que parece requerer a organização do citoesqueleto de actina. Sendo assim, como a Fak transloca para estruturas subcelulares como costâmeros e núcleo, podemos assumir que o citoesqueleto de actina também esteja envolvido na sua translocação ao longo de estruturas subcelulares dos miócitos cardíacos.

Trabalhos recentes têm demonstrado que a RhoA desempenha um papel importante na contratilidade miocardial, muito provavelmente devido a uma influência direta sobre o aparato contrátil, dependente da ativação da ROCK (HU & LEE, 2003). Esses dados estão de acordo com a indicação de que a Citocalasina D deva afetar a contratilidade do miócito cardíaco, por uma interação direta com o miofilamento de actina sarcomérica. Dessa maneira, a sinalização de

RhoA/ROCK deve, possivelmente, influenciar a atividade da Fak pelo seu efeito direto sobre a contratilidade dos miofilamentos.

Além de seu papel na ativação, seja direta ou indiretamente, de proteínas sinalizadoras citosólicas, muitos estudos têm fornecido evidências de que a RhoA desempenha um papel crucial no controle da transcrição gênica muscular durante o desenvolvimento da hipertrofia dos miócitos cardíacos, induzida pelo estiramento mecânico e por agonistas (AOKI *et al.*, 1998; AIKAWA *et al.*, 1999; YAMAKAWA *et al.*, 2000). Os resultados apresentados neste trabalho, confirmam a importância da atividade da RhoA na regulação da β-MHC em MVRN, induzida pelo estiramento, e também indicam que a influência da RhoA na expressão da β-MHC, em resposta ao estiramento, é mediada pela ativação da Fak. Esse fato pode ser explicado pela estreita dependência da ativação da Fak, induzida pelo estiramento, sobre os mecanismos de sinalização de RhoA e também pela nossa demonstração que a inibição da sinalização da Fak, pela presença de sequências antisenses de Fak e RhoA em MVRN, atenuou fortemente a expressão da β-MHC induzida pelo estiramento nessas células.

A análise por imunocitoquímica e microscopia confocal revelou que MVRN estirados por 2 hs mostram um padrão distinto de marcação nuclear, indicando que o estiramento mecânico induz a Fak a translocar para o núcleo dos miócitos cardíacos. Translocação similar para o núcleo foi recentemente reportada em miócitos cardíacos de ratos espontaneamente hipertensos infartados, sugerindo que a Fak possa desempenhar um papel importante na regulação do processo nuclear em resposta ao estiramento mecânico (YI *et al.*, 2003). Outras evidências da translocação nuclear da FAK foram obtidas em estudos que constataram a presença de fragmentos amino-terminais da FAK em núcleos de células endoteliais durante a apoptose (VAN De WALTER *et al.*, 1999; LEVKAU *et al.*, 1998), e em células apoptóticas de glioblastomas (JONES *et al*, 2001). Em células endoteliais, sabe-se que a clivagem da FAK, e conseqüente obtenção de fragmentos amino-terminais estão vinculadas à ruptura das adesões focais que ocorre durante a apoptose (GERVAIS *et al.*, 1998; LEVKAU *et al.*, 1998; LOBO & ZACHARY, 2000).

Em conjunto, estes achados vinculam a presença da FAK no compartimento nuclear com a viabilidade celular e indicam que a Fak possa ser não apenas uma importante proteína sinalizadora em eventos precoces de ativação de vias de sinalização, mas também uma proteína essencial na regulação da expressão gênica.

Quanto aos mecanismos que poderiam ser determinantes na translocação nuclear da FAK pouco se conhece, no entanto, apesar de não conter um sinal clássico para localização nuclear, é possível que a FAK seja capaz de se associar com proteínas carreadoras que transitam entre o citoplasma e o núcleo, e, assim, ser importada para tal compartimento e nele exercer suas funções, tal como uma variedade de proteínas citoplasmáticas (CLARK *et al*., 2002, YI *et al*, 2003).

Estudos adicionais são necessários a fim de ampliar o conhecimento sobre os mecanismos que determinam a translocação nuclear da FAK, bem como elucidar as funções desempenhadas por esta quinase neste compartimento.

Capítulo 3

As indicações de que a ROCK, a proteína efetora da RhoA, seria ativada pelo estímulo mecânico foram confirmadas inicialmente em nosso trabalho realizado em miocárdio de ratos, dando indícios da participação da proteína ativadora de RhoA, a Dbl3, antes mesmo que se pensasse na importância desta via de sinalização Dbl/Rho/ROCK na ativação da Fak, e sua participação na regulação da expressão gênica diferencial em células de miócitos cardíacos submetidas a estímulo hipertrofiantes.

Esse estudo forneceu evidências que a sobrecarga pressora induz uma rápida ativação da ROCK em miocárdio de ratos adultos. Como essa ativação foi concomitante com o aumento na associação das proteínas ROCK/RhoA e RhoA/Dbl3, nossos dados também indicaram que a rápida ativação da ROCK no miocárdio pode ser mediada pelo complexo Dbl3-RhoA. Os dados funcionais foram complementados com análises estruturais, utilizando técnicas de imunohistoquímica e imunohistoquímica estrutural, a fim de se obter informações acerca da distribuição e localização de ROCK e RhoA em miócitos cardíacos. Análises de microscopia confocal de imunofluorescência de secções de

ventrículos esquerdos de ratos adultos e miócitos cardíacos de ratos adultos isolados, mostraram que a sobrecarga pressora aumentou o aparecimento de agregados de ROCK, tão bem como de RhoA, regularmente arranjados ao longo do eixo longitudinal de miócitos cardíacos.

A marcação diferencial de ROCK e RhoA em miócitos cardíacos de corações submetidos a sobrecarga, foi confirmada por imunohistoquímica estrutural utilizando partículas de ouro, onde pôde ser visto o aparecimento de agregados de ROCK e RhoA em regiões específicas, tais como linha Z, estruturas tipo túbulo T, disco intercalar e áreas sarcolêmicas e subsarcolêmicas. De uma maneira geral, estes dados sustentam a idéia de que a carga induz uma rápida reunião e ativação do complexo de sinalização Dbl-Rho-ROCK em estruturas compromissadas com a transmissão de força em miócitos cardíacos de ratos adultos.

Nossa demonstração no presente trabalho que a sobrecarga pressora induziu as proteínas ROCK e RhoA a se co-localizarem e agregarem em estruturas subcelulares específicas, concomitante com sua associação detectada por ensaios de co-imunoprecipitação, sugere que a ativação delas seja dependente, não apenas de sua interação com proteínas ativadoras anteriores na cascata de sinalização, mas também, que seja dependente de seu recrutamento para um compartimento subcelular específico.

A ROCK contém múltiplos domínios, incluindo um quinase, um domínio de ligação à Rho e um C-terminal, que poderia direcionar esta enzima à membrana ou ao citoesqueleto de actina (ISHIZAKI *et al.*, 1996). Estudos realizados em diferentes modelos experimentais tem confirmado o direcionamento da ROCK para proteínas citoesqueléticas (FUJITA *et al.*, 1997; NAKANO *et al.*, 1999). A localização em estruturas subcelulares específicas poderia conferir à ROCK a habilidade para contribuir com mecanismos de sinalização que envolvessem a membrana celular e o citoesqueleto. Dessa maneira, poderíamos inferir que a ativação induzida pela sobrecarga e o direcionamento da ROCK e da RhoA para a linha Z, disco intercalar e área subsarcolêmica, ou seja, estruturas que suportam e

transmitem forças mecânicas, poderia indicar um papel para essa enzima na transdução mecanobioquímica em miócitos cardíacos.

Diversos trabalhos têm demonstrado o papel do complexo de sinalização RhoA-ROCK na ativação da regulação gênica, sarcomerogênese e hipertrofia do miócito cardíaco induzida por estiramento ou por agonistas (AIKAWA *et al.*, 1999; CLERK E SUGDEN, 2000; HOSHIJIMA *et al.*, 1998; KUWAHARA *et al.*, 1999; MORISSETE *et al.*, 2000; SAH *et al.*, 2000; SUEMATSU *et al.*, 2001; WEI *et al.*, 2001). Nossos dados indicam uma rápida associação RhoA-ROCK e ativação da ROCK em sítios específicos, sugerindo que essa enzima desempenha um papel em eventos iniciais disparados pelo aumento da carga de trabalho em miócitos cardíacos.

A ROCK parece contribuir para várias características da hipertrofia celular miocárdica, incluindo aumento no tamanho da célula, organização sarcomérica e indução da expressão de genes fetais como ANF e β-MHC (YANAZUME *et al.*, 2002). No entanto, ainda se desconhece o mecanismo pelo qual essa proteína consegue mediar esses efeitos, ou seja, se eles são influenciados indiretamente pelo papel da ROCK na organização citoesquelética induzida por estresse, ou pelo seu efeito direto nas múltiplas vias de sinalização.

Vários efetores da RhoA têm sido identificados ao lado da ROCK, incluindo a citron quinase, proteína quinase N, p140mDia e rotequina (KAIBUCHII *et al.*, 1999). Embora a contribuição relativa desses efetores de RhoA ainda não esteja clara, é possível que eles desempenhem papéis distintos no mecanismo de sinalização iniciado pela RhoA. Nesse contexto, estudos recentes (MORISSETE *et al.*, 2000) demonstraram que a proteína quinase N regula a transcrição gênica do ANF em miócitos cardíacos, através de um elemento responsivo a soro. Por outro lado, também tem sido mostrado (YANAZUME *et al.*, 2002) que a via Rho/ROCK contribui para a hipertrofia do miócito cardíaco induzida por agonistas adrenérgicos, através da ativação de ERK e GATA-4, sugerindo que os efeitos da RhoA sobre a regulação gênica são mediados por mecanismos e efetores múltiplos.

Nossos resultados indicam que a ativação de ROCK induzida pela sobrecarga é transiente, com um pico de sobrecarga pressora a 30 min, embora a associação Dbl-RhoA e, presumivelmente, a atividade de RhoA, esteja ainda aumentada com 60 min de sobrecarga pressora. Esses achados podem indicar que, após o período inicial, quando a ROCK é o maior efetor da RhoA, outros efetores poderiam ser ativados e desempenhariam um papel no efeito da RhoA na resposta miocardial à sobrecarga pressora. Essa hipótese necessita ser confirmada a partir de estudos adicionais envolvendo efetores conhecidos da RhoA, diferentes da ROCK, em animais submetidos ao mesmo modelo de sobrecarga pressora.

Novos estudos envolvendo a real participação das proteínas RhoA e da ROCK na fosforilação da Fak serão necessários, a fim de determinar se essa ativação se faz através da regulação positiva de proteínas efetoras, ou através da regulação negativa de proteínas capazes de inibir a ativação da Fak, como tirosinas fosfatases.

7. CONCLUSÃO

Os resultados apresentados neste trabalho fortalecem o papel da Fak como um sensor biomecânico em miócitos cardíacos, capaz de responder a pequenas alterações na carga. Também de grande importância, os dados obtidos no presente estudo demonstram que o complexo RhoA/ROCK é fundamental na ativação da Fak pelo estiramento mecânico em miócitos cardíacos, contribuindo para o entendimento, não somente sobre o mecanismo fundamental de ativação desta proteína no miócito cardíaco, mas também sobre a significância fisiológica do processo mediado pela Rho no miocárdio.

Nossos achados também sugerem que a cascata de sinalização iniciada por RhoA/ROCK/Fak/Src, em resposta à estimulação contínua pelo estiramento mecânico, coordena a maquinaria de sinalização celular que controla a expressão gênica associada com a hipertrofia do miócito cardíaco.

Baseado nos resultados obtidos, o presente estudo abre novas perspectivas de pesquisa quanto à descoberta do mecanismo de ativação da Rho, seu efeito direto sobre a regulação da Fak e sua relação com a resposta hipertrófica.

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9. ANEXOS

Em anexo encontram-se

1. Manuscrito relacionado a atividades paralelas desenvolvidas durante o período de tese.

FRANCHINI, K. G.; TORSONI, A. T.; SOARES, P. H. A.; SAAD, M. J. A. Early activation of the multicomponent signaling complex associated with adhesion kinase induced by pressure overload in the rat heart. **Circ Res.** 87: 558-565, 2000.

2. *I*- Confirmação de recebimento do artigo intitulado "RHOA/ROCK SIGNALING IS CRITICAL TO FAK ACTIVATION BY CYCLIC STRETCH IN CARDIAC MYOCYTES", submetido à revista American Journal Physiology: Heart and Circulatory Physiology, e *II*- apreciação do editor.

Early Activation of the Multicomponent Signaling Complex Associated With Focal Adhesion Kinase Induced by Pressure Overload in the Rat Heart

Kleber G. Franchini, Adriana S. Torsoni, Paulo H.A. Soares, Mario J.A. Saad

Abstract—Mechanical overload elicits functional and structural adaptive mechanisms in cardiac muscle. Signaling pathways linked to integrin/cytoskeleton complexes may have a function in mediation of the effects of mechanical stimulus in myocardial cells. We investigated the tyrosine phosphorylation and the assembly of the multicomponent signaling complex associated with focal adhesion kinase (Fak) and the actin cytoskeleton in the overloaded myocardium of rats. Pressure overload induced a 3-fold increase in Fak tyrosine phosphorylation within 3 minutes after a 60-mm Hg rise in aortic pressure. A pressure stimulus that lasted for 60 minutes was accompanied by a 5-fold increase in the amount of tyrosine-phosphorylated Fak, and a stimulus as low as 10 mm Hg doubled the amount of tyrosine-phosphorylated Fak in the myocardium within 10 minutes. Pressure overload also induced a time-dependent association of actin with Fak and an increase in the amount of Fak detected in the cytoskeletal fraction of the myocardium. These events were paralleled by c-Src activation and binding to Fak and by an association of Grb2 and p85 subunit of phosphatidylinositol 3-kinase with Fak. Erk1/2 and Akt, two possible downstream effectors of Fak via Grb2 and phosphatidylinositol 3-kinase, were also shown to be activated in parallel with Fak. These findings show that pressure overload induced a rapid activation of the Fak multiple signaling complex in the myocardium of rats, which suggests that this mechanism may have a role in mechanotransduction in the myocardium. (***Circ Res.* 2000;87:558-565.)**

Key Words: myocardium ■ signaling ■ rats ■ kinase

Mechanical stress is the major factor responsible for functional and structural adjustments of the myocardium in response to increased workload.[1] Mechanosensitive ion channels and the release of autocrine and paracrine factors have been suggested to act as potential mechanisms that link mechanical stimuli to cellular responses in the myocardium.[2–4]

Mechanical stimuli may also trigger cellular signaling mechanisms through the cytoskeleton via the elastic coupling to sites such as plasma membrane, internal organelles, or nucleus.[5–7] In addition, the filamentous cytoskeletal network provides a scaffold where signaling proteins can anchor and become involved in signal transduction pathways.[8,9] The activation of signaling systems associated with cytoskeleton in tissues is fundamentally dependent on the clustering of transmembrane integrins that act as linkers between extracellular matrix proteins and the intracellular cytoskeletal scaffold. 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. These regions are rich in a variety of signaling molecules, including focal adhesion kinase (Fak), c-Src family kinases, guanine nucleotide exchange factors, Ras family proteins, phosphatidylinositol 3 (PI3)-kinase, and mitogen-activated protein kinases.[8,9] After mechanical stimulation, integrin clustering and engagement lead to a marked increase in tyrosine phosphorylation and the recruitment of several cellular proteins to the actin meshwork, in particular including Fak.[10,11] The precise mechanism that links integrin to Fak activation is unknown, although it is clear that integrin clustering mediates Fak autophosphorylation, predominantly at Tyr397.[12] After autophosphorylation, additional tyrosine residues of Fak are phosphorylated through the action of c-Src family kinases that bind to Fak at Tyr397 via their Src homology 2 (SH2) domains. This leads to the binding of other SH2 domain proteins such as PI3 kinase[13] and the Grb2/Sos complex,[14] which can then activate signaling pathways that are involved in multiple cellular processes.

Multiple integrins are expressed in the heart and may participate in biological processes such as intracellular pH regulation and hypertrophic growth.[15–17] In addition, mechanical stimuli such as hypotonic cell swelling and pulsatile stretch have been shown to stimulate Fak tyrosine phosphorylation.[18,19] In the myocardium of cats, a 4-hour period of pressure overload leads to tyrosine phosphorylation and activation of c-Src and its redistribution from cytosol to the

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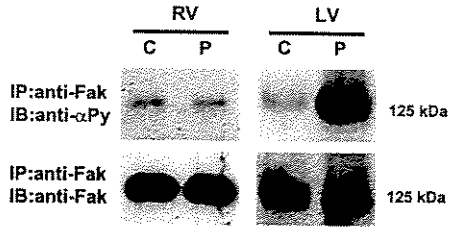

Figure 1. Load-induced p125^{Fak} tyrosine phosphorylation. p125^{Fak} was immunoprecipitated with anti-Fak polyclonal antibody from 200 μg protein from the left (LV) and right (RV) ventricles of unloaded and pressure-overloaded rat hearts (increases of ≈60 mm Hg in ascending aorta blood pressure during 60 minutes) and blotted with anti-phosphotyrosine (anti-αPy) or anti-Fak antibodies. The results represent 1 of 3 independent trials that gave nearly identical results. C indicates control; P, pressure overloaded; IP, immunoprecipitation; IB, immunoblotting.

cytoskeletal compartment.[20] However, in this study, the authors were unable to show substantial levels of tyrosine phosphorylation of Fak.

Although this evidence suggests that the Fak signaling complex is activated by and contributes to the adaptive myocardial responses to mechanical stimuli, a clear demonstration of this relationship is still lacking. In the present study, we examined the phosphorylation and activation of the Fak signaling complex in the rat heart during acute pressure overload produced through controlled constriction of the transverse aorta. Moreover, possible downstream pathways mobilized by the Fak signaling complex were explored.

Materials and Methods

Antibodies and Chemicals

Polyclonal rabbit anti-Fak, Grb2, Erk1/2, and monoclonal mouse anti-phosphotyrosine, anti–phospho-Erk1/2 (Thr202/Tyr204), and anti–c-Src antibodies were obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti–phospho-c-Src (Tyr416) was obtained from BioSource International, Inc. Polyclonal rabbit anti-p85 subunit of PI3 kinase was from Upstate Biotechnology. Polyclonal rabbit anti-Akt and anti–phospho-Akt (Ser473) were from New England Biolabs, Inc. Monoclonal mouse anti–nonsarcomeric α-actin was from Zymed. Affinity-purified rabbit anti-mouse IgG was from DAKO. [^{125}I]Protein A and [γ-^{32}P]ATP were from Amersham. Protein A–Sepharose 6 MB was from Pharmacia. All other reagents were from Sigma Chemical Co.

Animals

Adult male Wistar rats (n=60; weight 180 to 220 g) were obtained from the animal facilities of the university. All procedures and care of the rats were in accordance with institutional guidelines for the use of animals in research.

Pressure overload was induced in pentobarbital-anesthetized rats (50 mg/kg IP) through constriction of the transverse aorta with an adjustable clamp. After anesthesia was induced, the animals were maintained under controlled temperature and ventilation. The aortic and vagus nerves were sectioned bilaterally to minimize the influence of neural reflex on hemodynamics during aortic constriction. The transverse aorta was accessed through the second left intercostal space. A customized adjustable clamp was placed around this vessel, after which the thoracic cavity was closed. After stabilization (≈20 minutes), pressure overload protocols were started with adjustment of the aortic clamping while blood pressure signals from above and below the constriction were monitored. The experimental protocols included sustained (≈60 mm Hg; 3 to 60 minutes) and stepwise (from 10 to 30 mm Hg; 10 minutes) increases in the ascending aorta blood pressure. At the end of the blood pressure–recording period, the ventricles were rapidly removed, minced coarsely, and homogenized. Sham animals were prepared in the same way except for the aortic constriction.

Protein Analysis With Immunoblotting

Aliquots of whole extracts or immunoprecipitated proteins that contained equal amounts of total protein were treated with Laemmli's sample buffer and underwent SDS-PAGE. The nitrocellulose membranes with transferred proteins were incubated with specific antibodies and [^{125}I]Protein A. Band intensities were quantified through optical densitometry of the developed autoradiographs.

Isolation of Cardiac Cytoskeleton

Cardiac cytoskeletal preparations were obtained from 100 mg ventricular tissue as described previously.[20] Samples of the cytoskeletal fraction and of the soluble fraction obtained through ultracentrifugation underwent SDS-PAGE, were transferred to nitrocellulose membranes, and were blotted with anti-Fak antibody.

Immune Complex Tyrosine Kinase Reactions

Tyrosine kinase activity associated with the immune complex of the anti-Fak antibody was assayed with denatured rabbit muscle enolase used as substrate.[21] After SDS-PAGE, the proteins were transferred to nitrocellulose membranes, and the ^{32}P-enolase band (≈46 kDa) was visualized and quantified with autoradiography.

Statistical Analysis

The data are presented as mean±SEM. Differences between the mean values of the densitometric readings were tested with 1-way ANOVA for repeated measurements and Bonferroni's multiple-range test. A value of $P<0.05$ indicated statistical significance.

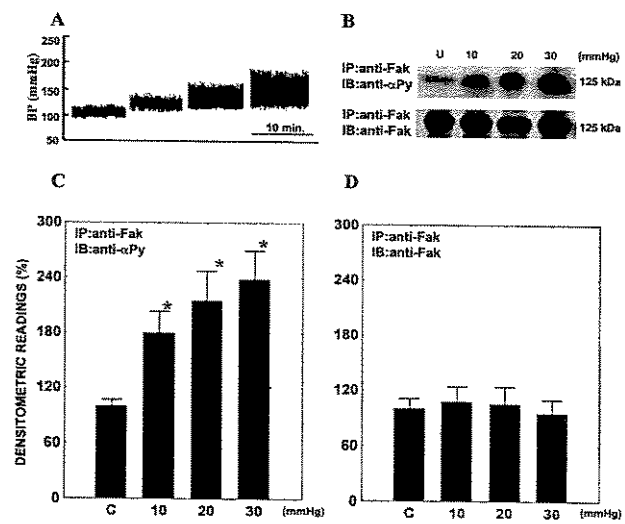

Figure 2. Relationship between pressor stimulus and p125^{Fak} tyrosine phosphorylation in rat heart. A, Representative stepwise increases in ascending aorta blood pressure (BP). Aortic constriction was adjusted to increase the ascending aorta mean blood pressure by 10, 20, and 30 mm Hg in individual rats. B, Representative blot showing p125^{Fak} tyrosine phosphorylation and the amount of p125^{Fak} recovered from unloaded (U) and pressure-overloaded myocardial tissue extracts. C, Average values (5 experiments) of the percent changes in p125^{Fak} tyrosine phosphorylation compared with control values quantified with scanning densitometry. D, Average values of the percent changes in the amount of p125^{Fak} recovered from heart homogenates. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting.

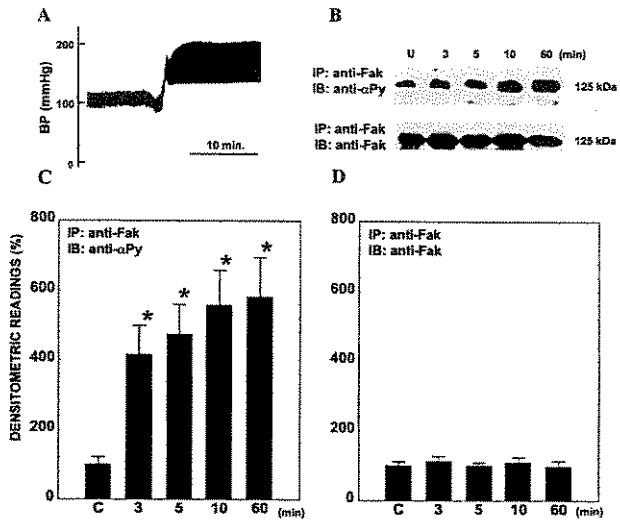

Figure 3. Time course of p125^{Fak} tyrosine phosphorylation induced by stable pressure increases. A, Representative example of a 20-minute aortic blood pressure (BP) increase (≈60 mm Hg). B, Representative blot showing p125^{Fak} tyrosine phosphorylation and the amount of p125^{Fak} recovered from myocardial tissue extracts of unloaded and pressure-overloaded hearts after 3 to 60 minutes. C, Average values (7 experiments) of the percent changes in p125^{Fak} tyrosine phosphorylation. D, Average values of the percent change in p125^{Fak} recovered from heart homogenates. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Pressure Overload Induces Tyrosine Phosphorylation and Activation of p125^{Fak} and p60^{Src}

The tyrosine phosphorylation of Fak is closely related to its kinase activity.[12,13] Figure 1 shows that acute pressure overload increased the p125^{Fak} phosphotyrosine content mainly in the left ventricle, whereas a comparable amount of this protein was found in both ventricles. Next, we studied p125^{Fak} tyrosine phosphorylation induced with stepwise increases in aortic blood pressure (Figure 2). p125^{Fak} phosphotyrosine content increased to ≈180% in response to pressure increases of 10 mm Hg. Additional increases were seen in hearts when the constriction increased aortic pressure by 20 and 30 mm Hg (to ≈220% and ≈240%, respectively). The time course of p125^{Fak} tyrosine phosphorylation was examined in hearts subjected to sustained increases in aortic pressure of ≈60 mm Hg (Figure 3). p125^{Fak} phosphotyrosine content increased 3-fold within 3 minutes and 5-fold within 60 minutes of continuous and stable pressure increases. Parallel immunoblots with anti-Fak antibody revealed that the amount of p125^{Fak} in the myocardium remained unaltered during this period.

After activation, Fak autophosphorylates Tyr397, creating a binding site for SH2 domain of c-Src. c-Src kinase is then activated with phosphorylation on Tyr416.[12] The activation of c-Src also depends on the dephosphorylation of constitutively phosphorylated Tyr527.[12] Coimmunoprecipitation assays with anti-Fak and anti–c-Src antibodies showed only a weak binding of p60$^{c\text{-}Src}$ to Fak in the myocardium of control rats (Figure 4A). Pressure overload increased the p60$^{c\text{-}Src}$ binding to Fak, in parallel with its tyrosine phosphorylation. We next examined whether pressure overload also induces tyrosine phosphorylation and activation of p60$^{c\text{-}Src}$. Immunoprecipitation experiments with anti–c-Src antibodies showed that the tyrosine phosphorylation of p60$^{c\text{-}Src}$ increased in overloaded compared with unloaded hearts (Figure 4B). This finding could imply either activation or inactivation, depending on whether the phosphorylation was at Tyr416 or Tyr527.[12] We then examined the c-Src tyrosine phosphorylation with a phosphospecific antibody against c-Src (Tyr416). As demonstrated in Figure 4C, pressure overload induced a rapid and sustained increase in phospho-c-Src (Tyr416), indicating that p60$^{c\text{-}Src}$ is activated in parallel with Fak. This occurred while the amount of p60$^{c\text{-}Src}$ remained constant (Figure 4D).

Further indication of load-induced Fak/c-Src association and c-Src activation was provided by experiments in which the kinase activity of the immune complex of anti-Fak antibody was tested against rabbit denatured muscle enolase, a substrate for c-Src but not for Fak.[21] Figure 5 shows that there was negligible tyrosine kinase activity in the immune complex of anti-Fak antibody in unloaded hearts. However, the enolase phosphorylation increased greatly (to ≈150% after 3 minutes and to ≈350% after 60 minutes of pressure overload) when incubated with immunoprecipitates of overloaded hearts.

Redistribution of p125^{Fak} to the Cytoskeletal Compartment Induced by Pressure Overload

Because the migration of Fak to the actin meshwork is a crucial event for Fak activation,[8,9] we performed experiments to detect the association of actin with p125^{Fak}. The membranes used to detect p125^{Fak} were stripped and blotted with antibody against nonsarcomeric α-actin. As shown in Figure 6A, overloaded hearts showed a time-dependent coimmunoprecipitation of actin with p125^{Fak}. The pressure-induced migration of p125^{Fak} to the cytoskeletal compartment was further suggested by experiments with cytoskeletal fraction preparations obtained with differential centrifugation. A substantial increase in the amount of p125^{Fak} was detected in the cytoskeletal fraction of homogenates from overloaded myocardial tissue (Figure 6B).

Pressure Overload Induces the Association of Fak With Grb2 and Activation of ERK1/2

Phosphorylation of Fak Tyr925 by c-Src confers a site for Grb2 binding, which potentially links integrin/Fak signaling to the Ras/mitogen-activated protein kinase pathway.[14] As shown in Figure 7A, acute pressure overload increased the amount of Grb2 binding to Fak in parallel with activation of the Fak/c-Src complex.

We next investigated whether activation of the Fak/c-Src complex was paralleled by the activation of Erk1/2, a possible downstream effector of Fak via Grb2 binding.[21] Western

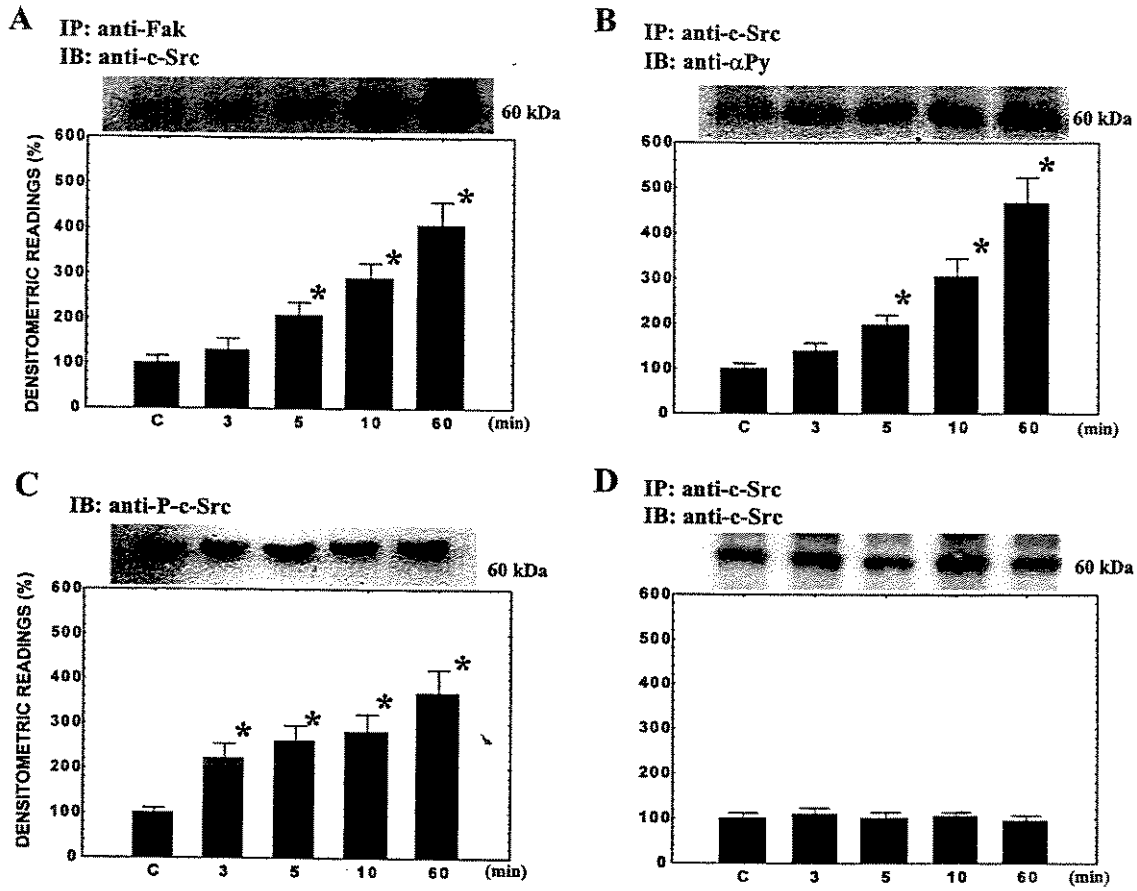

Figure 4. A, Average values (4 experiments) and representative blot showing the results of Fak/Src coimmunoprecipitation experiments. B, Representative blot and average (n=5) of the time course relationship of $p60^{Src}$ tyrosine phosphorylation induced by aortic pressure increases of ≈60 mm Hg detected with monoclonal antibody against c-Src phosphotyrosine residues. C, Pressure overload–induced c-Src activation. Representative blot showing the amount of $p60^{c\text{-}Src}$ detected by the phosphospecific antibody against c-Src (Tyr418). The graphic shows the average of 6 experiments. D, Representative blot of the amount of $p60^{Src}$ in the myocardium of unloaded and loaded hearts and the average of 5 experiments. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting.

blotting with anti–phospho-Erk1/2 (Thr202/Tyr204)–specific antibody revealed an increase and presumably an activation of this enzyme within 5 minutes (to ≈180%), with a maximal activation being achieved by 10 minutes (to ≈240%) of pressure overload. The values of phospho-Erk1/2 returned toward the baseline values after 60 minutes of pressure overload (Figure 7B). Western blotting with an antibody for Erk1/2 that detects both the phosphorylated and unphosphorylated forms of the kinases showed that myocardial Erk1/2 levels were similar in the heart studied at various periods after aortic constriction (Figure 7C).

Pressure Overload Induces the Association of Fak With p85 Subunit of PI3 Kinase and Activation of Akt

In addition of being a site for c-Src binding, Tyr397 of Fak has been identified as the major site for binding of PI3 kinase, whose inositol lipid products are key mediators of multiple intracellular signaling.[13,22] As shown in Figure 8A, pressure overload induced a rapid increase in the association of Fak with p85 subunit of PI3 kinase (to ≈180% within 3 minutes), increasing to ≈360% within 1 hour of aortic constriction. These results indicated that pressure overload induced a recruitment and a possible activation of this enzyme.

One of the multiple downstream signaling molecules regulated by 3′-phosphorylated phosphatidylinositides is the serine/threonine protein kinase Akt.[22] In many cases, the activation of Akt is initiated by the binding of 3 phosphoinositides to its pleckstrin homology domain, translocation from the cytoplasm to the plasma membrane, and subsequent phosphorylation by upstream kinases, including PDK1. In the present study, we also examined whether pressure overload activates Akt. Western blotting with anti-Akt (Ser473)-phosphospecific antibody revealed an increase and presumably an activation of this enzyme within 3 minutes (to ≈200%) after the beginning of pressure overload stimulus. The amount of phospho-Akt (Ser473) remained increased to ≈300% up to 1 hour of pressure overload (Figure 8B), whereas the amount of Akt remained unchanged during this period (Figure 8C).

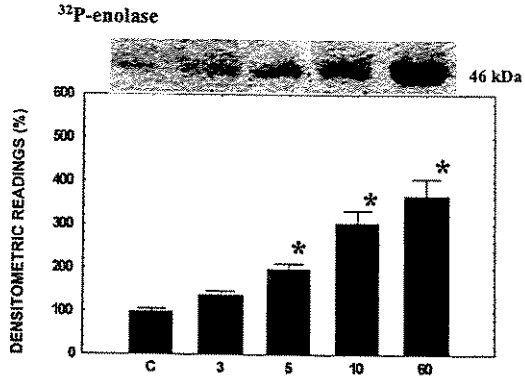

Figure 5. Tyrosine kinase activity of the $p125^{Fak}$ immune complex toward denatured rabbit muscle enolase. The kinase activity of $p125^{Fak}$ was measured in vitro in $p125^{Fak}$ immunoprecipitates of myocardial homogenates of hearts subjected to pressure overload (3 to 60 minutes) using acid-denatured enolase as a substrate. The ^{32}P-labeled enolase was quantified with scanning densitometry. *$P<0.05$ compared with unloaded hearts.

Discussion

The data presented here support the conclusion that load induces a rapid assembly and activation of the multicomponent signaling complex associated with Fak in the rat heart. The close relationship between increased load and Fak/c-Src activation and the concurrent activation of Erk1/2 and Akt, 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.

Load-Induced Activation and Assembly of the Fak Multicomponent Signaling Complex

Pressure overload induced a rapid increase (3 minutes) in the myocardial $p125^{Fak}$ phosphotyrosine content, preferentially in the left ventricle. Further increases were observed after 1 hour of augmented workload. This was accompanied by a load-induced association of c-Src with Fak, as indicated by the increase in the amount of $p60^{c\text{-}Src}$ detected in blots of immunoprecipitated Fak, and c-Src activation, as indicated by the increase in the amount of c-Src detected with phosphospecific antibody against c-Src (Tyr416) in overloaded compared with unloaded hearts. The idea that load induces a rapid activation of c-Src and its association with Fak was strengthened by the increased kinase activity detected in the immune complex of anti-Fak antibody toward the c-Src substrate enolase. Our study also provides evidence that load induces the association of the signaling molecules Grb2 and PI3 kinase to Fak and the migration of $p125^{Fak}$ to the cytoskeletal compartment. Presumably, this $p125^{Fak}$ migration was directed to the actin meshwork, as suggested by the time-dependent association of actin with Fak in overloaded hearts.

These results are in general consistent with data from noncardiac cells that demonstrate that mechanical stimulus activated the Fak/c-Src complex and its recruitment to the actin cytoskeletal meshwork, presumably at sites related to focal adhesion.[8,9] Cardiac myocytes contain structures known as costameres, which resemble focal adhesion complexes and have been suggested to be involved in the transduction of mechanical forces from cardiac myocyte surface.[23,24] Costameres are also rich in vinculin, talin, integrins, and a meshwork of actin that occur in register with Z lines.[23,24] However, a clear demonstration that Fak is localized or migrates to costameres on mechanical stimuli must be confirmed.

The clear relationship between the assembly and activation of the Fak multicomponent signaling complex with the load stimuli suggests that this mechanism may play a central role in the mechanotransduction during increased load in the myocardium. In this context, studies in cultured rat cardiomyocytes have shown that pulsatile stretch can activate Fak within 5 minutes after the beginning of the stretch.[19] As has

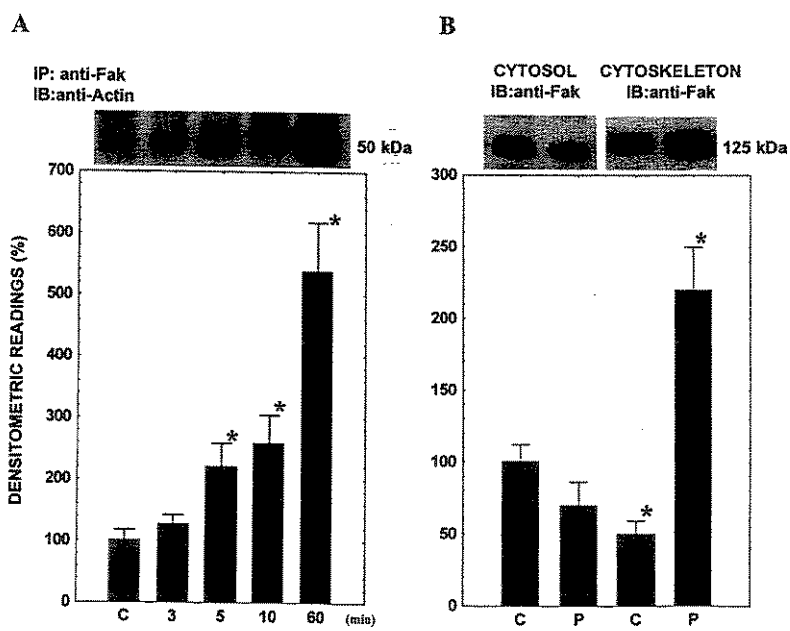

Figure 6. Pressure overload–induced Fak/actin association and redistribution of Fak from cytosolic to the cytoskeletal compartment. A, Representative blot and average values (5 experiments) of myocardial Fak/actin association induced by pressure overload. B, Representative blots and average values (4 experiments) from cytosolic and cytoskeletal fractions blotted with anti-Fak antibody. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting; P, pressure overloaded.

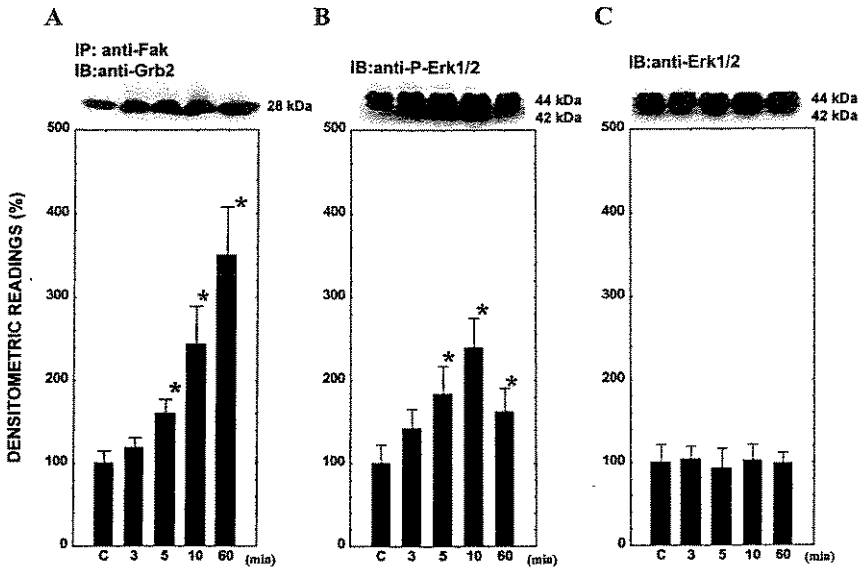

Figure 7. A, Representative blot and average values (6 experiments) of myocardial Grb2/Fak association induced by pressure overload. B, Pressure overload–induced Erk1/2 activation. Representative blot of the amount of Erk1/2 detected by the phosphospecific antibody against Erk1/2 (Thr202/Tyr204) and graphic showing the average of 5 experiments. C, Representative blot and average of Erk1/2 content in the myocardium homogenates. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting.

been shown in cultured cells,[10] it is possible that this rapid activation of the Fak/c-Src signaling complex is mediated via integrin, but Fak tyrosine phosphorylation and activation may also be elicited through a variety of nonintegrin cell surface receptors, including growth factor tyrosine kinases and G protein–coupled receptors.[9] However, Fak activation mediated by soluble factors has been shown to be short in duration, which favors the hypothesis that the activation of Fak seen in the present study is predominantly mediated via integrins.[8,9]

In contrast with the present results, previous studies[20] have failed to detect substantial phosphorylation of Fak in pressure-overloaded myocardium. One possible explanation for this discrepancy may be related to the fact that in previous studies, the tyrosine phosphorylation of Fak was examined after 4 hours of pressure overload, by which time the tyrosine phosphorylation of Fak may have vanished because of the actions of tyrosine phosphatases. However, this explanation is not consistent with data that show a persistent activation of nonreceptor tyrosine kinases for longer periods in overloaded myocardium.[20] Experiments with controlled aortic constriction for longer periods could clarify the difference between the results of the present study and those of previous studies. However, the maintenance of a stable and reliable preparation with aortic constriction for periods of >1 hour is difficult in anesthetized rats.

Load-Induced Activation of ERK1/2 and Akt

The activation of Fak has been shown to influence a variety of cellular functions, including the control of cell shape, growth, and survival.[8,9] In looking at possible downstream events regulated by the Fak signaling complex, we also showed that Erk1/2 and Akt were activated in parallel with activation of the Fak signaling complex. The rapid association of Fak with Grb2 and PI3 kinase, two intermediate steps between Fak and Erk1/2 and Akt activation, strengthened the idea that Fak/c-Src activation may be the upstream event of

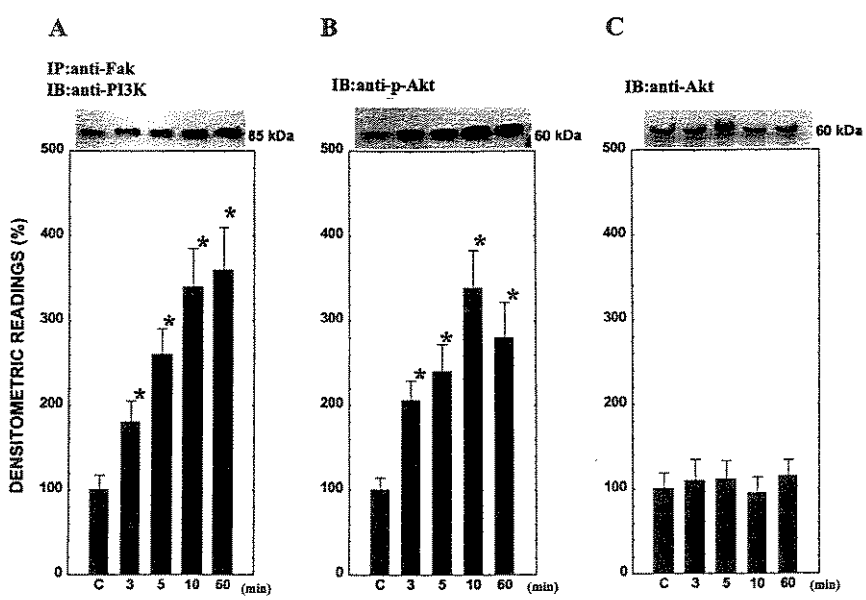

Figure 8. A, Representative blot and average (5 experiments) of myocardial p85 subunit of PI3 kinase/Fak association induced by pressure overload. B, Pressure overload–induced Akt activation. Representative blot of the amount of Akt detected by the phosphospecific antibody against Akt (Ser473) and graphic showing the average of 5 experiments. C, Representative blot and average of Akt content in the myocardium homogenates. *$P<0.05$ compared with unloaded hearts. IP indicates immunoprecipitation; IB, immunoblotting.

such a rapid activation of these kinases, as has been suggested previously.[21,25]

Erk1/2 regulates an extensive range of cellular processes, including gene transcription, cytoskeletal organization, metabolic homeostasis, cell growth, and survival.[26] The activation of Erk1/2 is likely to be an important pathway to the adaptation of myocardial cells to mechanical stimuli. To date, mechanical stretch of neonatal cardiac myocytes has been shown to rapidly (within 5 minutes) activate the Ras/Erk1/2 pathway.[27,28] Although the functional roles of these Erks in stretch-induced cardiac hypertrophy are unclear at the present, they may be important in the regulation of the expression of genes such as early responsive genes (eg, c-*fos*, c-*jun*, *egr*-1), the expression of which is rapidly and transiently upregulated in the myocardium and isolated cardiomyocytes subjected to mechanical stress. Moreover, the early activation of Erk1/2 has been suggested to contribute to the reexpression of fetal ventricle genes (eg, atrial natriuretic factor, β-myosin heavy chain, and skeletal muscle α-actin).[29]

Akt activation transduces signals that regulate multiple biological processes, including glucose metabolism, apoptosis, gene expression, and cell proliferation.[22] Studies have shown that Akt mediates the antiapoptosis effects of Fak.[25] In cardiac myocytes, it has been shown that Akt mediates β-adrenergic receptor–stimulated atrial natriuretic factor transcription.[30] To our knowledge, the present study is the first to show that Akt may be activated during the early response to pressure overload in the myocardium. The importance of the early activation of Akt during pressure overload can only be speculated. Fak-mediated activation of Akt is likely to be important for the overall response to increased load. Akt phosphorylates various intracellular substrates, thereby affecting metabolism,[31] protein synthesis,[32] cell survival/apoptosis,[33] and gene expression through the regulation of transcription factors,[34] which could mediate the cellular responses to increased load in the myocardium.

The present results do not exclude the possibility that mechanisms other than Fak/Src activation could be responsible for the activation of Erk1/2 and Akt during the early phase of cardiac response to pressure overload. To date, Erks and Akt have been shown to be activated through protein–tyrosine kinase and G protein–coupled receptors.[22,26] Therefore, further studies are necessary to confirm the importance of the Fak signaling complex in the activation of Erk and Akt.

In conclusion, pressure overload induced a rapid assembly of the Fak/Src signaling complex in the myocardium. This activation was shown to be roughly parallel to stimulus intensity and duration and to the activation of possible downstream pathways such as Erk1/2 and Akt activation. In consideration of the potential effects of this signaling system on multiple cellular functions such as ion transport, force transmission, metabolic pathways, intracellular molecular transport, survival, and gene expression, this complex may occupy a central position in the adaptive changes induced by increased load in the myocardium.

Acknowledgments

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