Endothelin-induced cardiac myocyte hypertrophy: role for focal adhesion kinase

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1Cardiovascular Institute and Departments of Medicine and Physiology, Loyola University Chicago, Maywood, Illinois 60153; and 2Department of Orthopedic Surgery, Washington University Medical School, St. Louis, Missouri 63110

Eble, Diane M., James B. Strait, Geetha Govindarajan, Jueren Lou, Kenneth L. Byron, and Allen M. Samarel. Endothelin-induced cardiac myocyte hypertrophy: role for focal adhesion kinase. Am J Physiol Heart Circ Physiol 278: H1695–H1707, 2000.—Endothelin-1 (ET) produces neonatal rat ventricular myocyte (NRVM) hypertrophy and activates focal adhesion kinase (FAK) in other cell types. In the present study, we examined whether ET activated FAK in NRVM and whether FAK was necessary and/or sufficient for ET-induced NRVM hypertrophy. Chronic ET-1 stimulation (100 nM, 48 h) increased protein-to-DNA and myosin heavy chain (MHC)-to-DNA ratios and stimulated the assembly of newly synthesized MHC into sarcomeres. ET-1 also induced the assembly of focal adhesions and costameres, as evidenced by increased phosphotyrosine, FAK, and paxillin immunostaining. Acutely, ET treatment rapidly increased tyrosine phosphorylation of FAK and paxillin. FAK was also activated by phorbol 12-myristate 13-acetate (2 µM, 5 min). Pretreatment with chelerythrine (5 µM) or rottlerin (10 µM) completely blocked ET-induced FAK phosphorylation, indicating that protein kinase C activation was upstream of ET-induced FAK activation. In contrast, ET-induced FAK activation was not affected by blocking calcium influx via L-type voltage-gated calcium channels. Adenoviruses (Adv) containing FAK and FAK-related nonkinase (FRNK) were used to specifically define the role of FAK in ET-induced hypertrophy. ET stimulation failed to increase total protein-to-DNA or MHC-to-DNA ratios or to stimulate sarcomeric assembly in myocytes infected with Adv-FRNK. However, Adv-FAK alone did not increase total protein-to-DNA or MHC-to-DNA ratios and failed to increase the number or size of myofibrils as evidenced by double immunofluorescence labeling for MHC and FAK. Thus, although FAK is necessary for ET-induced NRVM hypertrophy, other ET-generated signals are also required to elicit the hypertrophic phenotype.

Animal models of cardiac hypertrophy have demonstrated increased ET expression and secretion in the heart, and ET has been shown to be a potent stimulus for neonatal rat ventricular myocyte (NRVM) hypertrophy in vitro (34). Chronic ET stimulation produces increased cell size and protein synthesis and increased transcription of myosin light chain-2 and atrial natriuretic factor (ANF) as well as enhanced sarcomeric assembly (11, 17, 20, 32, 35). Over the past several years, cultured NRVM have been used to delineate the signaling pathways activated by ET. NRVM have been shown to contain predominantly ETA receptors (14, 21), which, when activated, stimulate a signaling cascade that involves several downstream serine/threonine protein kinases. ET has also been shown to activate a nonreceptor protein tyrosine kinase, focal adhesion kinase (FAK), in mesangial cells (13) and primary astrocytes (5). FAK is one component of the focal adhesion, a structural site at which integrin receptors tether extracellular matrix proteins to intracellular, cytoskeletal proteins (2, 3, 23). At the time of integrin clustering, FAK is activated and phosphorylated itself at tyrosine residue 397. This phosphorylated tyrosine provides a docking site for the protein tyrosine kinase Src and other Src-family protein tyrosine kinases to bind (28). Propagation of the signal continues as Src then phosphorylates other tyrosine residues within FAK and creates sites for still other protein tyrosine kinases and cytoskeletal proteins to bind. In this way signals are transduced via focal adhesions to downstream targets.

In nonmuscle cells, FAK phosphorylation is necessary for focal adhesion and stress fiber formation and may be important in cell adhesion and spreading. In cardiac myocytes, focal adhesions form part of the costameres, which are bandlike structures linking the Z disk to the sarcolemmal membrane (36). There appears to be a coordinated interplay between focal adhesions/costameres and the assembly and maintenance of sarcomeres. For example, normal organization and alignment of sarcomeres were altered in myocytes treated with anti-β1-integrin antibodies (15). In addition, contractile arrested myocytes not only disassemble myofibrils but also lose focal adhesion and costamere integrity with the loss of β1-integrin receptors and vinculin (9, 25, 31). Focal adhesion proteins, therefore, play an intimate role in the primary function...
of cardiac myocytes. In the present study, we hypothesize that ET signals through FAK activation to increase focal adhesion formation and to promote and/or stabilize sarcomere assembly. Therefore, we examined the role of FAK in ET-induced NRVM hypertrophy and further examined whether FAK is necessary and/or sufficient to induce sarcomeric assembly.

MATERIALS AND METHODS

Reagents. PC-1 tissue culture medium was obtained from BioWhittaker (Walkersville, MD). DMEM was obtained from Gibco-BRL (Grand Island, NY). Medium 199 (M199), Ca²⁺- and Mg²⁺-free and Mg²⁺-free Hanks’ balanced salt solution (HBSS), acid-soluble calf skin collagen, and antibiotic/antimycotic solution were obtained from Sigma Chemical (St. Louis, MO). Permanox chamber slides were obtained from Nunc (Naperville, IL). Tissue culture plates were obtained from Costar (Cambridge, MA). Sarcomeric myosin heavy chain (MHC) monoclonal antibody (MF20) was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, and the Department of Biological Sciences, University of Iowa). This MHC antibody recognizes both α- and β-MHC, which are both expressed in NRVM. FAK, paxillin, and phosphotyrosine antibodies were obtained from Transduction Laboratories (Lexington, KY), Santa Cruz Biotechnology, (Santa Cruz, CA), or Upstate Biotechnologies (Lake Placid, NY). Protein A and protein G beads were obtained from Calbiochem (San Diego, CA) and Sigma. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse Ig G were from Bio-Rad (Hercules, CA). All other reagents were of the highest grade commercially available and were obtained from Sigma and Baxter S/P (McGaw Park, IL).

NRVM isolation. Animals used in these experiments were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985]. NRVM were isolated from the hearts of 1- to 3-day-old Sprague-Dawley rat pups via collagenase digestion as previously described (25). Dissociated cells were preplated for 1 h in serum-free PC-1 medium, and then resuspended and seeded onto collagen-coated, plastic 35- or 100-mm dishes or onto chamber slides and were left undisturbed in a 5% CO₂ incubator for 14–18 h. Unattached cells were removed by aspiration, and the attached cells were maintained in a solution of DMEM-M199 (4:1) containing antibiotic/antimycotic solution. The medium was changed daily.

Cellular composition. NRVM were quantitatively scraped from the dishes and used for analysis of total protein by the Lowry method with crystalline human serum albumin as standard and for analysis of DNA with Hoechst 33258 dye and salmon sperm DNA as standard, as previously described (25). For quantitative analysis of α- and β-MHC content, cells were lysed in sample buffer, and then the concentrations of α- and β-MHC isoenzymes were assessed by SDS-PAGE and silver staining (25). MHC band intensity was quantified by laser densitometry and compared with the band intensity of purified MHC standards (0–300 ng). Results are the means of each treatment group for each cell collection (expressed as μg MHC/μg DNA).

Immunolocalization. Cells grown on chamber slides were fixed and permeabilized as previously described (9). Myocytes were then stained with antibodies to MHC, paxillin, phosphotyrosine, and FAK/FRNK (FAK-related nonkinase). Of note, the antibody (no. 06-543, UBI) used to detect FRNK was directed to the COOH terminus of FAK. Thus the staining for adenovirally expressed FRNK and endogenous FAK could not be distinguished. Appropriate FITC- or rhodamine-conjugated secondary antibodies were used to visualize the specific proteins. Fluorescently labeled cells were then viewed using a Zeiss model LSM 410 or LSM 510 laser scanning confocal microscope. Multiple optical sections ~1 μm thick were taken of each sample to eliminate out-of-focus fluorescence.

Immunoprecipitation and Western blotting. NRVM were plated at high density (1,600 cells/mm²) and cultured in medium containing the L-type calcium channel blocker nifedipine (10 µM) to inhibit spontaneous contractile activity. After 48 h, fresh nifedipine-free medium was applied for 1 h with or without various inhibitors, and then the myocytes were stimulated with ET (100 nM) or phosphor-12-myristate 13-acetate (PMA; 2 µM). At the indicated times, the myocytes were rinsed with cold PBS and then scraped in an ice-cold modified lysis buffer according to Schlaepfer and Hunter (29). Equal amounts of protein were immunoprecipitated with anti-FAK, -paxillin, or -phosphotyrosine antibodies, followed by the addition of protein A/G beads. Immune complexes were collected and washed three times and then boiled in 8% SDS sample buffer to solubilize the proteins. To quantify ET-induced FAK and paxillin phosphorylation, proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham, Arlington Heights, IL). The bands corresponding to phosphorylated FAK or paxillin were quantified by laser densitometry.

Subcellular fractionation. High-density NRVM cultures were scraped in homogenization buffer (2 mM EDTA, 2 mM EGTA, 10 µg/ml aprotonin, 10 µg/ml leupeptin, 500 µM sodium orthovanadate, 1 mM Pefabloc, and 20 mM Tris·HCl, pH 7.5), sonicated, and centrifuged (100,000 g, 1 h). The supernatant fraction (S) was considered the cytosolic fraction. The pellet was resuspended in 1% Triton X-100-containing buffer, sonicated, and centrifuged (10,000 g, 10 min). The supernatant fraction from this spin was considered the P₁ fraction, and the pellet from the second spin, the P₂ fraction. The S and P₁ fractions were lyophilized, and then all three fractions were resuspended in lysis buffer. Each fraction was immunoprecipitated with an anti-FAK monoclonal antibody and separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane. The blot was then probed with a monoclonal antibody directed to phosphorylated tyrosine residues.

Adenoviral constructs. A replication-defective adenovirus encoding FRNK (Adv-FRNK) was constructed using a 1.2-kb wild-type chick FRNK cDNA kindly provided by Dr. Thomas Parsons (University of Virginia, Charlottesville, VA). Replication-defective adenoviruses encoding wild-type chick FAK (Adv-FAK) and the β-galactosidase gene LacZ (Adv-βgal) were constructed as previously described (19). Adenoviruses were amplified and purified using HEK-293 cells (10). Preliminary experiments determined that a concentration of 50–100 particles of Adv-FRNK, Adv-FAK, or Adv-βgal per cell increased the expression of these proteins by over 40 times in 48 h (Western blot analysis) and infected virtually every myocyte (X-Gal staining) (data not shown).
RESULTS

ET induces cardiomyocyte hypertrophy. ET (100 nM, 48 h) increased levels of total protein-to-DNA, total MHC protein-to-DNA, and both α-MHC protein- and β-MHC protein-to-DNA ratios as well as the percentage of myosin that was β-MHC (Table 1). In contrast, ET had no effect on DNA content, indicating that growth occurred predominately via cell hypertrophy. Using immunofluorescence confocal microscopy, we found that ET increased cell size, MHC staining intensity, and sarcomeric organization compared with control myocytes (Fig. 1). Low-density control cells contained relatively few organized sarcomeres (Fig. 1a), whereas ET-treated myocytes contained many tightly packed myofibrillar arrays throughout the cell (Fig. 1b). Thus, in agreement with previous studies (11, 17, 20, 32, 35), chronic exposure to ET produced morphological and biochemical alterations indicative of cardiomyocyte hypertrophy.

ET causes focal adhesion formation. We next examined whether ET-induced sarcomeric assembly was accompanied by the formation of focal adhesions. Paxillin is an abundant cytoskeletal protein that has been found in focal adhesions in nonmuscle cells (39). Paxillin localized within control myocytes in distinct punctate areas around the edge of the cell (Fig. 1c). ET-treated myocytes showed a marked increase in the size of the punctate regions and in the distribution of paxillin staining within linear arrays along the sites of cell-substrate contact (Fig. 1d). Using an antibody that recognizes all tyrosine-phosphorylated proteins, we found that focal adhesions contain tyrosine-phosphorylated proteins, we found that focal adhesions contain tyrosine-phosphorylated proteins even under control conditions (Fig. 1e). However, with chronic ET stimulation, tyrosine-phosphorylated proteins increased, particularly along the cell periphery (Fig. 1f, arrows). Finally, we examined whether FAK localized in a similar distribution to paxillin in control and ET-treated myocytes. As shown in Fig. 1g, FAK was readily detected in focal adhesion structures as well as in a more diffuse staining pattern along the cell-substrate interface of control cells. Like paxillin, ET increased FAK staining along the cell periphery as well as within linear arrays along the sites of cell-substrate contact (Fig. 1h). Thus focal adhesions and specific proteins located within focal adhesions are increased in ET-treated NRVM.

Table 1. Endothelin-1 induces NRVM hypertrophy

<table>
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<th>Control</th>
<th>Endothelin-1 (100 nM, 48 h)</th>
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<tr>
<td>Total protein, µg/well</td>
<td>58 ± 5</td>
<td>68 ± 4*</td>
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<tr>
<td>DNA, µg/well</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Total protein/DNA, µg/µg</td>
<td>48 ± 1</td>
<td>57 ± 2*</td>
</tr>
<tr>
<td>Total MHC/DNA, µg/µg</td>
<td>2.2 ± 0.3</td>
<td>3.4 ± 0.3*</td>
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<tr>
<td>α-MHC/DNA, µg/µg</td>
<td>2.0 ± 0.3</td>
<td>3.7 ± 0.3*</td>
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<tr>
<td>β-MHC/DNA, µg/µg</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.2*</td>
</tr>
<tr>
<td>%β-MHC</td>
<td>54 ± 4</td>
<td>58 ± 4*</td>
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Values are means ± SE; n = 24–28 cell isolations. Quantitative analysis of total protein and DNA content as well as α- and β-myosin heavy chain (MHC) content was performed using standard biochemical assays. Data from the 2 groups were analyzed using a paired t-test where appropriate. *P < 0.05 vs. control.
Fig. 1. Effect of endothelin-1 (ET) on localization of myosin heavy chain (MHC), paxillin, tyrosine-phosphorylated proteins, and focal adhesion kinase (FAK). Neonatal rat ventricular myocytes (NRVM) were cultured in serum-free medium alone (a, c, e, and g) or in serum-free medium containing 100 nM ET (b, d, f, and h) for 48 h. Myocytes were then fixed, permeabilized, and stained using antibodies specific for MHC (a and b), the cytoskeletal protein paxillin (c and d), proteins containing phosphorylated tyrosines (e and f), and FAK (g and h). Myocytes were imaged by laser scanning confocal microscopy. Images depicted in a and b were acquired several micrometers above substratum, whereas images in c-h were obtained at cell-substrate interface. Arrows in f indicate regions of increased tyrosinylated proteins at cell periphery.
phorylation (Fig. 5). Of note, chronic stimulation with PMA induced a similar degree of hypertrophy and an increase in focal adhesion formation similar to that evident with chronic ET stimulation (data not shown).

Paxillin, an FAK substrate, is phosphorylated in response to ET treatment. As shown in Fig. 6, control NRVM contained rather high levels of phosphorylated paxillin. However, paxillin phosphorylation further increased within 2–5 min of ET stimulation and then slowly returned toward baseline by 1 h. After 24 and 48 h of continuous ET stimulation, paxillin phosphorylation was no different than in untreated control cells (data not shown). This time course was coincident with ET-induced FAK phosphorylation, suggesting that FAK functions in a signal transduction pathway leading to paxillin phosphorylation. Thus the increased paxillin staining in response to chronic ET treatment shown in phosphorylation (Fig. 5). Of note, chronic stimulation with PMA induced a similar degree of hypertrophy and an increase in focal adhesion formation similar to that evident with chronic ET stimulation (data not shown).

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Is FAK necessary for ET-induced neonatal rat ventricular myocyte hypertrophy? To further examine the role of FAK in eliciting specific aspects of the hypertrophic phenotype induced by ET, we created an Adv containing the COOH-terminal region of FAK, known as FRNK. FRNK has been shown in other cell types to

Fig. 2. Effect of ET on FAK phosphorylation. NRVM were stimulated with ET for indicated times (in min). Tyrosine-phosphorylated proteins (pTyr) were immunoprecipitated, size fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Blots were probed with an anti-FAK polyclonal antibody, and protein bands were visualized using enhanced chemiluminescence (ECL). Cell lysate from 3T3 murine fibroblasts served as a positive control for FAK. A: ET induced FAK tyrosine phosphorylation within 2 min. FAK activation then returned toward baseline by 1 h. Molecular mass is shown at left in kDa. STD, standard; IP, immunoprecipitate; WB, Western blot; Ab, antibody. B: quantitative analysis of blots from 7–17 different experiments. Results are expressed as means ± SE for each time point.

Fig. 3. Subcellular fractionation and detection of phosphorylated FAK in response to ET. NRVM were stimulated with ET for indicated times. Myocytes were lysed in absence of detergents and divided into 3 fractions: soluble (S), particulate 1 (P1), and particulate 2 (P2). Each fraction was immunoprecipitated with an anti-FAK monoclonal antibody, separated using SDS-PAGE, and transferred to nitrocellulose membrane. Blot was probed with a monoclonal antibody directed to phosphorylated tyrosine residues. ET caused FAK tyrosine phosphorylation predominantly in P2 fraction, which corresponds to myocyte cytoskeleton. Consistent with data shown in Fig. 2, FAK phosphorylation is rapidly induced by ET and then returns toward baseline by 1 h. Molecular mass is shown at left in kDa.

Fig. 4. Effect of protein kinase C (PKC) inhibitors on ET-induced FAK phosphorylation. NRVM were cultured in serum-free medium alone or in medium containing the PKC inhibitors chelerythrine or the PKC-δ isoenzyme-specific inhibitor rottlerin for 1 h (basal) and then stimulated with ET (100 nM, 5 min). Tyrosine-phosphorylated proteins were immunoprecipitated, size-fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Blots were probed with an anti-FAK polyclonal antibody, and protein bands were visualized using ECL. ET-induced FAK phosphorylation was inhibited by PKC-specific inhibitors, suggesting that FAK activation is downstream of PKC activation. Data are means ± SE for 4–17 different experiments. *P < 0.05 vs. basal control levels of FAK phosphorylation.
compete with FAK for binding to focal adhesions and thus inhibit FAK-dependent signaling (22). Adv-FRNK expression increased to a maximum 48 h after infection (Fig. 7A) and inhibited ET-induced FAK phosphorylation compared with uninfected or Adv-βgal-infected myocytes (Fig. 7B). In other experiments, we found that Adv-FRNK also inhibited ET-induced paxillin phosphorylation compared with uninfected myocytes (0.9 ± 0.1 vs. 2.4 ± 0.2-fold increase in ET-induced paxillin phosphorylation in FRNK-infected and uninfected NRVM, respectively; n = 4 experiments; P < 0.05). Thus Adv-FRNK appears to act as a "dominant-negative" inhibitor of FAK-dependent signaling.

We next examined whether Adv-FRNK inhibited ET-induced NRVM hypertrophy (Table 2). Although infection with Adv-βgal alone appeared to increase the total protein-to-DNA ratio above that of uninfected control myocytes (Table 1), ET stimulation caused an additional, significant increase in total protein-to-DNA and MHC-to-DNA ratios. However, ET stimulation failed to increase total protein-to-DNA or MHC-to-DNA ratios in Adv-FRNK-infected myocytes (Table 2). Thus FAK signaling appears to be necessary for ET-induced total protein and MHC accumulation.

Double immunofluorescence labeling for MHC and FAK/FRNK was then used to specifically examine whether FAK was necessary for ET-induced sarcomeric assembly. Low-density myocytes were infected (1 h) with Adv-FRNK (100 particles/cell) or, to control for adenoviral infection, with Adv-βgal (100 particles/cell). Cells were then cultured in fresh, serum-free medium

![Fig. 5. Effect of ET and phorbol 12-myristate 13-acetate (PMA) on FAK phosphorylation. NRVM were cultured in serum-free medium alone for 1 h and then stimulated with ET (100 nM) or PMA (2 µM) for 5 min.](image)

![Fig. 6. Effect of ET on paxillin phosphorylation. NRVM were cultured in serum-free medium alone (1 h) and then stimulated with ET (100 nM) for indicated times (in min). Tyrosine-phosphorylated proteins were immunoprecipitated, size-fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed with paxillin antibody, and protein bands were visualized using ECL.](image)
control and Adv-FAK treatment increased FAK phosphorylation in ECL. Cell lysate from 3T3 murine fibroblasts served as a positive FAK polyclonal antibody, and protein bands were visualized using nitrocellulose membrane. Blots were probed with an anti-FAK antibody, immunoprecipitated, size-fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Molecular mass is shown at indicated times after infection. In control NRVMs, FAK-related nonkinase (FRNK) was markedly increased by 48 h.

Table 2. Overexpression of FRNK inhibits ET-induced NRVM hypertrophy

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<tr>
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<th>Adv-βgal</th>
<th>Adv-FRNK</th>
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<tr>
<td>Total protein, µg/well</td>
<td>62 ± 7</td>
<td>62 ± 7</td>
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<tr>
<td>DNA, µg/well</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Total protein/DNA, µg/µg</td>
<td>62 ± 7</td>
<td>62 ± 7</td>
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<tr>
<td>Total MHC/DNA, µg/µg</td>
<td>59 ± 4</td>
<td>59 ± 3</td>
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<tr>
<td>α-MHC/DNA, µg/µg</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
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<tr>
<td>β-MHC/DNA, µg/µg</td>
<td>0.1 ± 1.3</td>
<td>0.1 ± 1.3</td>
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<tr>
<td>γ-MHC</td>
<td>53 ± 3</td>
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Values are means ± SE; n = 11–13 cell isolations. FRNK, focal adhesion kinase (FAK)-related non-kinase; ET, endothelin-1; NRVM, neonatal rat ventricular myocyte; Adv-βgal or Adv-FRNK, adenoviruses encoding β-galactosidase gene or FRNK. Data obtained from standard biochemical assays were analyzed using a paired t-test or Wilcoxon signed rank test where appropriate. *P < 0.05, respective ET-treated vs. untreated cells infected with Adv-βgal or Adv-FRNK.

for an additional 48 h in the presence or absence of ET (100 nM). Myocytes were fixed and immunofluorescently labeled for FAK/FRNK and MHC and then viewed by laser confocal microscopy (Fig. 8). To demonstrate the effects of the adenoviruses and ET on sarcomeric assembly, we acquired images several micrometers above the substratum so that adhesion plaques were not optically visualized. However, identical laser intensity and gain/offset settings were used to visualize each slide so that the intensity of staining could be compared. As shown in Fig. 8A, Adv-βgal-infected myocytes contained low levels of FAK staining and few assembled sarcomeres (Fig. 8B), which was expected in these noncontractile cells (9, 31). ET treatment of Adv-βgal-infected myocytes increased cell size and stimulated sarcomeric assembly, as evidenced by increased MHC staining intensity and the appearance throughout the cytoplasm of numerous well-developed myofibrils (Fig. 8D). Adv-FRK-infected myocytes stained intensely with the FAK antibody (Fig. 8E) but, like the unstimulated Adv-βgal-infected cells, had few assembled sarcomeres. In contrast, the Adv-FRK-infected myocytes (Fig. 8, E–H) failed to respond to ET stimulation in that there was no substantial difference in cell size or sarcomere assembly (Fig. 8H). Taken together, these biochemical and morphological data demonstrate that overexpression of FRNK inhibited ET-induced NRVM hypertrophy and sarcomeric assembly.

Is FAK sufficient to cause NRVM hypertrophy? To determine whether FAK could induce NRVM hypertrophy, we overexpressed wild-type chick FAK in NRVM by using Adv-FAK (19). A concentration of 50–100 particles/cell was found to be sufficient to produce marked FAK overexpression within the myocytes (data not shown). Using this concentration of adenovirus, a time course of expression was established. As shown in Fig. 9, A and B, FAK overexpression occurred within 10 h of infection and reached a sustained maximum at 24 h. Compared with uninfected myocytes or myocytes infected with Adv-βgal, Adv-FAK-infected myocytes contained approximately four- to fivefold higher levels of phosphorylated FAK (Fig. 9C). Thus myocytes infected with Adv-FAK expressed activated FAK by 24 h.

We next examined whether myocytes infected with Adv-FAK were hypertrophied compared with uninfected myocytes or myocytes infected with Adv-βgal. The plating density was lowered for these studies so that Adv-FAK-specific growth of the myocytes could be more readily detected. Myocytes were infected with the respective adenoviruses or left uninfected in serum-free medium. After 1 h, the medium was changed, and FAK phosphorylation was inhibited in Adv-FRNK-infected cells.
Fig. 8. Overexpression of FRNK inhibits ET-induced sarcomeric assembly. Low-density NRVM were infected (1 h) with 100 particles/cell of Adv-βgal (A–D) or Adv-FRNK (E–H). Myocytes were fixed and permeabilized after an additional 23 h of culture in serum-free medium alone (A, B, E, and F) or in medium containing 100 nM ET (C, D, G, and H). Myocytes were double stained using antibodies for FAK/FRNK (A, C, E, and G) or MHC (B, D, F, H). The antibody used to detect FRNK was an antibody directed to the COOH terminus of FAK. Thus staining for adenovirally expressed FRNK and endogenous FAK could not be distinguished. Myocytes were imaged by laser scanning confocal microscopy. Images were acquired several micrometers above substratum.
the myocytes were cultured in serum-free medium for an additional 47 h. Adv-FAK infection had no obvious effect on cell viability, and Adv-FAK did not induce spontaneous contractile activity in these quiescent, low-density cultures. Adv-FAK infection did not increase total protein, total DNA, or the total protein-to-DNA ratio compared with uninfected myocytes or myocytes infected with Adv-βgal (Table 3). In fact, the total MHC-to-DNA ratio, the β-MHC-to-DNA ratio, and the percentage of total MHC protein that was β-MHC were all significantly reduced with Adv-FAK infection compared with controls.

Using double immunofluorescence labeling for MHC and FAK, we examined whether myocytes overexpressing FAK contained more assembled sarcomeres. The low-density uninfected control myocytes (Fig. 10, A and B) and Adv-βgal-infected myocytes (Fig. 10, C and D) contained few assembled sarcomeres and low levels of FAK staining, which was expected in these noncontractile myocytes (9, 31). In Adv-FAK infected cells, the FAK staining was intense and appeared diffusely throughout the myocyte cytoplasm (Fig. 10E). However, whereas MHC staining was readily detected in Adv-FAK infected myocytes (Fig. 10F), there was no substantial difference in myofibrillar organization compared with uninfected control myocytes (Fig. 10B) or Adv-βgal-infected myocytes (Fig. 10D). Thus overexpression of FAK alone was not sufficient to induce NRVM hypertrophy or sarcomeric assembly.

**DISCUSSION**

Cardiac myocytes rely on focal adhesions and costameres for the assembly and maintenance of sarcomeres (15). Our laboratory recently demonstrated (9, 31) that spontaneously contracting NRVM contain many focal

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**Table 3.** Overexpression of FAK is insufficient to induce NRVM hypertrophy

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<th></th>
<th>Control</th>
<th>Adv-βgal</th>
<th>Adv-FAK</th>
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<tr>
<td>Total protein, µg/well</td>
<td>35 ± 10</td>
<td>41 ± 12</td>
<td>41 ± 13</td>
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<td>DNA, µg/well</td>
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<td>Total protein/DNA, µg/µg</td>
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<td>35 ± 5</td>
<td>32 ± 4</td>
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<tr>
<td>Total MHC/DNA, µg/µg</td>
<td>1.9 ± 1.4</td>
<td>2.1 ± 0.5</td>
<td>1.2 ± 0.2*†</td>
</tr>
<tr>
<td>α-MHC/DNA, µg/µg</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
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<td>β-MHC/DNA, µg/µg</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.5 ± 0.1*†</td>
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<tr>
<td>%β-MHC</td>
<td>50 ± 5</td>
<td>50 ± 6</td>
<td>38 ± 3*†</td>
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Values are means ± SE; n = 9 cell isolations. Data obtained from standard biochemical assays were analyzed using repeated-measures one-way ANOVA or repeated-measures ANOVA on ranks where appropriate. *P < 0.05 vs. Control; †P < 0.05 vs. Adv-βgal.
adhesions and focal adhesion/costamere components such as FAK, vinculin, and $\beta_1$-integrin receptors. Contractile-arrested myocytes treated with L-type calcium channel blockers not only disassemble myofibrils but also lose focal adhesion and costamere integrity with the loss of vinculin, $\beta_1$-integrin receptors, and FAK from focal contact sites (9, 25, 31, 33). Restoration of contractile activity caused reassembly of focal adhesions and costameres that temporally preceded sarcomeric assembly. Thus focal adhesion and costamere formation play an intimate role in maintaining contractile function of cardiac myocytes.

In the present study, we found that FAK is present in a Triton X-100-insoluble fraction of the cell corresponding to the cytoskeleton and is rapidly activated by ET. FAK has been shown to associate with the cytoskeleton on fibronectin stimulation of NIH/3T3 fibroblasts (30). The present results using NRVM demonstrate that FAK is already present within a detergent-insoluble fraction, where it is rapidly phosphorylated on ET.

Fig. 10. Overexpression of FAK does not promote sarcomeric assembly. Low-density NRVM were left uninfected (A and B) or were infected (1 h) with 100 particles/cell of Adv-\(\beta\)gal (C and D) or Adv-FAK (E and F). After an additional 23 h of culture in serum-free medium, myocytes were fixed and permeabilized. Myocytes were double stained using antibodies specific for FAK (A, C, and E) or MHC (B, D, and F). Myocytes were imaged by laser scanning confocal microscopy. All images were acquired several micrometers above substratum.
stabilization. However, it should be pointed out that our fractionation scheme does not distinguish whether FAK is associated only at focal adhesions or whether it is also associated with other cytoskeletal elements. Furthermore, previous studies have shown that phenylephrine, ET, and ANG II all induce sarcomeric assembly within 0.5–4 h following agonist stimulation (1, 8). Thus the rapid (2 min) ET-induced FAK phosphorylation observed in our studies must precede sarcomeric assembly and may be a necessary initial component in focal adhesion formation.

Cardiac myocytes are highly dependent on the influx of calcium not only for contractile activity but also for the assembly and maintenance of sarcomeres (4). However, in the present study, basal and ET-induced FAK phosphorylation were found to be independent of calcium influx through L-type voltage-gated calcium channels. It is possible that a small amount of calcium released from inositol 1,4,5-trisphosphate-sensitive stores was sufficient for FAK phosphorylation. Nonetheless, because calcium influx is required for sarcomeric assembly, there must be additional calcium-sensitive events acting downstream of, or parallel to, FAK activation. We have also identified PKCs as potential upstream regulators of FAK in NRVM. Not only did PMA activate FAK but the PKC inhibitor chelerythrine completely blocked ET-induced FAK phosphorylation. In addition, our results with the PKC-δ isoenzyme inhibitor rottlerin (12) suggest that the novel calcium-independent PKCs are in some way necessary for this response.

Using a replication-defective FRNK adenovirus, we have shown that FAK signaling is necessary for ET-induced NRVM hypertrophy. In some cell types FRNK is endogenously expressed at low levels as a separate 41- to 43-kDa protein (26). FRNK overexpression using a replication-competent avian retrovirus has been shown to inhibit FAK tyrosine phosphorylation and to delay chick embryo fibroblast spreading on fibronectin (22). In addition, FRNK overexpression decreased tensin and paxillin phosphorylation (22). In the present report, ET-induced FAK and paxillin phosphorylation was inhibited in NRVM infected with Adv-FRNK, and no hypertrophy or sarcomeric assembly was observed after chronic ET stimulation. Nevertheless, additional studies are needed to characterize precisely which intermediate signaling pathways were affected by FRNK overexpression and which are required for eliciting specific aspects of the hypertrophic phenotype.

Unlike the Adv-FRNK experiments, the Adv-FAK experiments described in Fig. 10 and Table 3 are more difficult to interpret. Instead of inducing NRVM hypertrophy as we hypothesized, FAK overexpression alone did not promote sarcomeric assembly, had no effect on the total protein-to-DNA ratio, and actually reduced β-MHC protein compared with uninfected or Adv-βgal-infected myocytes. These changes occurred despite the fact that FAK overexpression resulted in an increase in the amount of phosphorylated FAK that was comparable to that observed in ET-stimulated cells. Clearly, FAK overexpression alone was not sufficient, in the absence of other trophic stimuli, to produce NRVM hypertrophy. However, the mechanisms responsible for these unexpected results are unclear. One potential explanation is that we used wild-type FAK rather than a constitutively active form of FAK (6). Wild-type FAK overexpression resulted in a four- to fivefold increase in active FAK (Fig. 9C) but a 40-fold increase in total FAK (Fig. 9B), which was distributed not only in focal adhesions but throughout the myocyte cytoplasm (Fig. 10E). It is conceivable that nonphosphorylated FAK actually displaced endogenous, active FAK from focal adhesions and thus interfered with basal FAK-dependent signaling. Furthermore, in a previous study we showed that transient transfection of high-density, spontaneously contracting NRVM with an expression vector encoding wild-type FAK along with rat β-MHC and ANF promoter-luciferase constructs resulted in a two- to fourfold increase in luciferase activities (9). This was a substantial increase above the already high levels of ANF and β-MHC promoter activities observed in these high-density cultures. Thus FAK overexpression under these conditions could indeed stimulate the transcription of fetal genes associated with the hypertrophic phenotype. Interestingly, FAK overexpression did not significantly transactivate ANF or β-MHC promoter activity in verapamil-arrested cells, indicating that wild-type FAK overexpression alone was not sufficient to stimulate ANF or β-MHC transcription in the absence of [Ca 2+] i transients or other trophic signals. The Adv-FAK data described in the present report provide important, complementary information. FAK overexpression in low-density, noncontracting NRVM was also insufficient to induce total protein or MHC accumulation or to induce sarcomeric assembly. Determining which signaling molecules are necessary and/or sufficient to induce NRVM hypertrophy has been the subject of several recent studies (1, 16, 24, 37, 38, 40). Clearly, other signaling components that are stimulated by ET A-receptor activation in addition to FAK must be necessary to produce sarcomeric assembly and NRVM hypertrophy.

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