Focal adhesion kinase mediates MEF2 and c-Jun activation by stretch: Role in the activation of the cardiac hypertrophic genetic program

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Received 20 December 2004; received in revised form 5 May 2005; accepted 9 May 2005
Available online 14 June 2005
Time for primary review 16 days

Abstract

Objective: We have previously reported that myocyte enhancer factor-2 (MEF2) transcription factors and c-Jun are rapidly activated by pressure overload and that these events are involved in the early activation of the myocardial hypertrophic genetic program. In this study, we investigated whether focal adhesion kinase (FAK) mediates the activation of MEF2 and c-Jun by mechanical stress in isolated neonatal rat ventricular myocytes (NRVMs).

Methods: NRVMs were subjected to cyclic stretch up to 4 h and studied by immunoblotting, reverse transcriptase-polymerase chain reaction, laser confocal analysis, and reporter gene and electrophoretic mobility shift assays. Analysis was extended to NRVMs transfected with FAK-antisense oligodeoxynucleotide, treated with FAK/Src inhibitor PP2 or JNK/c-Jun inhibitor SP600125.

Results: Cyclic stretch increased c-Jun expression, JNK/c-Jun phosphorylation, and MEF2-DNA binding activity in NRVMs. Reporter gene assays indicated that the MEF2 site is critical to c-jun transcription in stretched cells. FAK-antisense transfection abolished MEF2 and c-jun promoter activation, while either FAK-antisense or PP2 treatment inhibited the stretch-induced c-Jun expression and JNK/c-Jun phosphorylation. Finally, treatment of NRVMs with the specific JNK/c-Jun inhibitor SP600125 significantly reduced the stretch-induced increase of atrial natriuretic factor promoter activity.

Conclusion: The present data indicate that FAK regulates the activation of MEF2 and JNK/c-Jun pathways, which in turn have a key role in the early activation of the hypertrophic genetic program by mechanical stress in cardiac myocytes.

Keywords: Hypertrophy; Signal transduction; Gene expression; Stretch

1. Introduction

Mechanical stress induced by hemodynamic overload plays a prominent role in the development of myocardial hypertrophy and maladaptive myocardial remodeling [1]. These phenotypic changes are accompanied by complex reprogramming of genes that contribute to substantial alterations of myocardial motor unit composition and energy metabolism [2,3]. Early mediators of hypertrophic transcriptional program include the transient activation of immediate-early genes, which encode transcription factors, such as c-Jun and c-Fos [2,3]. Several lines of evidence support the notion that the activation of these early genes is important to the subsequent transcription of the fetal gene program (ie, α-skeletal-actin, β-MHC, and atrial natriuretic peptide) which in turn plays a key role in adaptive growth as well as maladaptive changes of cardiac myocytes in response to mechanical stress [3,4,5].

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doi:10.1016/j.cardiores.2005.05.011
The transcriptional regulation of immediate-early genes is mediated by transcription factors such as Sp1, CTF, activator protein-1 (AP-1), and myocyte enhancer factor 2 (MEF2) [6]. Binding of the AP-1 complex (c-Fos/ATF or c-Jun/c-Jun dimer) or MEF2 in the c-jun promoter has been shown to result in the stimulation of its transcription [6,7]. To date, we have previously shown that the load-induced transcriptional regulation of c-jun in the myocardium is mediated by MEF2 transcription factors [8]. In addition, load-induced c-Jun expression in cardiac myocytes is also controlled by c-Jun N-terminal kinase (JNK), via increased protein stabilization caused by JNK-induced c-Jun phosphorylation at serine-63 [5]. Overall, these data support the notion that load-induced c-Jun expression involves a complex web of signaling mechanisms acting at transcriptional and post-translational levels. However, the mechanisms acting as upstream mediators of load-induced MEF2 or JNK activation and c-Jun expression in cardiac myocytes are yet unclear.

New understanding of signaling molecules associated with the various constitutive elements of contractile and cytoskeletal proteins are beginning to elucidate the mechanisms of mechano-sensing and mechano-transduction mechanisms in cardiac myocytes. Among the candidate molecules, much attention has been given to Focal Adhesion Kinase (FAK), a tyrosine kinase which is rapidly activated in cardiac myocytes by mechanical stress [9,10,11]. In addition, FAK is a critical component of the early up-regulation of ANF transcription induced by mechanical stress [11], implying that stretch-induced FAK activation controls cellular signaling machinery that coordinate load-induced gene expression in cardiac myocytes.

In the present study, we investigated whether FAK mediates the activation of MEF2 and c-Jun by mechanical stress in isolated neonatal rat ventricular myocytes (NRVMs) and whether these events are involved in the early activation of the myocardial hypertrophic genetic program.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Reagents

Polyclonal anti-FAK (sc-558), MEF2 (sc-313), JNK (sc-571), c-Jun (sc-1694), ERK (sc-153) and monoclonal anti-p-JNK (sc-6254), p-c-Jun (sc-822) and p-ERK (sc-7383) antibodies were from Santa Cruz. Polyclonal anti-p-FAK was from Biosource. All other grade chemicals were from Sigma.

2.2. Cell culture and stretching

Primary cultures of NRVMs from 1- to 2-day-old Wistar rats were prepared as previously reported [11]. 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) at 500,000/well. After 48 h, the medium was replaced with serum-free DMEM and 6 h later, NRVMs were stretched in a Flexercell FX-3000 strain unit to 115% of resting length at a frequency of 1 Hz for variable periods.

2.3. Preparation of nuclear extracts

This procedure was carried out as previously described [12]. Cells were collected in buffer A without Nonidet-P 40 (NP-40) [10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/mL of aprotinin]. Then the cells were pelleted by centrifugation for 5 min at 1000 rpm (4 °C) and the pellet was resuspended in 200 μL of buffer A with 0.1% NP-40 added. After incubation on ice for 15 min, the suspension was passed through a 26.5 G needle ten times and the nuclei were pelleted by centrifugation for 1 min at 12,000 g and 4 °C. The nuclear pellet was washed with buffer A twice and then incubated at 4 °C for 30 min in 40 μL of 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2.5 μg/mL of aprotinin. Nuclear debris was pelleted by centrifugation for 10 min at 4 °C and 12,000 g, and the supernatant of nuclear proteins was used for analysis.

2.4. Electrophoretic mobility shift assays (EMSA)

EMSA were performed as previously described [8]. MEF2 (GATCGCTCTAAAAATACCCCTGTGCG) and NF-κB (AGTTGAGGGACTTTCCCAGGC) DNA-binding site oligonucleotides were from Santa Cruz. The oligonucleotides were end-labeled with [32P]ATP (Amersham) and T4 polynucleotide kinase. The probes (5,7 μmol/L in final reaction volume) were incubated with 20 μg of nuclear extracts in a 20-μL reaction containing 1 μg poly(dl-dC), 50 mM NaCl, 5 mM MgCl2, 10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, and 2% glycerol for 20 min at room temperature. For competition studies, the extracts were incubated with 100 × excess of unlabeled oligonucleotides (specific — MEF2 DNA-binding oligo; unspecific — NF-κB DNA-binding oligo). For immuno-gel shift assays, the extracts were incubated with 1 μL of anti-MEF2 antibody 45 min before electrophoresis at 4 °C. The samples were then analysed on a non-denaturing 6% polyacrylamide gel and run at 400 V for 90 min at 4 °C. The dried gels were exposed and the bands were visualized by autoradiography.
2.5. Plasmid transfection and dual reporter gene assays

Plasmids containing the positions —225 to +150 of the murine c-jun promoter fused to the firefly luciferase gene (pJC6G3L, wild type or WT-c-jun) with mutations at the MEF2 (pJSXG3L or MEF2-mut-c-jun) and AP1-like (or ATF-1) (pJTXG3L or AP1-mut-c-jun) sites were generated by Dr. Ron Prywes (Columbia University, New York, NY) [13]. Rat ANF promoter luciferase reporter gene (ANF-LUC) was obtained from Dr. Mona Nemer (Institut de Recherches Cliniques de Montréal, Canada). NRVMs were co-transfected with 2 µg of ANF-LUC or c-jun promoter plasmids and 0.1 µg of the internal control SV40-renilla luciferase using Lipofectamine (Gibco) in serum-free medium for 24 h. NRVMs were washed with DMEM and maintained in DMEM containing 10% serum for 18 h, and then for 6 h in serum-free medium, stretched, lysed with Passive Lysis Buffer and assayed for luciferase activities as described by the manufacturer (Promega). All firefly luciferase values were normalized to renilla activities.

2.6. FAK-antisense oligodeoxynucleotide (ODN) transfection

This procedure was performed as previously described with minor modifications [8]. 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'-CGGCTAACCGAAGCTGTCACGCACGA-3') was used as control. All bases were obtained from Life Technologies and were phosphorothioate-protected. Forty eight hours after being plated or 24 h after c-jun promoter reporter genes transfections, NRVMs were serum-starved for 6 h and transfected with 1 µmol/L antisense or sense ODN and 12 µL lipopectamin in serum-starved DMEM without antibiotics (final volume — 1mL) for 6 h. NRVMs were washed with DMEM and maintained in DMEM containing 10% serum for 18 h, and then for 6 h in serum-free medium, stretched, and harvested for analysis.

2.7. FAK/Src and JNK/c-Jun pharmacological inhibition

Thirty minutes before stretching, cells were treated with the FAK/Src inhibitor PP2 (1 µmol/L) or the JNK/c-Jun inhibitor SP600125 (10 µmol/L), which were purchased from Calbiochem.

2.8. Isolation of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

NRVMs were homogenized in Trizol Reagent, and total RNA was isolated by precipitation with isopropl and then digested for 10 min with DNAs to remove any contaminating DNA as previously described [8]. A 6-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 rat c-jun gene (5'-GACCTTCTACGACGTGTCG-3' and 5'-CACGCACGCTACTGAGGC-3') or human beta-actin gene (5'-TTCTGAGCTCGGTGCTGC-3' and 5'-GCTACATATGCTACGACAGA-3'). Oligonucleotides were synthesized by Life Technologies. The amplification conditions consisted of denaturing at 94 °C for 2 min, annealing at 45 °C for 1 min, and extension at 72 °C for 2 min. The number of cycles was 25. PCR products were size-fractionated by agarose gel electrophoresis. After staining with ethidium bromide, DNA bands were visualized with a UV transilluminator.

2.9. Immunoblotting

Cells were lysed in assay lysis buffer (1% Triton, 10 mM Tris–HCl, pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 µg/mL aprotinin, 1 mM PMSF, 0.25 mM sodium orthovanadate). Insoluble material was removed by centrifugation for 20 min at 11,000 g, and samples were boiled with loading buffer and resolved by SDS-PAGE. Immunoblotting was performed using primary antibodies (against FAK, FAK-pY397, JNK, p-JNK, c-Jun, p-c-Jun, MEF2, ERK1/2 and p-ERK1/2) overnight at 4 °C, followed by exposure to [125I]Protein A (Amersham). Bands corresponding to protein were quantified by optical densitometry.

2.10. Confocal immunofluorescence analysis

This procedure was performed as previously described with minor modifications [8]. Cells were cultured and after the experimental protocol, were washed with PBS and fixed with paraformaldehyde containing 4% sucrose. The primary antibody used was anti-MEF2 (Santa Cruz). As a negative control, the cell samples were incubated without primary antibody. Appropriate FITC (Cy2)-conjugated secondary antibodies and phalloidin-rhodamine were used to visualize the specific proteins. The coverslips were inverted onto microscope slides, mounted in Vectashield (Vector Labs, Inc.) and sealed using nail polish. Fluorescently-labeled cells were then viewed using a laser scanning confocal microscope Zeiss.

2.11. Statistical analysis

Data are presented as mean±S.E.M. 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. Comparisons of luciferase activity among c-jun promoter constructs used a non-parametric Kruskal–Wallis test followed by a Wilcoxon signed rank test for pairwise comparisons.
3. Results

3.1. Cyclic stretch increases c-Jun expression and phosphorylation in NRVMs

Data shown in Fig. 1A, B summarize the influence of cyclic stretch in c-Jun expression and phosphorylation in NRVMs. c-Jun expression in NRVMs, evaluated by immunoblotting with anti-c-Jun antibody, increased by 2-fold at 30 min and remained stable up to 2 h of cyclic stretch (Fig. 1A). However, c-Jun phosphorylation at Ser-63 was markedly increased (~3.5-fold) as soon as 10 min and remained elevated, although at lower levels (~2-fold), up to 2 h of cyclic stretch (Fig. 1A). c-jun transcript (Fig. 1B) was increased after 10 min (~3-fold), peaked at 30 min (~5.2-fold) and remained elevated (~2.2-fold) up to 2 h of cyclic stretch.

3.2. MEF2 transcription factors control stretch-induced c-jun transactivation

To examine whether cyclic stretch induces transcriptional regulation of c-jun, NRVMs were transfected with firefly luciferase-fused plasmids containing wild-type c-jun promoter (WT-c-jun), a c-jun promoter mutated for the MEF2 site (MEF2-mut-c-jun), or a c-jun promoter mutated for the AP1-like site (AP1-mut-c-jun) (Fig. 2A). Cyclic stretch lasting for 2 h induced a 2-fold increase in the luciferase activity.
activity of cells transfected with WT-c-jun plasmid, but no change in luciferase activity was seen in cells stretched for 30 min (Fig. 2B), suggesting that the enhanced expression of c-jun transcripts detected in NRVMs at 30 min of cyclic stretch is mediated by post-transcriptional rather than transcriptional mechanisms. At 2 h of stretch, increased luciferase activity (~1.8-fold) was still seen in NRVMs transfected with AP1-mut-c-jun, while no change occurred in stretched cells transfected with MEF2-mut-c-jun. Noticeable, basal luciferase activity in extracts of non-stretched NRVMs transfected with AP1-mut-c-jun and MEF2-mut-c-jun were ~20-fold less than that of cells transfected with WT-c-jun (Fig. 2B).

Next we examined the expression and localization of MEF2, as well as whether it can be activated by cyclic stretch in isolated NRVMs. As shown in Fig. 3A, cyclic stretch lasting from 10 min up to 2 h produced no changes in MEF2 protein levels in NRVMs. Confocal microscopy imaging (Fig. 3B) showed that the anti-MEF2 antibody stained exclusively the nuclei of such cells, whether they were stretched or not. To address whether cyclic stretch activates MEF2, NRVMs nuclear extracts were analyzed by EMSA with an oligonucleotide containing the consensus binding DNA sequence for MEF2 (Fig. 3C). A consistent increase in DNA binding activity of MEF2 was observed after 1 and 2 h of cyclic stretch. The specificity of the DNA probe for MEF2 binding was confirmed by competition assays with unlabeled oligonucleotides, cold oligonucleotide containing consensus sequence for NF-κB and by supershift assay with the anti-MEF2 antibody. In general, these data reproduce in isolated cardiac myocytes our previous data obtained with overloaded rat left ventricle [8] and strengthened the notion that MEF2 is activated and plays an important role in c-jun promoter transactivation by mechanical stress.

3.3. FAK is an upstream regulator of stretch-induced MEF2 activation in NRVMs

FAK has been shown to be activated and to coordinate the activation of hypertrophic genetic program (atrial natriuretic factor) by mechanical stress in cardiac myocytes [11]. Accordingly, Fig. 4A shows representative immunoblots demonstrating that FAK is rapidly activated from 10 min up to 2 h after the onset of cyclic stretch (Fig. 4A). Because MEF2 transcription factors are important downstream effectors of the early gene activation in response to mechanical stimuli we investigated whether FAK and MEF2 are coordinately regulated during the initial response of NRVMs to cyclic stretch. For this purpose, NRVMs were
transfected with FAK-antisense ODN. The effectiveness of FAK-antisense ODN to reduce FAK protein expression was demonstrated by immunoblotting and laser confocal analysis (Fig. 4B and 5). Antisense ODN transfection reduced FAK protein expression by \( \sim 72\% \) (Fig. 4B), an effect confirmed by immunohistochemical analysis (Fig. 5). The specificity of this procedure was indicated by the fact that MEF2 expression remained unaltered in cells transfected with FAK antisense ODN and that transfection with sense ODN produced no detectable change on FAK or MEF2.
Fig. 6. Effect of FAK ODNs on stretch-induced MEF2 and c-jun promoter activation. A, Representative EMSA and average values of densitometric readings ($n=3$) using a MEF2 consensus oligonucleotide. Arrow indicates protein-DNA complex. *$P<0.05$ compared to non-stretched cells. B, Average values ($n=6$) of luciferase activity normalized to renilla luciferase activity in-WT-c-jun-transfected NRVMs subjected to cyclic stretch and transfected with FAK ODNs. C, Non-stretched and ST, 2-h-stretched cells. FAK antisense transfection inhibited stretch-induced c-jun promoter activation.

Fig. 7. Effect of FAK inhibition on stretch-induced JNK and c-Jun activation. Representative blots ($n=5$) of extracts from NRVMs treated with FAK ODNs performed with A, anti-phospho-JNK and anti-JNK or B, anti-phospho-Ser-63-c-Jun and anti-c-Jun antibodies. Representative blots ($n=5$) of NRVMs extracts treated with PP2 performed with C, anti-phospho-JNK and anti-JNK or D, anti-phospho-Ser-63-c-Jun and anti-c-Jun antibodies. C, Non-stretched and ST, 30-min-stretched cells. NT, not transfected with ODNs. *$P<0.05$ compared to non-stretched cells. A.U., Arbitrary Units.
expression and on stretch-induced FAK activation in NRVMs (Fig. 4B). Moreover, the lack of change in the morphology of antisense ODN-transfected NRVMs supports the notion that this procedure induced no deleterious effect in these cells (Fig. 5C, D).

To evaluate the role of FAK signaling on stretch-induced MEF2 binding activity, we performed EMSA with nuclei extracts of NRVMs transfected with FAK antisense ODN. As shown in Fig. 6A, transfection of FAK antisense inhibited stretch-induced MEF2 activation. We then evaluated the role of FAK signaling on MEF2-induced c-jun promoter activation in cells transfected with wild-type c-jun promoter reporter gene (WT-c-jun). Transfection of NRVMs with FAK antisense ODN ablated the stretch-induced activation of the c-jun promoter (Fig. 6B) at a rate comparable to that of the mutation of MEF2 element. Transfection with FAK sense ODN had no effect on stretch-induced c-jun promoter activation.

3.4. FAK is an upstream regulator of stretch-induced JNK/c-Jun phosphorylation in NRVMs

In order to investigate whether FAK signaling plays a role in stretch-mediated JNK/c-Jun phosphorylation, NRVMs transfected with FAK antisense and sense ODNs were stretched for 30 min and cell extracts were analyzed by immunoblotting with phosphospecific antibodies (Fig. 7). As shown in Fig. 7A, transfection of NRVMs with FAK antisense ODN inhibited stretch-induced JNK phosphorylation. This was paralleled by a consistent attenuation in c-Jun phosphorylation and expression induced by cyclic stretch (Fig. 7B). Transfection with FAK sense ODN did not affect JNK and c-Jun phosphorylation or c-Jun expression in stretched NRVMs (Fig. 7A and B).

To further evaluate the role of FAK signaling on JNK/c-Jun activation, we performed experiments using the pharmacological FAK/Src inhibitor PP2 [11]. As shown in Fig. 7C and D, PP2 treatment (1 μmol/L), markedly reduced the stretch-induced JNK/c-Jun phosphorylation as well as c-Jun expression, strengthening the idea that FAK is an upstream regulator of stretch-induced activation of JNK/c-Jun pathway in NRVMs and that this event is dependent on a cooperation of FAK and Src signaling.

3.5. JNK/c-Jun pathway regulates stretch-induced ANF promoter activity

We have previously demonstrated that the stretch-induced ANF promoter activation in NRVMs is dependent on FAK activation [11]. Because our data indicate that FAK signaling plays a critical role in the regulation of JNK and c-Jun phosphorylation in stretched cardiac myocytes, we next examined the role of JNK/c-Jun pathway on the activation of ANF promoter by cyclic stretch in NRVMs. In order to address this issue, NRVMs were transfected with the reporter gene ANF-LUC, pre-treated with the specific pharmacological JNK/c-Jun inhibitor SP600125 (10 μmol/L) and stretched for 4 h. As shown in Fig. 8A, a 4-h stretching protocol increased the ANF-LUC luciferase activity of NRVMs by ~8-fold. SP600125 treatment did not change the ANF-LUC basal activity compared with untreated cells but significantly reduced the stretch-induced increases of ANF-LUC activity in NRVMs.

To evaluate the effectiveness and specificity of SP600125 treatment on JNK/c-Jun pathway, cell extracts were analyzed with antibodies against phospho-c-Jun, c-Jun, phospho-ERK1/2 and ERK1/2. The results shown in Fig. 8B demonstrate that SP600125 treatment efficiently inhibited
the load-induced phosphorylation of c-Jun, but had no effect on stretch-induced ERK1/2 activation. Otherwise, SP600125 treatment attenuated the increase in c-Jun protein induced by mechanical stress (Fig. 8B).

4. Discussion

Stretch-induced hypertrophic growth of cardiac myocytes involves the coordinated activation of interconnected signaling modules and transcription factors which culminate in sequential activation of immediate early and fetal genes. Distinct regulatory pathways can be critical for the functioning of the entire complex web of signaling involved in the hypertrophic growth of cardiac myocytes in response to mechanical stress. The understanding of critical pathways has remained an area of active interest specially to devise possible targets to limit cardiac hypertrophy and prevent heart failure in clinical setting. Here we show that FAK, a tyrosine kinase implicated in the sensing of mechanical stress, controls the sequential activation of the immediate early gene c-Jun and the fetal gene ANF in cardiac myocytes in response to cyclic stretch by distinct activation of MEF2 transcription factor and JNK.

4.1. Regulation of c-Jun activation by cyclic stretch

The regulation of c-Jun occurs through the control of its concentration and phosphorylation within the cell [5]. Our present data extend to NRVMs previous demonstration in rat heart [5,8], that mechanical stress induces c-Jun expression by a complex combination of transcriptional, post-transcriptional and post-translational mechanisms. c-Jun protein and mRNA transcript increased as soon as 30 min after the onset of cyclic stretch. No change was observed in c-jun promoter activity at the same period suggesting that the early increases of c-Jun protein and c-jun mRNA transcripts are post-transcriptional events. The later increases of c-jun expression were otherwise paralleled by consistent increases of wild-type c-jun promoter activity, suggesting a transcriptional regulation of the gene. Moreover, the strict dependence of c-jun promoter activity on MEF2 promoter site as well as the parallel increase of MEF2 DNA-binding activity further supported the notion that c-Jun expression in the late period is regulated by transcriptional events mediated by MEF2 transcription factors.

The data of the present study also indicate that unlike MEF2 site, AP1-like consensus sequence of c-jun promoter does not seem to directly regulate the stretch-induced transactivation of c-jun. This accords with previous demonstrations that MEF2 mediates the increased expression of c-jun in overloaded rat left ventricle [8] and in differentiating myoblasts [14]. However, the finding here that the absolute luciferase activity increased less in cells transfected with AP1-mut-c-jun than those transfected with WT-c-jun plasmid might suggest a permissive role of AP1-like site in the MEF2 regulation of c-jun promoter activity in stretched cardiac myocytes. Notably, basal luciferase activity in extracts of non-stretched NRVMs transfected with AP1-like and MEF2 mutated constructs were ~20-fold less than that of cells transfected with wild-type c-jun promoter plasmid. These results differ from those reported in non-myocyte cells in that, cells transfected with mutated c-jun promoter plasmids showed similar luciferase activity when compared with cells transfected with wild-type constructs [13,15]. Differences in cellular models as well as in experimental conditions might account for such inconsistencies among the studies. It is important to mention that in the present study, cells were serum-starved for only 6 h while starvation lasted for 24–30 h in aforementioned reports. Serum is known to activate c-jun promoter through MEF2 and AP-like sites [14,15]. Thus, the shorter period of serum starvation in our experiments might account for residual activation of wild-type c-jun promoter that could be responsible for higher basal luciferase activity of these cells as compared with those transfected with MEF2 and AP1-like mutated plasmids.

c-Jun abundance has also been demonstrated to be dependent on the regulation of protein stability conferred by phosphorylation mediated by JNKs, which decreases its ubiquitination [16]. The recent demonstration that JNKs activation by endothelin is necessary to stabilize c-Jun for efficient up-regulation of c-jun protein supports the importance of such mechanism in hypertrophying cardiac myocytes [17]. Accordingly, we showed here that the JNK inhibitor SP600125, besides inhibiting Ser-63 c-Jun phosphorylation, also attenuated the increase in c-jun protein induced by mechanical stress, suggesting that post-translational mechanisms might contribute to enhance c-Jun expression in response to mechanical stress. However, the lack of a complete blockade of stretch-induced c-Jun up-regulation in SP600125 treated cells indicates that c-jun mRNA translation also accounts for the increase of c-Jun protein content in stretched cardiac myocytes.

4.2. Regulation of MEF2 by mechanical stress

Our present data provide evidence that cyclic stretch activates both DNA-binding and transcriptional activity of MEF2 as indicated by the results of EMSA and reporter gene assays with c-jun promoter constructions. These findings are in keeping with previous demonstration that MEF2 transcription factors are activated in rat myocardium by mechanical stress [8,18]. Multiple signaling systems can stimulate MEF2 activity by altering its phosphorylation and association with other transcriptional cofactors [19,20]. Notably, MEF2 factors are phosphorylated by MAP kinases, which augment their transcriptional activity [20]. In this regard, we have previously shown that ERK5 may be a mediator of MEF2 activation induced by mechanical stress in rat myocardium [8]. A role for Gq protein coupled receptors to MEF2 activation by hypertrophic stimuli has
been suggested [8], but the upstream mechanisms mediating MEF2 activation by mechanical stress in cardiac myocytes remains largely unknown. Here, we showed that transfection of cardiac myocytes with FAK antisense abolished stretch-induced activation of MEF2 DNA-binding and the stretch-induced activation of c-jun promoter, supporting the notion that FAK is a key upstream regulator of signaling pathways leading to MEF2 activation in cardiac myocytes in response to mechanical stress.

Although the intermediate signaling pathways linking FAK to MEF2 were not explored in the present study, some downstream FAK effectors could explain, in part, its influence on MEF2. For instance, FAK activation has been shown to promote efficient and robust stimulation of the MAP kinase pathway and protein kinase C [21]. Accordingly, data from other sources have shown that such kinases are activated and play a role in the expression of genes induced by mechanical stress in cardiac myocytes [22,23]. However, further studies are necessary to elucidate the signaling pathways whereby FAK regulates MEF2 activation in response to mechanical stress.

4.3. Regulation of the hypertrophic genetic program by FAK

Previous studies showed that both FAK [11] and JNK [24] control the expression of the fetal gene ANF in cardiac myocytes in response to mechanical stress. Our present study provides novel evidence that JNK/c-Jun pathway is an important downstream signaling effector of FAK in the regulation of the ANF promoter activity induced by cyclic stretch. These data are in line with previous demonstration that FAK signaling may contribute to JNK activation by mechanical stress in distinct cell types [25]. 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. Several studies [9–11] have consistently shown that FAK is implicated in the activation of ERK1/2 in response to mechanical stress in cardiac myocytes. Accordingly, a linkage between ERK1/2 pathway and ANF expression has been shown in myocardial cell hypertrophy [26].

Collectively, the results of the present study suggest a model of synergistic control of the expression of immediate early gene and the fetal gene ANF by FAK through regulation of MEF2 and JNK/c-Jun in response to mechanical stress (Fig. 9). It is tempting to speculate that FAK might be a central coordinator of the activation of the genetic hypertrophic program in cardiac myocytes in response to mechanical overload.

In conclusion, the present observations expand our understanding of the signaling mechanisms activated by mechanical stress in cardiac myocytes. Our results indicate that FAK coordinates distinct signaling mechanisms which culminate with a complex activation of MEF2 transcription factors, immediate early genes and ANF in cardiac myocytes in response to cyclic stretch. Further studies are needed to elucidate whether such signaling web and early signaling mechanisms are critical to the phenotypic changes of cardiac myocytes in response to mechanical stress.

Acknowledgements

This study was sponsored by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP (Proc. 00/05137-4, 01/11698-1, 00) and Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (Proc. 521098/97-1).

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