A role for focal adhesion kinase in cardiac mitochondrial biogenesis induced by mechanical stress


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Submitted 29 March 2010; accepted in final form 11 October 2010

Mechanical stress induced by hemodynamic overload plays a prominent role in the development of left ventricular hypertrophy and in the transition from the functional and clinical compensated state to heart failure in common forms of cardiac disease (12). At the cellular level, the hypertrophic growth of cardiomyocytes is the initial dominant response to hemodynamic overload while the progression to dysfunction and clinical heart failure are related to degeneration and loss of cardiomyocytes as well as myocardial fibrosis (5). Increases in the number and activity of mitochondria provide the energy indispensable for the anabolic effect of hypertrophic growth, but, in the settings of chronic overload, changes of mitochondria function can be detrimental, contributing to degeneration of hypertrophic cardiomyocytes (14, 25, 27, 28).

Several studies have implicated the transcriptional coactivator peroxisome proliferator-activated receptor coactivator (PGC)-1α as a regulator of genes responsible for the maintenance of mitochondrial oxidative capacity and energy transduction either at baseline or in response to increased energy demand in adult hearts (1, 7, 28). Accordingly, the levels of PGC-1α gene expression correlate with the mitochondrial oxidative capacity in both healthy and failing rat hearts subjected to chronic pressure overload (11). As a result of cardiac-specific overexpression of PGC-1α the number and size of mitochondria are increased in mice cardiomyocytes, concomitant with upregulation of genes associated with mitochondrial biogenesis. However, cardiac-specific overexpression of PGC-1α in adult mice is associated with abnormalities in the mitochondria ultrastructure and cardiomyopathy (13, 22). Interestingly, deficiency in PGC-1α is also associated with accelerated dysfunction of the mechanically overloaded mice left ventricle, despite the lack of changes in the number of cardiac mitochondria (1, 2, 15). PGC-1α exerts control over nuclear and mitochondria transcription factors, including nuclear respiratory factors (NRF-1, NRF-2) and mitochondrial transcription factor A (Tfam) (29). PGC-1α activation of NRF-1 and NRF-2 expression, as well as its direct coactivation of NRF-1, induces the expression of mitochondrial and nuclear genes central to mitochondrial biogenesis (23). However, there is no clear understanding of the upstream signaling mechanisms involved in the activation of mitochondria transcriptional cascade in response to the many conditions associated with increased energy demand.

Sustained mechanical stress, as other stimuli that increase energy demand, enhances the functional mass of mitochondria in cardiomyocytes (20). However, the mechanisms linking mechanical stress to the enhanced mitochondrial biogenesis of cardiomyocytes are yet unclear. Evidence has emerged that focal adhesion kinase (FAK) plays a critical role in linking events initiated by mechanical stress to the hypertrophic responses in cardiomyocytes (3, 18, 26). Accordingly, depletion of FAK has been shown to prevent the left ventricular hypertrophy induced by pressure overload in mice (3, 19). In particular, several ultrastructural abnormalities were found in cardiac mitochondria of the FAK conditional knockout mice model, suggesting a possible coupling between signaling mediated by FAK and mitochondrial biogenesis (19). Furthermore, marked upregulation of proteins of mitochondrial respiration and the...
content of contractile proteins can be induced by FAK overexpression in rat soleus muscle (6), supporting its involvement in the mechanisms that control mitochondrial biogenesis in skeletal muscle fibers. Given these results, we hypothesized that a coupling between FAK and the PGC-1α/NRF cascade might connect mechanical stimuli and mitochondrial biogenesis in cardiomyocytes. To test this hypothesis, we examined whether FAK depleton induced by RNAi would interfere in the mitochondrial biogenesis of cardiomyocytes undergoing prolonged cyclic stretch. This was assessed in a neonatal rat ventricular myocyte model cultured in silicone plates as well as in the left ventricle of mice subjected to mechanical stress induced by transverse aortic constriction.

MATERIALS AND METHODS

Antibodies and chemicals. Bioflex (Flex I) collagen (type I) culture plates were from Flexcell International; Dulbecco’s modified Eagle’s medium (DMEM), OPTI-MEM, and FBS were from GIBCO-BRL; pancreatic and collagenase type II were from Worthington. Polyclonal rabbit antibodies against FAK (sc558), phosphorylated FAK (sc11765), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc25778), PGC-1α (sc13067), NRF-1 (sc33771), and cytochrome oxidase subunit IV (COX-IV, sc58348) were from Santa Cruz Biotechnology. Trizol, Phenol, and Super Script II were from Invitrogen.

Super Signal west Pico Cheluminescent Substrate and Ampliscrbe T7 high-yield transcription were from Epicentre. HEPES, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, and rhodamine-conjugated phalloidin and BSA (fraction V) were from Sigma. Lipofectamine Plus was from Life Technologies. Nitrocellulose membrane (Hybond ECL, 0.45 μm) was from Amersham; protein A-Sepharose 6MB was from Pharmacia; primers for GAPDH, atrial natriuretic peptide (ANP), Tnα, D-loop, and 18S and the TaqMan reagent kit were from Applied Biosystems, and the SyBr Green Reagents Kit and Mito Tracker were from Invitrogen.

Transverse aortic constriction in mice. Housing, handling, and experimental procedures in mice and neonatal rats followed the recommendations written in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health [DHEW Publication No. (NIH) 85–23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD] and were approved by the Animal Care and Use Committee of the State University of Campinas. Swiss mice (6–8 wk old) were subjected to procedures such as jugular vein catheterization, aortic banding, and echocardiographic examination under anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). For the injections of small-interfering RNA (siRNA), the jugular vein was cannulated with a 27-gauge needle. Transconstriction systolic gradient output was connected to an analog-to-digital board and this to a GP4A Stemtech amplifier (Stemtech). The amplifier was connected to a COBE transducers (Arvada), replaced with serum-free DMEM and incubated for 24 h under 95% air-5% CO2 before the experimental procedure.

Cell stretching. NRVM cultured in Bioflex were changed to serum-free medium and stretched in a Flexcell FX-3000 strain unit by 10% above the initial length at a frequency of 1 Hz (0.5 s of stretch and 0.5 s of relaxation) for up to 12 h. Control nonstretched NRVM were cultured in Bioflex in serum-free medium. At the conclusion of the experimental protocols, cells were scraped from membranes, lysed, and used as total extracts or cellular fractions for immunoblotting, immunoprecipitation, or pull-down assays. Some cell cultures were fixed for analysis by fluorescence microscopy.

Subcellular fractionation. NRVM were washed in 0.5 μL of buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 1 mM PMSF, and 0.1 mg/ml aprotinin, pH 7.9), scraped thoroughly, and left on ice for 10 min. The extracts were centrifuged at 3,000 rpm for 10 min 4°C to obtain the precipitates containing the nuclear fraction. The supernatant, which was enriched with soluble proteins, was saved as a cytosolic fraction at −70°C. The pellet containing the nuclear fraction was resuspended in 188 μL of buffer B (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (vol/vol), 1 mM PMSF, and 0.1 mg/ml aprotinin, pH 7.9) and 12 μL of 5 M NaCl to give 300 mM NaCl. This suspension was homogenized with 20 full strokes in Dounce and left on ice for 30 min before centrifugation at 24,000 g for 20 min at 4°C, and the supernatant was saved as nuclear extracts. Protein concentrations of the cytosolic and nuclear extracts were obtained by Bradford assay.

Protein analyses by immunoblotting. Extracts of NRVM or neonatal rat left ventricle were harvested in buffer (100 mM Tris-HCl, pH 7.4; 100 mM NaHPO4; 100 mM sodium fluoride; 10 mM EDTA; 10 mM Na3VO4; 2 mM PMSF; 0.2 mg/ml aprotinin, and 10% Triton X-100) and cleared by centrifugation at 11,000 rpm, 4°C. Extracts containing equal amounts of total protein (50 μg) were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk solution for 30 min, the membranes were incubated with primary antibodies in 3% nonfat milk solution overnight at 4°C. Proteins were detected using ECL. Quantification of bands was performed using ImageJ (NIH). Signals from phosphoproteins were normalized to the total protein obtained from blots with specific antibody. Blots were stripped and probed with GAPDH antibody to confirm equal loading.

Immunoprecipitation. Aliquots of NRVM nuclear extracts containing 50 μg protein were used for immunoprecipitation with specific antibody (2 μg/μl) at 4°C overnight, followed by the addition of protein A-Sepharose 6MB for 2 h. The pellets were washed three times in ice-cold buffer (0.5% Triton X-100, 100 mm Tris (pH 7.4), 10 mm EDTA, and 2.0 mm sodium vanadate). After being washed, the pellet was resuspended in Laemmli’s sample buffer (25) containing 10 mM DTT and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (10% bis-acrylamide) followed by electrotransfer of proteins from the gel to the nitrocellulose membrane which were preincubated in blocking buffer (5% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20 for 2 h) and incubated
with the appropriate antibody diluted in blocking buffer (3% BSA instead of nonfat dry milk) overnight at 4°C and washed for 15 min with the blocking buffer without BSA.

**siRNA synthesis and treatment.** Protocols for the synthesis of siRNA targeted to mouse and rat FAK gene, transfection of NRVM, and injections in adult mice were performed as previously reported (3, 19).

**Immunofluorescence staining of NRVM.** Cultured cardiomyocytes were fixed in a solution containing 4% paraformaldehyde (pH 7.4). After being washed with 100 mM glycine solution (pH 7.4), cardiomyocytes were permeabilized with 1% Triton X-100 and rinsed with PBS containing 3% wt/vol BSA. Primary antibody against FAK was used in a dilution of 1:100 and incubated overnight at 4°C. After being washed, cells were incubated with secondary antibody labeled with FITC for 2 h at room temperature, washed, and mounted on glass slides. The cardiomyocytes were analyzed by fluorescence microscopy (Leica DMi4000B). For the analysis of cell surface area, cultures were fixed and immunostained with phalloidin, and the nuclei were identified using DAPI. Cell surface area was obtained by the analysis of individual cardiomyocytes with the software Leica QWin. At least 250 cells were counted for each treatment. To examine the effects of prolonged stretch in the morphology of cardiomyocytes, cells underwent continuous cyclic stretch (10%) for 12 h.

**Mitochondria labeling.** NRVM were plated at $1 \times 10^5$ cells/well and allowed to attach for 3 days before the cyclic stretch protocol (10%, 12 h). At the end of the cell-stretching protocol, NRVM were

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**Fig. 1.** Cyclic stretch activates mitochondrial transcription cascade in neonatal rat ventricular myocytes (NRVMs). Immunoblots and graphs ($n = 5$ cultures for each time point) showing relative changes in peroxisome proliferator-activated receptor coactivator (PGC)-1α (A) and nuclear respiratory factor (NRF)-1 (B) induced by cyclic stretch (2–12 h) with respect to values of nonstretched (NS) cells. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblots for the same samples demonstrate equal protein loading. C: graph ($n = 5$ cultures for each time point) shows the relative changes of mitochondrial transcription factor A (Tfam) normalized by GAPDH transcripts [relative quantity (RQ)] from samples of stretched compared with nonstretched NRVM. D: representative immunoblot and average (5 cultures) changes of PGC-1α protein in the cytosolic and nuclear extracts of stretched (ST) compared with nonstretched (NS) NRVM. E: representative anti-NRF-1 immunoblot from samples of total extract and anti-PGC-1α precipitates from NS and ST NRVM (pool of 3 cultures). Assay performed with beads only was used as control for the coimmunoprecipitation assay. *$P < 0.05$ vs. NS.
Fig. 2. Cyclic stretch induces mitochondrial biogenesis in NRVMs. A: graph shows the changes in D-loop normalized by 18S transcripts of ST compared with NS NRVM (n = 6 cultures in each experimental group). B: representative immunoblot and graph indicating the change in the expression of subunit IV of cytochrome oxidase in NS and ST NRVM (n = 3 cultures in each experimental group). C: graph indicating change in the area of Mito Tracker green-stained mitochondria in ST compared with NS NRVM (n = 250 cells in each experimental group). D and E: fluorescence images of NS and ST (12 h) NRVM loaded with Mito Tracker green. F: changes of the RQ of atrial natriuretic peptide (ANP) with respect to GAPDH transcripts in ST compared with NS NRVM (n = 5 cultures in each experimental group). G: graph indicates changes in the average surface area in ST compared with NS NRVM (n = 250 cells in each experimental group). H and I: representative examples of NS and ST NRVM stained with rhodamine-conjugated phalloidin. *P < 0.05 vs. NS.
exposed for 30 min to live cell-staining solution containing mitochondrial membrane potential dye (Mito Tracker Green) before formaldehyde fixation. Two hundred fifty cells for each group were analyzed using a x40 objective using the Leica QWin Pro version 3.41. We then estimated the relative area occupied by mitochondria by assessing the green fluorescence intensity with respect to cell surface area of individual cardiomyocytes.

**RNA and DNA extraction.** DNA extraction was performed by standard phenol/chloroform procedure. For gene expression quantification, total RNA (2 μg) was treated with DNase I (RNase-free) and reverse-transcribed with random hexamers using SuperScript II reverse transcriptase to generate cDNA.

**Real-time PCR.** Aliquots (4 μg) of total RNA were used for cDNA synthesis with the Superscript preamplification system according to the manufacturer’s instructions. Probes for ANP were sense 5’-CCATCACCAAGGGCTTCTTC-3’, antisense-5’-GTGTTG-GACACCGCAGTGTATAC-3’, GAPDH, sense 5’-GGCATTGCTCT-CAATGACAA-3’, antisense 5’-GGTTCTTACCTAAGGCCATCA-3’, and 18S, sense 5’-ATCTCGAGGTAACGGGCTTAATC-3’; and 18S, sense 5’-TAGAGGGACAGGCGGCTTC-3’, antisense 5’-CGCTGAGC CAGTCAGTGT-3’. The primers and probes for GADPH, 18S, TaqMan Reagents kit, and SyBr Green Reagents kit were used according to the manufacturer’s instructions. PCR amplifications were carried out in 96-well plates with the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems), and the results were analyzed with a 7500 System SDS Software (Applied Biosystems). Samples were analyzed in triplicate, and mRNA quantities were normalized against GAPDH (primer-probe mix from Applied Biosystems) or GAPDH/18S (primer-probe mix from Invitrogen). Reactions were carried out using the following conditions: an initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Determination of ATP levels of NRVM.** Cultured cardiomyocytes were washed with cold saline and ground to a powder under liquid nitrogen. ATP was extracted from powdered cell suspended in 1 ml of extraction buffer (50 mmol/l K2HPO4 and 25 mmol/l citric acid; pH 4.5). The mixture was placed in a water bath (80°C) during 2 min to

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Fig. 3. Focal adhesion kinase (FAK) is activated and interacts with PGC-1α in the nuclei of NRVM. A: representative examples of immunoblots performed with antibodies targeted to FAK and phosphorylated (p) FAK. Graph indicates the changes in the values of the FAK-to-pFAK ratio in ST (2–12 h) compared with NS NRVM (n = 6 cultures in each time point). B-E: imaging data of NS and ST (2 h) NRVM double-labeled with anti-FAK antibody and phalloidin.

F: representative anti-FAK immunoblot from samples of total extract and anti-FAK precipitates from nuclear and cytosolic extracts of NS and ST NRVM (pool of 4 cultures). Control for the immunoprecipitation assay was obtained with beads only. G: representative anti-NRF-1 immunoblot from samples of total extract and anti-FAK precipitates from nuclear and cytosolic extracts of NS and ST NRVM (pool of 4 cultures). Control for the immunoprecipitation assay was obtained with beads only. H: anti-PGC-1α immunoblotting from pull-down assays performed with recombinant COOH-terminal FAK and NRVM nuclear extracts compared with immunoprecipitates of anti-FAK antibody (pool of 4 cultures). *P < 0.05 vs. NS. #P < 0.05 vs. NS.
deactivate any biological process that could degrade the ATP. After vortex mixing, samples were added with an additional 3 ml of extraction buffer, and then 1 ml of the suspension was centrifuged (8,000 rpm, 4 min). Samples of 200 μl of this suspension were mixed with 20 μl of 2-chloroacetaldehyde solution and heated at 80°C for 20 min. Aliquots of 25 μl of the reaction mixture were then resolved by high-performance liquid chromatography (HPLC). The fluorescence of derivatized ATP was detected with excitation and emission wavelengths set at 280 and 420 nm, respectively. Chromatographic separation of ATP was achieved at room temperature, using a reversed-phase Cosmosil 5C18-MS column (150 × 4.6 mm ID; 5 μm particle size) with a Cosmosil guard column (5C18-MS 10 × 4.6 mm). The column was equilibrated and eluted under gradient conditions using a flow rate of 1.0 ml/min. The standards and samples were separated using a gradient mobile phase consisting of methanol and extraction buffer, which was prepared immediately before passing through a 0.45-μm filter. The gradient condition was 0 – 4 min with 2% methanol; 4 – 12 min with linear gradient 2–15% methanol; a 17- to 18-min reconditioning step of the column was 2% methanol isocratic for 2 min. The chromatographic run time for each analysis was 20 min. Aliquots of 25 μl were injected in the HPLC system.

Statistical analysis: The statistical analysis of the immunoblottings, quantitative real-time PCR, and immunofluorescence staining were performed with the actual reading values (i.e., densitometric readings, relative quantity with respect to GAPDH transcripts, and immunofluorescence intensity, respectively). Differences among the mean ± SE values were tested with ANOVA and Bonferroni’s multiple-range test. The level of significance was set at P < 0.05. Data are presented as the relative changes in respect to values of nonstretched NRVM, which were considered to be one for the various experimental data.

RESULTS

Cyclic stretch induces mitochondrial biogenesis in NRVM. To establish whether cell stretching might induce the mitochondrial transcription cascade in NRVM, we initially examined the expression of PGC-1α and NRF-1 in cells exposed to cyclic stretch over 2–12 h. Data shown in Fig. 1A indicate that the levels of PGC-1α were already significantly increased 2 h following cell stretching and remained increased over the next 12 h. The level of NRF-1 increased in a manner consistent with its induction by PGC-1α (Fig. 1B). Western blotting with antibody targeted to GAPDH was used as a loading control for immunoblottings of PGC-1α and NRF-1. Furthermore, we measured the transcripts of Tfam, which were found to be increased after 6 and 12 h following cell stretching (Fig. 1C).

To obtain further evidence regarding the activation of the transcriptional regulatory pathways of mitochondria in stretched NRVM, we examined the subcellular distribution of PGC-1α and the association between PGC-1α and NRF-1 in the nuclear extracts of stretched compared with nonstretched NRVM. PGC-1α was most present in the nuclear fraction of both nonstretched and stretched NRVM (Fig. 1D). Cyclic stretch lasting for 2 h resulted in marked increases of PGC-1α in the nuclei of NRVM. Immunoblots performed with antibodies targeted to myosin and histone H-1 were used as controls to confirm the extract enrichment of cytosolic and nuclear proteins, respectively. Data from communoprecipitation experiments indicated that cyclic stretch enhanced the association between PGC-1α and NRF-1 in the nuclei of NRVM (Fig. 1E). Comparisons with the amount of NRF-1 expressed in the whole extracts of NRVM indicated that the fraction of NRF-1 associated with PGC-1α is markedly increased in the nuclei of stretched compared with nonstretched NRVM. Moreover, comparisons of the amount of NRF-1 in anti-PGC-1α precipitates with the amount of NRF-1 in total extracts indicate that

Fig. 4. FAK mediates the stretch-induced hypertrophy and mitochondrial biogenesis in NRVM. A: FAK protein expression in NS and ST NRVM treated with small-interfering RNA (siRNA) targeted to FAK (siFAK) compared with cells treated with si-green fluorescent protein (GFP) (n = 6 cultures in each experimental group). B: PGC-1α protein expression in NS and ST NRVM treated with siFAK compared with cells treated with siGFP (n = 6 cultures in each experimental group). C: NRF-1 protein expression in NS and ST NRVM treated with siFAK compared with cells treated with siGFP (n = 6 cultures in each experimental group). D: coimmunoprecipitation assays of PGC-1α and NRF-1 performed with nuclear extract of NS and ST NRVM treated with siFAK or siGFP (n = 6 cultures in each experimental group). *P < 0.05 vs. siGFP NS. #P < 0.05 vs. siGFP ST.
Fig. 5. Depletion of FAK attenuates the stretch-induced mitochondrial biogenesis and hypertrophy in NRVM. A: graph shows the changes in D-loop normalized by 18S transcripts of ST compared with NS NRVM treated with siRNA targeted to FAK or GFP (n = 6 cultures in each experimental group). B: representative immunoblot and graph indicating the changes in the expression of subunit IV of cytochrome oxidase in NS and ST NRVM treated with siRNA targeted to FAK or GFP (n = 3 cultures in each experimental group). C-F: representative examples of NS and ST NRVM treated with siFAK and siGFP labeled with Mito Tracker. G: graph indicating change in the area of Mito Tracker green-stained mitochondria in ST compared with NS NRVM treated with siRNA targeted to FAK or GFP (n = 250 cells in each experimental group). H: amount of ATP in NS and ST NRVM treated with siFAK or siGFP (n = 6 cultures in each experimental group). I: changes of the RQ of ANP with respect to GAPDH transcripts in ST compared with NS NRVM treated with siRNA targeted to FAK or GFP (n = 6 cultures in each experimental group). J: graph indicates changes in the average surface area in ST compared with NS NRVM treated with siRNA targeted to FAK or GFP (n = 250 cells in each experimental group). *P < 0.05 vs. siGFP NS. #P < 0.05 vs. siGFP.
60% of total NRF-1 is associated with PGC-1α in stretched cardiomyocytes. The specificity of the NRF-1/PGC-1α coimmunoprecipitation was confirmed in assays with beads only.

To determine whether the upregulation of the mitochondrial transcription cascade is reflected in an increased mitochondrial biogenesis, we quantified the relative mtDNA vs. genomic DNA, the expression of the mitochondrial component of COX-IV, and the relative area occupied by stained mitochondria in stretched compared with nonstretched cells. Data shown in Fig. 2A indicate that cell stretching (12 h) induced the relative amount of mitochondrial DNA (mtDNA) to double over the baseline values. Parallel increases occurred in the amount of COX-IV (Fig. 2B) and in the relative area occupied by stained mitochondria in stretched NRVM (Fig. 2C). Figure 2, D and E, shows representative examples of Mito Tracker-labeled nonstretched and stretched NRVM I, respectively.

Next, we confirmed the previous demonstration (18) that prolonged cell stretching induces hypertrophic growth of NRVM, as assessed biochemically by quantitation of ANP and morphologically by quantitation of NRVM surface area. Consistent with previous studies, ANP transcripts (Fig. 2F) and the cell surface area (Fig. 2G) were enhanced in response to cyclic stretch in NRVM. Figure 2, H and J, shows representative examples of phalloidin-stained nonstretched and stretched NRVM, respectively.

FAK is activated and interacts with PGC-1α in the nuclei of NRVM. A role for FAK has been reported in the hypertrophic growth of cardiomyocytes in response to mechanical stress (3, 18, 26). Figure 3A shows that cyclic stretch lasting from 2 to 12 h enhanced FAK phosphorylation at Tyr397 but not FAK expression in NRVM. Supplemental Fig. 1S (Supplemental data for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.) shows that cyclic stretch markedly enhanced this association in parallel with the increases in PGC-1α expression in NRVM (Fig. 3G). The specificity of the FAK/PGC-1α coimmunoprecipitation assay was confirmed by assays with beads only. Comparisons of the amounts of nuclear and cytosolic PGC-1α precipitated by anti-FAK antibody with those found in total NRVM extracts indicate that a minor fraction (~30%) of total PGC-1α associated with FAK either in nonstretched or stretched NRVMs.

The interaction between FAK and PGC-1α was further suggested by the demonstration here that the COOH-terminal domain of FAK, but not the FERM or the kinase domains, is precipitated by PGC-1α from nuclear extracts of NRVM. Comparison of the amount of PGC-1α precipitated by the FAK COOH-terminal domain with that immunoprecipitated by anti-FAK antibody is shown in Fig. 3H.

FAK mediates the stretch-induced hypertrophy and mitochondrial biogenesis in NRVM. To determine whether signal-mediated by FAK is required for the activation of mitochondrial transcription cascade in response to cyclic stretch, NRVM were depleted of FAK by RNA interference. Consistent with previous studies (18), treatment of cells with siRNA targeted to FAK markedly reduced FAK protein expression in nonstretched and stretched NRVM compared with cells treated with siRNA targeted to green fluorescent protein (Fig. 4A).

Fig. 6. FAK depletion attenuates load-induced left ventricular hypertrophy. Data from sham-operated (SO) and aortic-banded mice [1–21 days after transverse aortic constriction (TAC)]. A: RQ of FAK transcript in the left ventricle of aortic-banded (TAC) and SO mice treated with siFAK or siGFP (n = 9 hearts in each experimental group). B: bar graph shows the average values of the left ventricle-to-body mass ratio from TAC and SO mice treated with siFAK or siGFP compared with siGFP counterparts. C: echocardiographic values of posterior wall thickness (LVWT) from the groups of mice shown in B. D: RQ of ANP with respect to transcripts of GAPDH as a relative change compared with calibrator sample (SO, siGFP) obtained by real-time PCR. Groups are the same as in B. *P < 0.05 vs. SO. #P < 0.05 vs. TAC siGFP with the corresponding group.
FAK depletion reduced the basal levels of PGC-1α and NRF-1 as well as their upregulation in stretched NRVM (Fig. 4, B and C). In addition, FAK depletion inhibited the increases in PGC-1α and NRF-1 association induced by cyclic stretch in the nucleus of NRVM (Fig. 4D).

FAK depletion did not change the basal levels of mtDNA (Fig. 5A), COX-IV expression (Fig. 5B), or the relative area occupied by stained mitochondria in NRVM (Fig. 5, C-G) but suppressed their increases in stretched NRVM. Given that mitochondria are the major source of energy in cardiomyocytes, we next examined whether the attenuation of mitochondrial biogenesis induced by FAK depletion was accompanied by changes in the amount of ATP in NRVM. Data shown in Fig. 5H indicate that cyclic stretch reduces the levels of ATP in NRVM in culture by ~20%. FAK silencing did not change the amount of ATP in nonstretched NRVM. However, FAK depletion added to the effect of cell stretching by further reducing the ATP levels in stretched NRVM.

Depletion of FAK was shown to attenuate the increases in the amount of ANP transcripts (Fig. 5I) and in the size (Fig. 5J) of NRVM induced by cyclic stretch.

FAK depletion attenuates the increases of mitochondrial transcription cascade and biogenesis in overloaded left ventricle of mice. To investigate if signaling mediated by FAK can affect mitochondrial transcription cascade and biogenesis in the overloaded left ventricle, FAK was transiently depleted in mice left ventricle by a previously validated (3) in vivo RNA interference-based approach. We confirmed the transient depletion of FAK induced by siRNA in the left ventricle of TAC mice by quantitating the transcripts of FAK in mice left ventricle (Fig. 6A). FAK depletion was shown to transiently attenuate the load-induced left ventricular hypertrophy while FAK was depleted, as evidenced by gravimetry (Fig. 6B), M-mode echocardiography (Fig. 6C), and the expression of myocardial ANP (Fig. 6D), as previously demonstrated (3).

Next, we examined if PGC-1α and NRF-1 can be regulated by pressure overload in mice left ventricle and if FAK signaling mediates these changes. As shown in Fig. 7A, the amount of PGC-1α increased 3.5-fold in the left ventricle of 7-day TAC over the control mice. Upregulation of PGC-1α persisted over 21 days after TAC. The level of NRF-1 also increased in a manner consistent with its induction by PGC-1α (Fig. 7B) after TAC, indicating the activation of mitochondrial transcription cascade in overloaded left ventricle. Data in Fig. 7, A and B, also indicate that FAK knockdown transiently suppressed the load-induced upregulation of PGC-1α and NRF-1 in mice left ventricle. Western blotting with antibody targeted to GAPDH (Fig. 7C) was used as a loading control for immuno-blottings of PGC-1α and NRF-1. We then examined whether mechanical stress can affect mtDNA replication in mice left ventricle. As shown in Fig. 7D, pressure overload progressively increased mtDNA in mice left ventricle up to 21 days following TAC. Depletion of FAK transiently suppressed the load-induced increases in mtDNA replication in the left ventricle up to 15 days after TAC.

**DISCUSSION**

Previous studies in isolated cardiomyocytes and in animal models of heart diseases have yielded evidence of FAK as an important mediator of cardiomyocyte response to mechanical
stress (3, 10, 18, 19, 26). New findings of the present study indicate a role for FAK in the signaling mechanisms that regulate the mitochondrial biogenesis in cardiomyocytes in response to mechanical stress.

**FAK and mitochondrial biogenesis in cardiomyocytes.** Depletion of FAK was shown here to attenuate the hypertrophic responses of NRVM and mice left ventricle to mechanical stress, as reported previously (3, 18, 19). This effect has been attributed to defective activation of extracellular signal-regulated kinase 1/2, phosphatidylinositol 3-kinase/protein kinase B, and mammalian target of rapamycin/S6K signaling systems in response to mechanical stress (9, 10). Apart from mediating the hypertrophic response, FAK was shown here to mediate the increases in the mitochondrial biogenesis in response to cyclic stretch. Moreover, data from biochemical studies led us to conclude that FAK stimulates the stretch-induced mitochondrial biogenesis by upregulating the mitochondria transcription cascade, namely PGC-1α, NRF, and Tfam. In line with this conclusion, depletion of FAK potentiated the reduction of the ATP levels induced by cyclic stretch in the model system of cultured NRVM. Notably, comparable reductions in cardiomyocyte ATP levels were reported in cardiomyocytes that lack PGC-1α (1, 14).

Evidence supported by data from coimmunoprecipitation and pull-down assays indicated that FAK may complex with PGC-1α in the nuclei of stretched cardiomyocytes, implying a direct influence of FAK in the transcriptional events modulated by PGC-1α in stretched cardiomyocytes. Consistent with this, FAK has been shown to relocate to the nucleus of stretched cardiomyocytes and to regulate chromatin remodeling and transcription in muscle cells (8, 17). Given that the levels of PGC-1α are subjected to a positive autoregulatory loop, it is tempting to speculate that the lack of PGC-1α upregulation in cardiomyocytes depleted of FAK would reflect the disruption of a nuclear complex involving FAK and PGC-1α.

**Effects of FAK depletion in overloaded left ventricle.** Despite the lack of appropriate hypertrophic response, function was found to be preserved in the overloaded left ventricle transiently depleted of FAK, as previously demonstrated (3). This would suggest that depletion of FAK may attenuate the adverse effects of pressure overload on left ventricular function. The beneficial effects of FAK depletion may be partially mediated by an attenuation in the interstitial fibrosis in overloaded myocardium (3). Accordingly, signaling mediated by FAK has been shown to play a critical role in the myocardial fibrogenesis that occurs in response to mechanical overload (4, 16). However, depletion of FAK may also attenuate intrinsic abnormalities of overloaded cardiomyocytes. In line with this, FAK depletion has been demonstrated to attenuate the hypertrophic growth induced by mechanical stress without affecting cell viability or contractile function of overloaded cardiomyocytes (3, 18). Our findings here that depletion of FAK did not affect the function of overloaded left ventricle, despite the attenuation in mitochondrial biogenesis, may suggest that modulation of load-induced mitochondrial biogenesis would contribute to the beneficial effects of FAK depletion in overloaded left ventricle. Indeed, available data imply massive activation of mitochondrial biogenesis as a mechanism that is detrimental to cardiac function (13). Increased mitochondrial mass could reduce the contractile efficiency by displacing sarcomeres, but it may also lead to myocardial oxidative stress, which would induce cell loss in the early period of pressure overload (22, 24, 28). Conceivably, FAK depletion may contribute to mitigate those functional and structural abnormalities of overloaded cardiomyocytes by modulating the excessive mitochondrial biogenesis. Intriguingly, however, cardiomyocytes depleted of FAK were shown here to be unable to sustain the ATP levels when subjected to mechanical stress, which is expected to be detrimental rather than beneficial to contractile function and cell viability. We speculate that the reductions of ATP levels in response to cyclic stretch might be related to peculiarities of the cultured cardiomyocyte system model, which may be susceptible to stimuli that enhance the energy demand. To date, hypertrophying cardiomyocytes have a high oxygen demand that may not be met by cell culture techniques (21). However, this issue will need to be explored in intact left ventricle to confirm whether the attenuation in mitochondrial biogenesis after FAK depletion is sufficient to reduce the ATP levels of mechanically stimulated cardiomyocytes.

In conclusion, the data from this study provide a rationale for a distinct role of FAK in mediating the activation of mitochondrial transcription cascade in response to mechanical stress. Notably, our data support the notion that the negative modulation of mitochondrial biogenesis by FAK depletion may be dependent on a disruption of a nuclear complex involving FAK and PGC-1α. Future studies should establish if the modulation of mitochondrial mass results in beneficial effects to overloaded left ventricle.

**DISCLOSURES**

No conflicts of interest are declared by the authors.

**REFERENCES**


