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Cellular localization of integrin isoforms in phenylephrine-induced hypertrophic cardiac myocytes

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Cardiac hypertrophy is characterized by remodeling of the extracellular matrix (ECM). Integrins are cell-surface molecules that link the ECM to the cellular cytoskeleton where they play roles as signaling molecules and transducers of mechanical force. To clarify the possible roles of integrins in cardiac myocyte hypertrophy, we investigated the cellular localization and expression of ECM proteins and integrins in both normal cardiac myocytes and phenylephrine-induced hypertrophic myocytes. Addition of phenylephrine (PE) to cultured neonatal cardiac myocytes induced sarcomeric organization, increase in cell size, and synthesis of the hypertrophic marker, atrial natriuretic factor (ANF). In particular, fibronectin and collagen underwent dramatic localization changes during PE-induced cardiac hypertrophy. Significant changes were noted in the cellular localization of the respective collagen and fibronectin receptors, integrin α1 and α5, from diffuse to a sarcomeric banding pattern. Expression levels of integrins were also increased during hypertrophy. Treatment with okadaic acid (OA), an inhibitor of protein phosphatase 2A (PP2A), resulted in inhibition of hypertrophic response. These results suggest that dephosphorylation of integrin β1 may be important in the induction of cardiac hypertrophy. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS — extracellular matrix (ECM); integrins; atrial natriuretic factor (ANF); okadaic acid (OA); cardiac hypertrophy

INTRODUCTION

Mechanical stress on the heart due to either pressure or volume overload may result in hypertrophy of the myocardium to enable optimization of cardiac performance. Hypertrophic responses are characterized by the enlargement of myocardial cells and accumulation of sarcomeric proteins.[1,2] Several cardiac myocyte hypertrophy factors have been identified, including phenylephrine (PE: α1-adrenergic agonist), noradrenaline, endothelin-1, angiotensin II and cardiotrophin-1.[3,4] Among these factors, the atrial natriuretic factor (ANF) gene product that is re-expressed in the ventricles during the hypertrophy process, is one of the most conserved and well-characterized markers of hypertrophic response, and may contribute to a reduction in cardiac preload and afterload.[3]

Many integrin α and β subunits are expressed during heart development and are critically involved in normal developmental processes. Integrins may function as mechanotransducers by forming an ECM–cytoskeleton linker to transduce mechanical stress into chemical signals. Signals initiated from the extracellular matrix (ECM) play important roles in the normal development of cardiac muscle, maintenance of normal function and disease.[5–7] Disruption of ECM networks leads to abnormal heart function, and eventually heart failure.[8,9] Enlargement of cell size in cardiac hypertrophic myocytes is accompanied by both remodeling of the ECM and expression of integrin counter receptors.[10,11] ECM proteins and their receptor integrins play unique roles in transmitting the hypertrophy signal from the external cellular environment to internal environment, consequently regulating this process. For example, adhesion of ventricular myocytes to ECM proteins is necessary for the

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adrenergic induction of ANF.[12–14] Additionally, overexpression of β1 integrin results in the upregulation of ANF expression.[11] Specifically, hypertrophic cardiac myocytes display increased synthesis of collagen, fibronectin and laminin.[5,15] Fibronectin is upregulated in the adult heart during pressure overload, myocardial infarction and spontaneous hypertension.[16,17] Therefore, changes in physical stress on the myocardium alter ECM composition, along with changes in the expression of the integrin counter receptors of matrix proteins. Specific interactions between ECM proteins and their receptor integrins may contribute to overall hypertrophic growth or phenotypic changes. Although ECM and integrin counter receptors influence remodeling in neonatal to adult heart transition and hypertrophy signaling,[18–20] limited information is available on changes in the distribution of these proteins during the transition from normal to hypertrophic myocytes. In this study, we investigate the cellular localization of ECM and integrin isoforms in PE-induced cardiac myocyte hypertrophy. Our results demonstrate that changes in the expression and cellular localization of ECM proteins (depending on enlargement of cell size) are correlated with expression of their respective integrin counter receptors in cardiac hypertrophic myocytes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), complete Freund's adjuvant, incomplete Freund's adjuvant, and 0.25% trypsin (w/v) were obtained from Gibco BRL (Grand Island, NY). Monoclonal anti-α-actinin antibody was purchased from Sigma Chemical Co. (Clone EA-53). Rabbit anti-integrin α1, α3 and α5 subunit polyclonal antibodies were purchased from Chemicon International Inc. (Temecula, CA) and collagen type IV goat serum was purchased from Santa cruz Biotechnology, Inc. (California). Phospho-threonine (p-Thr) monoclonal antibodies were obtained from Zymed Laboratories Inc. (San Francisco). Horseradish peroxidase (HRP)-labeled anti-mouse, rabbit, or goat immunoglobulin (IgG) and fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse or rabbit IgG were from Jackson ImmunoResearch Lab. (West Grove, PA). Okadaic acid was obtained from Sigma Chemical Co. (St. Louis, MO).

Cardiac myocytes culture

Neonatal rat ventricular myocytes were isolated according to the method of Burgess *et al.*[9] Briefly, hearts from 12–25 neonatal rats (1–2 days old) were excised and placed in Ads buffer (116 mM NaCl, 20 mM HEPES, 10 mM NaH_2PO_4, 5.5 mM glucose, 5 mM KCl, 0.8 mM $MgSO_4$, pH 7.4). The tissues were trimmed and then transferred to fresh Ads buffer. The minced tissues were incubated in enzyme dissociation solution and subjected to mechanical digestion (Ads buffer containing 65 units ml^{-1} collagenase Type II and 0.6 mg ml^{-1} pancreatin) at 37°C for 20 min and this process was repeated three times. To reduce the contaminants including fibroblasts and red blood cells, the supernatant of the first enzyme dissociation period was discarded. Cells collected from the second and third dissociation periods were subjected to centrifugation through a discontinuous Percoll (GIBCO-BRL, Grand Island, NY) gradient of 1.050, 1.062 and 1.082 g ml^{-1}, respectively. The band at the 1.062/1.082 g ml^{-1} interface was collected by centrifugation and used as a source of purified myocytes. These myocytes were resuspended in DMEM supplemented with 10% (w/v) FBS and then plated on collagen-coated dishes (50 μg ml^{-1}) at a density of 250 cells mm^{-2}. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of antibodies against atrial natriuretic factor (ANF)

To obtain the ANF gene, reverse transcription-polymerase chain reaction (RT-PCR) was performed with rat heart RNA. PCR was performed with the ANF-specific primers 5′-CCACTGCAG-CATGGGCTCCTTCT-3′ and 5′-GCAGAATTC GCTGTTATCTTCGGT-3′, the PCR product was cloned into TA vector, and subcloned into pRSET A vector containing a poly-histidine sequence. The ANF plasmid construct was introduced to BL-21 (DE3). The overexpressed ANF protein in bacterial cells was purified and used to immunize rabbit four times.

Total RNA isolation and Northern blot analysis

Total RNAs were isolated from cultured ventricular myocytes using TRI Reagent (Sigma, St. Louis, MO) as described in the manufacturer's protocol. After denaturation in formamide and formaldehyde, equal amounts of total RNA were size-fractionated by electrophoresis on 1% agarose gel. The fractionated

RNA was transferred onto a nylon membrane (Bio-Rad) by capillary action in 20× SSC. The nylon membrane was then baked at 80°C for 1.5–2 h and the RNA was permanently attached to the membrane by UV illumination for 2 min. Hybridization was performed in a polyethylene heat-sealable bag containing 10 ml of prehybridization solution (5.5 ml of 1 M $NaPO_4$, pH 7.5, 20 µl of 0.5 M EDTA, 100 µl of salmon sperm DNA (10 mg ml^{-1}), 1 ml of 10% (w/v) gelatin, 3.5 ml of 20% SDS). The nylon membrane in hybridization solution was preincubated for 1 h at 65°C before addition of the hybridization probe, and then hybridized with ^{32}P-labeled DNA probes prepared with an oligo-labeling kit (Pharmacia Biotech, Uppsala, Sweden). After 15 h incubation at 65°C, the membrane was washed three times in 0.1% SDS, 0.2× SSC at 65°C. The membrane was exposed to X-ray film for several days at −70°C. P5B (18S rRNA) was used to detect 18S rRNA as a control.

Immunofluorescence

Cardiac myocytes grown on collagen-coated coverslips (50 µg ml^{-1}) were fixed with 3.5% paraformaldehyde for 10 min and washed in phosphate-buffered saline (PBS). They were then permeabilized with 0.5% Triton X-100 in PBS, pH 7.4, for 5 min and then incubated for 45 min at room temperature with appropriate combinations of antibodies that had been diluted with PBS containing 1% BSA. The coverslips were then washed with PBS and mounted with 90% glycerol and 0.1% *o*-phenylenediamine in PBS.[21] Immunofluorescence was analysed under a Leica DMRBE microscope equipped with a 100× or 63× objective lens and filters for epifluorescence. Fluorescence micrographs were taken on T-max P3200 film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation

Cardiac myocytes grown on collagen-coated culture dishes were washed three times with cold PBS and extracted for 1 h at 4°C in 1 ml of extraction buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium ortho-vanadate, 10 mM leupeptin, 1.5 mM pepstatin, and 10 µg ml^{-1} aprotinin. The lysates were centrifuged for 10 min at 10,000 *g*. The protein concentrations of the supernatant were determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) and samples containing 600 µl of total protein were taken for immunoprecipitation with anti-β1A Ab, after which they were incubated for an additional 4 h at 4°C with protein A-agarose beads (Pharmacia Biotech, Uppsala, Sweden). Immune complexes were treated with SDS-sample buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol and 0.006% bromophenol blue, and subjected to SDS-PAGE.

Electrophoresis and immunoblotting

For immunoblot analysis, cells treated as described above were lysed in a lysis buffer containing 1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris, pH 7.4, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin and 10 µg ml^{-1} aprotinin, and then centrifuged for 5 min to remove insoluble material. Protein concentrations in the resultant lysate were measured using the BCA method. For electrophoresis, 8% polyacrylamide gels were used. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Blots were blocked with 3% BSA, 0.1% Tween 20 in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h. The membranes were incubated first with phospho-threonine Ab or integrins Ab and then incubated in TBS plus 0.1% Tween 20 (TBST) containing an HRP-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Lab.). Blots were washed thoroughly with TBST, and finally developed using ECL reagents. In some cases, blots were stripped by heating them to 65°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7) and re-probed.

RESULTS

Confirmation of hypertrophy induction by phenylephrine

To examine whether PE induces cardiac myocyte hypertrophy, we examined enlargement of cell size, increase in ANF expression and organization of the sarcomeric structure of cardiac myocytes treated with PE. Freshly isolated neonatal rat vetricular myocytes were cultured in growth medium containing 10% FBS for 24 h, and starved in serum-free medium for a further 24 h period. Following starvation, cells were incubated in serum-free medium with or without PE (100 µM). After PE treatment for 24 or 48 h, the expression of ANF was examined by Northern blotting and immunofluorescence analyses. ANF expression was dramatically increased in PE-treated

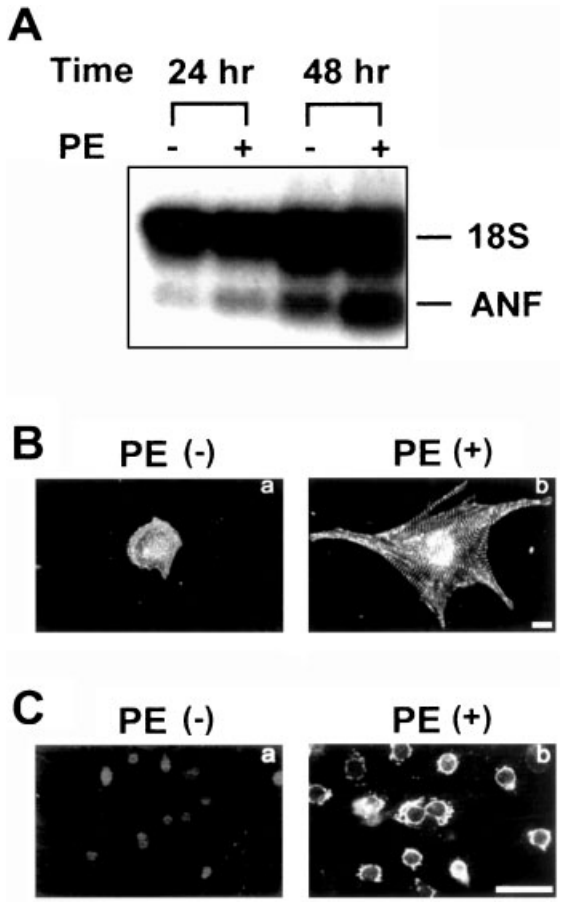

Figure 1. Hypertrophy induction by PE treatment in primary cultured cardiac myocytes. Cardiac myocytes were cultured for 1 day, and serum-starved for 24 h. (A) After treatment with or without 100 μM PE for 24 or 48 h, total RNA was isolated and subjected to Northern Blot analysis. (B) Expression of ANF was increased in PE-treated cardiac myocytes in a time-dependent manner. Cardiac myocytes were treated with (+) or without (−) 100 μM PE for 48 h, stained with anti-α-actinin Ab and visualized using TRITC-conjugated anti-mouse IgG. (C) Cell sizes increased and sarcomeric structure was well organized in PE-induced hypertrophic myocytes. Cultured cardiac myocytes were stained with specific polyclonal anti-ANF Ab after PE treatment for 48 h. Bars, 10 μm

myocytes (Figure 1A). Immunofluorescence studies using anti-ANF antibody further confirmed ANF protein synthesis (Figure 1C). Immunostaining studies with anti-α-actinin antibody demonstrated that PE-treated cardiac myocytes displayed well-developed sarcomere structure and significant increase in cell size (Figure 1B). In contrast, myocytes which were not treated with PE did not exhibit any significant characteristics of hypertrophy in terms of changes in cell size, ANF induction or sarcomere formation (Figure 1). These results indicate that PE treatment readily induces hypertrophy of cardiac myocytes *in vitro*.

Changes in cellular localization of fibronectin and collagen in PE-treated cardiac myocytes

We investigated the expression and cellular localization of the ECM proteins, fibronectin and collagen, in PE-induced hypertrophic myocytes. The cellular localization of fibronectin was dramatically modified from diffuse to sarcomeric banding patterns near the Z-line, corresponding to an increase in cell size of PE-treated cardiac myocytes. In contrast, no sarcomeric banding pattern was observed in cells not treated with PE, even in 8-day cultured myocytes in normal growth medium (Figure 2a and e). Notably, collagen was localized with a sarcomere banding pattern, both in PE-induced cardiac myocytes and 8-day cultured myocytes (Figure 2d and f). However, no specific distribution patterns of collagen or fibronectin were observed in cells not treated with PE. These

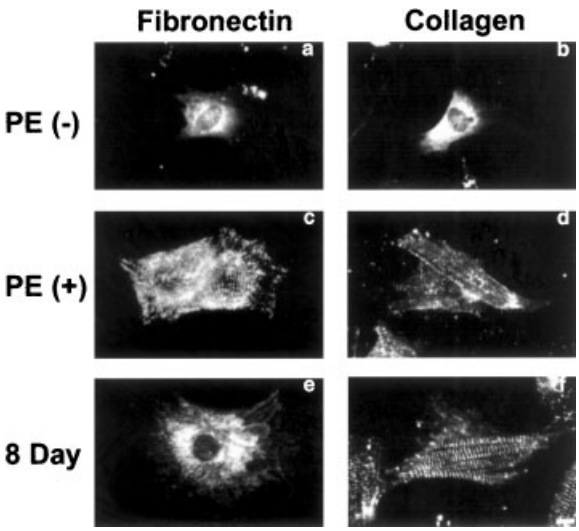

Figure 2. Cellular localization of fibronectin and collagen in PE-treated cardiac myocytes. After cardiac myocytes were cultured for 1 day in growth medium and serum-starved for 24 h, the first group (a and b) was cultured without 100 μM PE for 48 h, the second (c and d) was with 100 μM PE for 48 h and the third (e and f) with growth medium for 7 days. After culture, cells were stained with anti-fibronectin (a, c and e) or anti-collagen type IV (b, d and f) Abs and incubated with TRITC-conjugated anti-mouse IgG (for fibronectin) or TRITC-conjugated anti-goat IgG (for collagen type IV). Fibronectin and collagen were stained with a sarcomeric banding pattern in hypertropic cardiac myocytes (c and d) and cells (8 days old) cultured in normal growth medium (e and f) but not in untreated cells (a and b)

results suggest that both fibronectin and collagen play specific roles in the hypertrophic growth of myocytes.

Expression of integrin isoforms during PE-induced cardiac hypertrophy

To examine whether the changes in cellular localization of the ECM proteins, fibeonectin and collagen, are accompanied by alterations in the distribution of integrin, we investigated the cellular localization and expression of integrin isoforms during PE-induced cardiac myocyte hypertrophy.

In untreated myocytes, most integrins (α1, α3A, α5 and β1A) exhibited diffuse patterns on the cell surface. In contrast, integrins α1, α3A, α5 and β1A localized at the Z-line of the sarcomere in PE-induced hypertrophic myocytes (Figure 3b, e, h, and k). In 8-day cultured myocytes in normal medium, α3A, and β1A were localized with sarcomeric banding pattern, although integrin α1 and α5 remained unchanged as diffuse patterns on the cell surface (Figure 3c and f). Immunoblot analyses demonstrated a large increase in integrin α1 and α5 expression during hypertrophic growth (Figure 4). Changes in cellular localization and protein synthesis of integrins α1 and α5 corresponded to alterations in collagen and fibronectin during hypertrophic growth of myocytes, in agreement with the finding that integrins α1 and α5 are collagen and fibronectin receptors, respectively. Integrin α3A appeared to be localized in the sarcomere in PE-induced hypertrophic myocytes, indicating a potential candidate for collagen receptor.

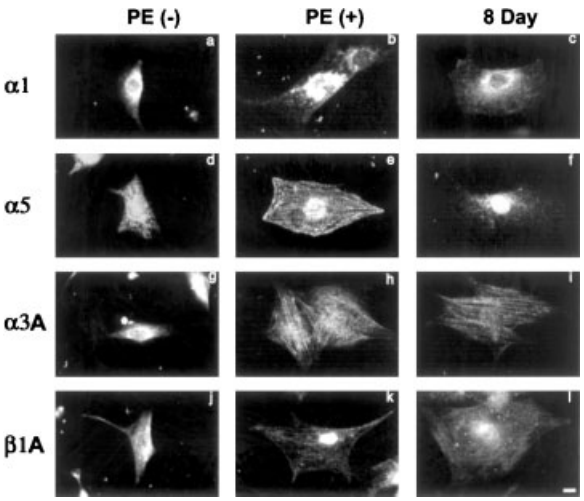

Figure 3. Cellular localization of integrin isoforms in hypertrophic cardiac myocytes. Neonatal cardiac myocytes were cultured for 1 day, and serum-starved for 24 h. After PE treatment for 48 h, cells were stained with specific Abs against integrin α1 (a, b, and c), α5 (d, e, and f), α3A (g, h, and I), and β1A (j, k and l). Integrin α1 and α5 localize at Z-lines in sarcomeres of hypertrophic myocytes (b and e) but not in 8-day myocytes cultured in normal growth medium (c and f) or untreated myocytes (a and d). In contrast, integrin α3A and β1A staining revealed well-organized sarcomeric structures in PE-induced hypertrophic cells (h and k) and 8 day-cultured myocytes (i and l). Bar, 10 μm

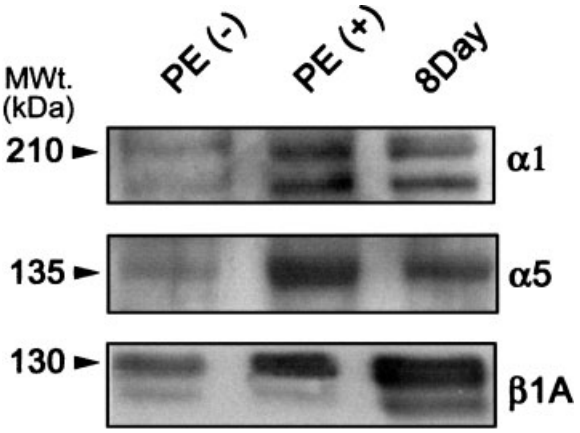

Figure 4. Expression of integrins in PE-induced hypertrophic and normal cardiac myocytes. Cardiac myocytes were cultured with or without PE for 48 h or in 10% FBS for 8 days. Cell lysates were immunoblotted with anti-integrin α1, α5 and β1A antibodies. The expression of integrin α1, α5 and β1A increased during hypertrophic induction and normal heart development

Inhibition of PE-induced hypertrophic growth by okadaic acid

Okadaic acid (OA), a serine/threonine protein phosphatase (PP2A) inhibitor, induces increased levels of integin β1 phosphorylation. The degree of integin β1 phosphorylation is important in cell growth, proliferation and differentiation. Accordingly, we examined changes in integrin β1 phosphorylation in hypertrophic growth. Myocytes were cultured in the presence of PE with or without OA (10 nM) for 48 h, and stained with anti-α-actinin antibody (Figure 5A). The sarcomere structure stained with anti-α-actinin antibody was clearly observed in PE-treated cells (Figure 5A-b), whereas okadaic acid (10 nM) significantly inhibited the formation of sarcomere (up to 70%) in PE-treated cells (Figure 5A-c and B). As shown in Figure 5C, treatment with this compound also prevented dephosphorylation of β1A integrin, thus inhibiting integrin β1 activity. This suggests that phosphorylation of integrin β1 may be regulated during hypertrophic growth of cardiac myocytes.

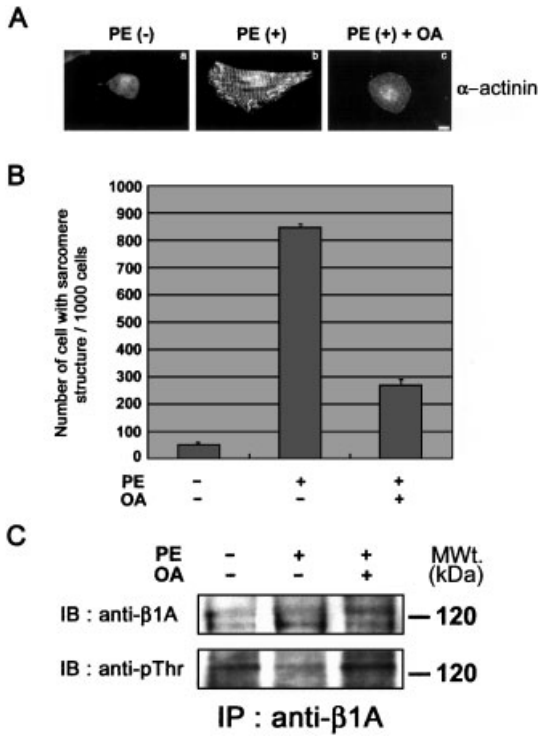

Figure 5. Inhibition of hypertrophic growth and β1A integrin dephosphorylation by okadaic acid. Cardiac myocytes were cultured for 1 day, serum-starved for 24 h, and treated with or without 100 μM PE or 100 μM PE plus 10 nM okadaic acid (OA) for 48 h (A and B) or 100 μM PE plus 20 nM okadaic acid (OA) for 48 h (C). Cells were stained with anti-α-actinin Ab (A-a, -b, and -c). The sarcomere structure was clearly observed in PE-treated cells (A-b). However, sarcomere formation was inhibited by OA (+) PE treatment (A-c). After treatment with PE (+), without PE (−) or PE with OA (PE + OA), cardiac myocytes were stained with anti-α-actinin Ab and cells with sarcomere structure were counted (B). OA treatment dramatically reduced the number of cells with sarcomere structure. Cell lysates were immunoprecipitated (IP) using β1A integrin antibody, and immunoblotted (IB) with anti-phosphothreonine antibody. After stripping, the membrane was re-probed with anti-β1A antibody (C). Inhibition of PP2A by okadaic acid (OA) led to an increase in the phosphorylation of β1A integrin

DISCUSSION

The present study involves an investigation into the cellular localization of several ECM and integrin subunits to elucidate the mechanism of integrin-mediated hypertrophic signaling. Cellular localization of fibronectin and collagen altered from diffuse to sarcomeric banding patterns during PE-induced hypertrophic growth, while no such changes were observed in normal developing cardiac cells (Figure 2). ECM and integrin as ECM receptor also displayed changes in cellular localization and protein synthesis levels in PE-induced hypertrophic cells, suggesting that integrin–ECM interactions play pivotal roles in cardiac myocyte hypertrophic growth. Inhibition of integrin-mediated hypertrophic signaling by OA further supports this theory.

PE-stimulated hypertrophy in cardiac myocytes is used as a model for cardiac hypertrophy. Prolonged exposure to α1 adrenergic agonists (such as PE) leads to a variety of hypertrophic alterations in neonatal rat ventricular myocytes, including increased cell size, sarcomeric protein assembly and specific changes in gene expression.[22–24] Increase in cell size may require the formation of an elastic stress-tolerant network inside or outside the cell, which is essential for the adaptation of the heart. This reflects the synthesis and redistribution of ECM proteins in hypertrophic myocytes and rearrangement and increase in myofibrillar proteins, particularly myosin and actin.[25–28] The effects of PE on myocytes included a rapid increase in the accumulation of the main constituents of the extracellular matrix, fibronectin and collagen. These results are concordant with previous reports that in the rat heart subjected to sudden pressure overload, the FN gene is upregulated at the early developmental stage of cardiac hypertrophy.[29] The specific organization of the collagen matrix in the heart serves as a scaffolding site for interconnecting myocytes, and functions by placing limits on myocyte movement and ensuring proper alignment of these cells.[30,31] With respect to increased expression and formation of a three-dimensional network, our immunofluorescence experiments demonstrated that the sarcomeric banding patterns of FN and collagen were visible on the surfaces of PE-treated cardiac myocytes. The cellular localization of collagen and fibronectin lateral to the Z-disk may be associated with cardiac mechanics by which the Z-disk transmits mechanical stress across the sarcolemma in conjunction with the contractile function of cardiac muscle. These results are consistent with the theory that FN plays a key role as a mechanical transducer in PE-induced myocardiac hypertrophy.

The association of ECM with cardiac myocytes in hypertrophic induction appears to be partly regulated by integrins on the cell surface. The expression of integrins in association with the synthesis of ECM components is more evident in cardiac hypertrophy; for instance, the elevation of α1 integrin correlates with increased collagen synthesis.[5] Our results demonstrate an increase in the levels of integrin α1 and α5 in PE-induced cardiac hypertrophy. Furthermore, in this study, α5 remained as a diffuse pattern

on the surface of myocytes in the early stages of development, following which molecules were physically localized into specific sites like Z-disks during hypertrophic growth, suggesting that $\alpha 5$ is likely to perform distinct functions depending on the hypertrophic growth. The $\alpha 1$ integrin also seems to actively participate in hypertrophy and associates with collagen in the Z-band to maintain contractile functions, form focal adhesion and induce cytoskeleton reorganization during hypertrophy. In PE-induced cardiac myocyte hypertrophy, increased expression of integrins $\alpha 1$ and $\alpha 5$ may be associated with an increase in their respective ligands, specifically collagen and fibronectin.

A number of reports suggest that integrin $\beta 1$ is important for signal transduction.[32,33] The binding of signal proteins to integrin $\beta 1$ cytoplasmic domains leads to the recruitment of signal and structural proteins, forming a complex hierarchical structure that directs cytoskeletal assembly and signal transduction. The results of knock-in/knock-out studies suggest that $\beta 1$ integrins act as mechanotransducers, and thereby function in the maintenance of myofibrillar organization during heart development.[34,35] However, little is known about the integrin $\beta 1$ phosphorylation and dephosphorylation mechanism, which may regulate different signals from integrins. The $\beta 1$ integrin is negatively and positively regulated by the ILK and protein phosphatase 2A cycle, respectively.[36] ILK is responsible for the phosphorylation of $\beta 1$ cytoplasmic domain, while PP2A performs the reverse dephosphorylation reaction. In our experiments, OA, a specific inhibitor of PP2A, inhibited hypertrophic growth in cardiac myocytes, which resulted in disorganized sarcomere structure. This may be due to an increase in integrin $\beta 1$A phosphorylation, suggesting that the hypertrophic signal in cells is at least partly regulated by the phosphorylation/dephosphorylation state mediated by the ILK-PP2A cycle. Addition of cytostatin, a PP2A inhibitor, results in loss of cell adhesion and disorganization of focal adhesion components,[37] further supporting the idea that regulation of phosphorylation/dephosphorylation of $\beta 1$A is an important step in the induction of cardiac hypertrophy.

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