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Mechanism of Oleic Acid-Induced Myofibril Disassembly in Rat Cardiomyocytes

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Abstract This study investigated the mechanism of oleic acid (OA)-induced disassembly of myofibrils in cardiomyocytes. OA treatment disrupted myofibrils, as revealed by the disorganization of several sarcomeric proteins. Since focal adhesions (FAs) are implicated in myofibril assembly, we examined structural changes in FAs after OA treatment. Immunofluorescence studies with antibodies against FA proteins (vinculin, integrin β1D, and paxillin) showed that FAs and costameres disintegrated or disappeared after OA treatment and that the changes in FA proteins occurred prior to myofibril disassembly. The effects of OA on FAs and myofibrils were reversed after removal of OA. OA decreased expression of integrin β1D, paxillin, vinculin, and actin, and induced tyrosine dephosphorylation of FA kinase (FAK) and paxillin. These effects were blocked by pretreatment with sodium orthovanadate, a protein tyrosine phosphatase (PTP) inhibitor. This inhibitor also prevented OA-induced myofibril disassembly, indicating the involvement of PTP in myofibril disassembly. Furthermore, OA increased protein levels of PTP-PEST. The upregulation of this phosphatase correlated with the tyrosine dephosphorylation of paxillin and FAK, which are targets for PTP-PEST. In addition, OA decreased RhoA activity and the phosphorylation of cofilin, a downstream target of RhoA. Cofilin dephosphorylation increased its actin-severing activity and led to the depolymerization of F-actin, which might provide another potential mechanism for OA-induced myofibril disassembly. J. Cell. Biochem. 102: 638–649, 2007.

Key words: oleic acid; cardiomyocyte; myofibril disassembly; focal adhesion; PTP-PEST; RhoA; cofilin

Intracellular lipid deposition in cardiomyocytes is associated with reduced myocardial contractile function [Tomita et al., 1990; Oyamada et al., 2001; Christoffersen et al., 2003]. Contractile activity is modulated by many factors, including gap junctions, myofibril assembly, and the intracellular calcium ion concentration. Recently, we showed that OA causes a reduced contraction rate and gap junction disassembly in cultured neonatal cardiomyocytes [Huang et al., 2004], which may contribute to cardiac arrhythmias. Whether OA also induces myofibril structural changes in cardiomyocytes remains to be studied.

In cardiac muscle, the cortical myofibrils are connected to the membrane-extracellular matrix via FAs and costameres [Borg et al., 2000]. In vitro and in vivo analyses have suggested that FAs and costameres are required for the assembly and maintenance of the structural integrity of the contractile apparatus, which modulates contractility [Ross and Borg, 2001]. Tyrosine phosphorylation of FA-associated proteins plays an important role in regulating FA assembly. Since integrin lacks intrinsic kinase activity, FA kinase (FAK) has been identified as the key cytoplasmic tyrosine kinase that transmits integrin-mediated signals in several cell types [Schlaepfer et al., 1999;

Abbreviations used: OA, oleic acid; FA, focal adhesion; FAK, focal adhesion kinase; PTP, protein tyrosine phosphatase; PKC, protein kinase C; HBSS, Hank's balanced saline solution; MEM, minimal essential medium; BSA, bovine serum albumin; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

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Salazar and Rozengurt, 2001]. Clustering of integrins in the plasma membrane caused by extracellular matrix molecules stimulates autophosphorylation of FAK at Tyr397 and creates a binding site for Src kinase [Schaller et al., 1994]. Binding of FAK to Src kinase induces tyrosine phosphorylation of p130CAS, paxillin, and Shc, which, in turn, phosphorylate several FA proteins and induce the formation of FAs and the cytoskeleton [Burridge et al., 1992; Thomas et al., 1999]. Since OA induces contractile dysfunction in cardiomyocytes, we hypothesized that it might affect the structural integrity of FAs and costameres by modulating the tyrosine phosphorylation of their associated proteins. The balance of cellular tyrosine phosphorylation is controlled by the coordinated actions of protein tyrosine kinases and PTP. Tyrosine phosphorylation of FA proteins is negatively regulated by PTP. For example, PTP-PEST (a cytosolic PTP expressed in various mammalian tissues) [Yang et al., 1993] has been shown to induce tyrosine dephosphorylation of FAK and paxillin, which may lead to FA disassembly [Tamura et al., 1998; Angers-Loustau et al., 1999]. In contrast, Src kinase is activated by phosphorylation on a single tyrosine residue, Tyr416, which modulates the assembly of FAs through the recruitment of FA adaptor proteins [Laser et al., 2000].

A small G-protein, RhoA, participates in various cellular functions such as cell growth, migration and development by triggering actin stress fibers and FA formation [Jaffe and Hall, 2005]. In cardiomyocytes, RhoA plays an important role in assembly of myofibrils and FAs [Wang et al., 1997; Aoki et al., 1998]. Inactivation of RhoA by C3 exoenzyme results in the loss of integrin-based costameres and the disassembly of myofibrils in cardiomyocytes [Wang et al., 1997]. In contrast, activation of RhoA by transfection with constitutively active form of RhoA increases myofibril formation and organization in cardiomyocytes [Aoki et al., 1998]. RhoA mediates actin cytoskeleton organization by its downstream effector cofilin, an actin-severing protein responsible for depolymerization of F-actin. RhoA activation induces subsequent phosphorylation of Rho kinase, LIM-kinase and cofilin, and decreases cofilin activity, which promotes actin filament assembly [Maekawa et al., 1999]. In order to obtain a better insight into the mechanism of OA-induced contractile dysfunction, the present study was designed to investigate the effects of OA on the distribution and expression of myofibrillar and FA proteins, and to identify the role of FAK, Src kinase, PTP-PEST and RhoA signaling in OA-induced changes in contractile apparatus in cultured neonatal rat cardiomyocytes.

MATERIALS AND METHODS

Cell Culture

Cardiomyocyte cultures were prepared from 1- to 2-day-old Wistar rats as described previously [Zheng et al., 1996]. Rat ventricles were removed, minced in Ca^{2+}/Mg^{2+}-free Hank's balanced saline solution (HBSS; Gibco, Grand Island, NY), and incubated for 15 min at 37°C with 0.5 mg/ml of collagenase type II (Sigma, St. Louis, MO) and 0.6 mg/ml of pancreatin (Sigma) in HBSS. The dissociated cell suspension was collected and mixed with an equal volume of ice-cold plating medium consisting of minimal essential medium (MEM; Gibco) containing 10% fetal bovine serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The residual tissue fragments were repeatedly digested (3–4 times) until completely dissociated. The combined cell suspensions from all digestions were centrifuged at 1,000 g for 10 min at room temperature, resuspended in plating medium, and preplated for 1 h at 37°C in a 10 cm culture dish in a CO_2 incubator. After fibroblast attachment, the cardiomyocytes in the supernatant were collected and plated at a density of 10^5 cells/ml for coverslips and 10^6 cells/ml (high density) for 35 mm collagen-coated dishes for one day, then the medium was changed to growth medium (MEM containing 10% calf serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine). All experiments were performed on day 2 cultures. Day 2 cultures on coverslips were 80% confluent, while those in dishes were 100% confluent. The percentage of cardiomyocytes was greater than 90%, as determined by the proportion of cells showing spontaneous contraction or staining positive for the muscle-specific protein, titin.

Drug Treatment

Unless otherwise stated, day 2 cultures were incubated with a mixture of 100 μg/ml of OA (Sigma) and 400 μg/ml of bovine serum albumin (BSA, Sigma) or with BSA alone for 6, 18, or 24 h at 37°C. To evaluate the role of PTP in FA disruption and myofibril disassembly following

OA treatment, we pretreated the cells for 30 min with the indicated concentrations of the tyrosine phosphatase inhibitor, sodium orthovanadate (30 and 50 μM; Sigma) before incubation with OA for 24 h; one set of controls was incubated with orthovanadate alone for 24 h.

Antibodies

The primary antibodies were rat polyclonal antibodies against α-actinin and M-protein, and mouse monoclonal antibodies against titin [Wang et al., 1998], α-actin (Sigma), myosin heavy chain (MF-20; Developmental Studies Hybridoma Bank, Iowa City, IA), vinculin (Sigma), integrin β1D (Abcam, Cambridgeshire, UK), FAK, paxillin (Pharmingen, San Diego, CA), phosphotyrosine (4G10: Upstate Biotechnology Inc., Lake Placid, NY), β-tubulin (BioVision Technologies, Mountain View, CA), PTP-PEST (Exalpha Biologicals, Watertown, MA), and RhoA (Cytoskeleton, Denver, CO). Rabbit polyclonal antibodies included anti-FAK phosphorylated at Tyr397, anti-paxillin phosphorylated at Tyr31 (both from Biosource International, Camarillo, CA), anti-Src kinase phosphorylated at Tyr416 (Cell Signaling Technology, Danvers, MA), anti-cofilin phosphorylated at Ser-3 and anti-cofilin (Cell Signaling). The secondary antibodies were FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rat IgG (both from Sigma), biotin-conjugated goat anti-mouse IgG antibodies (preabsorbed with rat IgG) (Vector, Burlingame, CA), alkaline phosphatase-conjugated goat anti-rabbit IgG and anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blot Analysis

Cardiomyocytes were scraped off the culture dish in lysis buffer (0.15% Triton X-100, 10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, 25 mM HEPES, pH 6.9) and sonicated, and then the protein concentration of the lysate was determined using a protein assay kit (Bio-Rad, Hercules, CA). Protein samples (50 μg per lane) were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and the proteins transferred to nitrocellulose membranes (Schleicher & Schuell BioSciences, Boston, MA). Membrane strips were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris, pH 8.2), then incubated overnight at 4°C with primary antibodies. After washes with TBS-0.1% Tween, the strips were incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibodies (1:7,500 dilution), and then bound antibody was visualized using substrate solution (3.3 mg/ml of nitroblue tetrazolium and 1.65 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5). The blots were photographed and the density of each band quantified by densitometric scanning using a Gel-Pro analyzer (Media Cybernetics, Inc., Houston, Texas) and expressed relative to that of the band in the control group (100%). All experiments were repeated at least three times.

Immunofluorescence

After various treatments, cardiomyocytes were fixed for 10 min at room temperature in 10% formalin in phosphate-buffered saline (PBS), pH 7.4, then non-specific binding sites were blocked with PBS containing 5% non-fat milk and 0.1% Triton X-100. For single labeling, the cells were incubated for 1 h at 37°C with the primary antibodies listed above, washed with PBS (3 × 5 min), and reacted for 1 h at 37°C with FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-mouse IgG. For double-labeling studies, the cells were incubated for 1 h at 37°C with a mixture of rat anti-α-actinin and mouse anti-vinculin antibodies, then, after a brief wash, were incubated with 1 h at 37°C with a mixture of FITC-conjugated goat anti-rat IgG antibodies pre-absorbed with mouse IgG and biotin-conjugated goat anti-mouse IgG antibodies pre-absorbed with rat IgG, followed by avidin-conjugated Texas red (Vector) for another 30 min at 37°C. After washing in PBS, the cells were mounted using 3% n-propyl gallate and 50% glycerol in PBS, and examined using a Zeiss epifluorescence microscope (Carl Zeiss, Oberkocheu, Germany), images being captured and digitized using a Nikon DIX digital camera (Nikon, Tokyo, Japan).

RhoA Activity Assay

The RhoA activity assay was performed according to the manufacturer's instructions (Cytoskeleton). Active (GTP-bound) RhoA was pulled-down from cell lysates with Rhotekin-conjugated agarose beads for 1 h at 4°C. The beads were collected by centrifugation and washed with buffer. Active RhoA was recovered from the beads by boiling for 5 min in SDS-

sample buffer, separated by 15% SDS-PAGE, and detected by Western blotting analysis using an anti-RhoA antibody.

Statistics

Densitometric scans were performed on three separate blots from different experiments and the results expressed as the mean ± S.D. Student's t test was used to analyze differences, P values of <0.05 and <0.01 being considered significant and highly significant, respectively.

RESULTS

OA Causes Disassembly of Myofibrils

To explore OA-induced contractile dysfunction, the effects of OA on the structural integrity of the contractile apparatus were investigated. Our previous study showed that the viabilities of cardiomyocytes were not affected by OA treatment as examined by MTT test and propidium iodide (a cell death indicator) vital staining [Huang et al., 2004]. Cultured cardiomyocytes were treated with various concentrations of OA for 24 h, and then the distribution of α-actinin (a Z-line marker) was examined by immunofluorescence. In the BSA-treated control group, the myofibrillar Z-lines were arranged in linear arrays (Fig. 1A). In contrast, in OA-treated cells, about 40% the cells showed marked morphological changes in Z-line architecture, this effect being dose-dependent (Fig. 1B–D). After treatment with OA, α-actinin staining became punctate (Fig. 1B), while, after treatment with higher concentrations of OA, randomly orientated Z-line staining was more obvious (Fig. 1C, D). In addition to targeting α-actinin, OA also induced disassembly of I-band, A-band, titin doublets and M-band as revealed by immunofluorescence staining for α-actin, myosin heavy chain, titin, and M-protein (data not shown).

OA Induces Disruption of FAs and Costameres in Cardiomyocytes

In striated muscle cells, formation of FAs and costameres is required for myofibril assembly and maintenance of myofibril integrity. To determine whether FA integrity was involved in OA-induced myofibril disassembly, cells were treated with BSA or with OA for 24 h, and double-labeled with antibodies against α-actinin and vinculin (FA marker). In the control cells, vinculin staining was seen as rod-shaped or oval spots (typical FA structures) at the ventral sarcolemma (Fig. 2B, arrows) and, in the en face view, as rib-like structures (costameres) (Fig. 2B, arrowheads). After 24 h of OA treatment, vinculin staining appeared as tiny dots and costameres were no longer identifiable (Fig. 2D). FA disorganization was consistently accompanied by myofibril disassembly (Fig. 2C). To examine whether the effects of OA on the

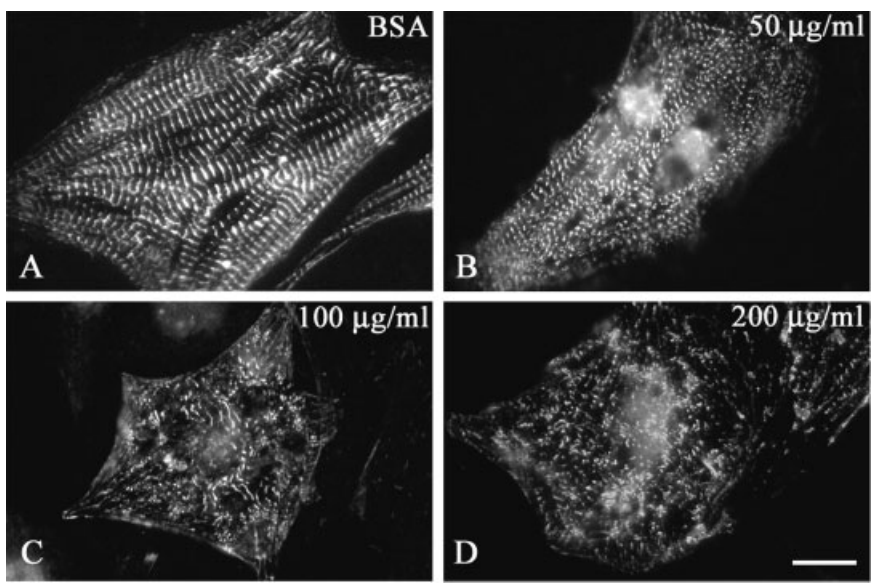

Fig. 1. Dose-response effect of OA on the organization of myofibrillar Z-lines in rat cardiomyocytes. Cells were treated for 24 h with BSA alone (**A**) or with the indicated concentration of OA (**B**, **C**, **D**), and labeled with antibody against α-actinin, a Z-line marker. Bar = 15 μm.

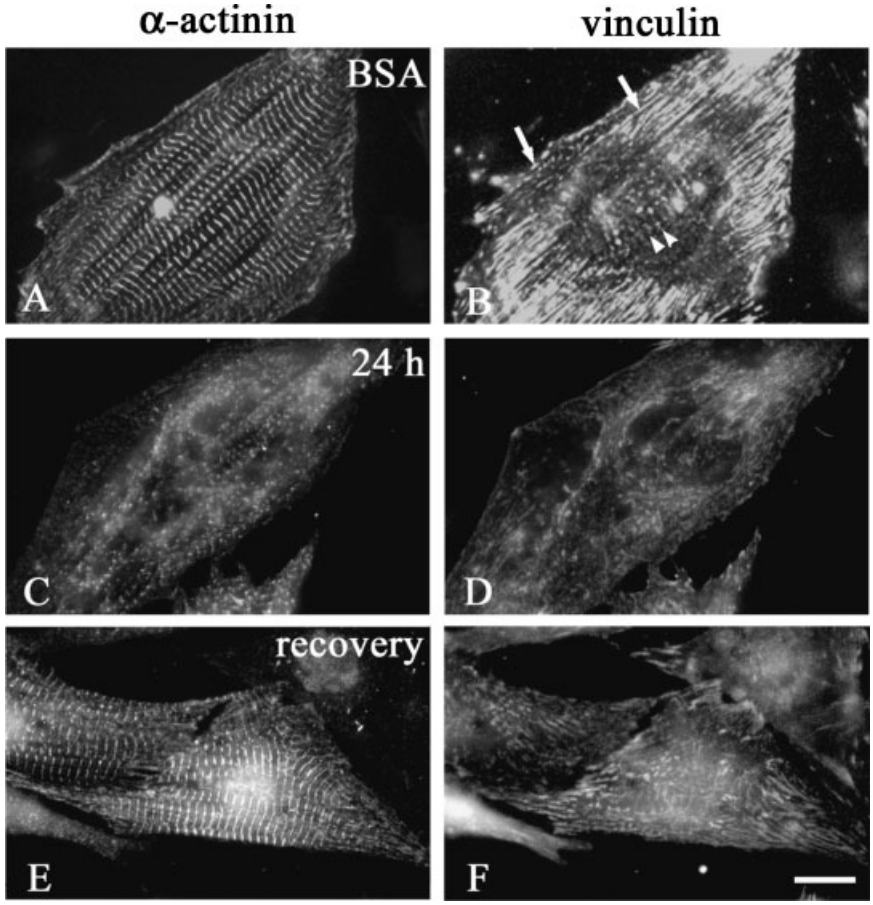

Fig. 2. The effect of OA on vinculin distribution in rat cardiomyocytes. Cells were treated with BSA alone for 24 h (**A**, **B**) or with 100 μg/ml of OA for 24 h (**C**, **D**). In the recovery experiment (recovery, **E**, **F**), after incubation of the cells for 24 h with 100 μg/ml of OA, the medium was replaced with fresh culture medium containing BSA for another 24 h. The cells were double-labeled with anti-α-actinin (A, C, E) and anti-vinculin (B, D, F) antibodies. The arrows in B indicate FAs at the ventral sarcolemma and the arrowheads indicate costameres. Bar = 15 μm.

disassembly of myofibrils and FAs were reversible, cardiomyocytes were treated with OA for 24 h, then incubated with fresh culture medium containing BSA for another 24 h. About 50% of the OA-affected cardiomyocytes resumed normal myofibril arrangement after incubation in BSA-containing fresh medium. In these "recovery" cells, α-actinin and vinculin displayed typical myofibrillar and FA staining (Fig. 2E, F) comparable to that in the BSA-treated control group (Fig. 2A, B). FAs can also be visualized by immunostaining for integrin β1D and paxillin. In BSA-treated cardiomyocytes, integrin β1D staining was seen at FAs (arrows in Fig. 3A) and costameres (arrowheads in Fig. 3A), while paxillin staining exhibited the typical patched pattern of FAs at the cell periphery (Fig. 3C, arrows). After incubation with OA for 24 h, the staining of integrin β1D at FAs became weak whereas that at costameres was not detected (Fig. 3B), and paxillin staining was seen as a dotted pattern in the cytoplasm (Fig. 3D).

OA Decreases Phosphotyrosine Staining of FA Proteins

Tyrosine phosphorylation of FA proteins is required for FA assembly. In order to evaluate the phosphorylation status of FA proteins after OA treatment, immunofluorescence staining with anti-phosphotyrosine antibody was carried out. Phosphotyrosine immunoreactivity was mainly seen at the FAs in control cells (Fig. 3E) and as weak, punctate staining throughout the cytoplasm in OA-treated cells (Fig. 3F), indicating a decrease in phosphotyrosine levels in FA proteins. In parallel to the morphological observations, the time-course of the effect of OA on protein levels of phosphorylated FAK and paxillin was evaluated by

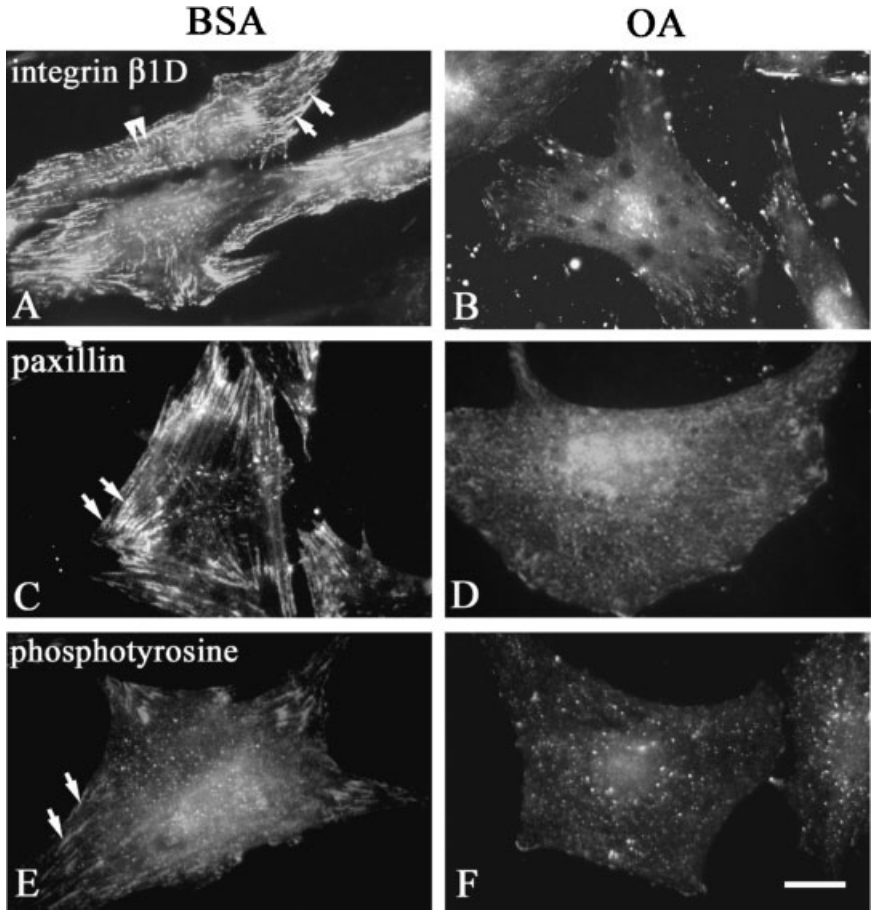

Fig. 3. Effect of OA treatment on the distribution of integrin β1D, paxillin, and phosphotyrosine in rat cardiomyocytes. Cells were treated for 24 h with BSA alone (**A**, **C**, **E**) or with 100 μg/ml of OA (**B**, **D**, **F**), then labeled with antibodies against integrin β1D (A, B), paxillin (C, D), or phosphotyrosine (E, F). The arrows in A, C, and E indicate FAs and the arrowheads in A indicate costameres. Bar = 15 μm.

Western blotting. The results showed that, after OA treatment, tyrosine phosphorylation of FAK and paxillin and protein levels of paxillin, but not FAK, were reduced in a time-dependent manner (Fig. 4). Notably, OA induced a 40% decrease in FAK phosphorylation within 6 h (Fig. 4B). OA also decreased protein levels of integrin β1D, paxillin, vinculin and α-actin. OA treatment did not affect the protein levels of the internal standard, β-tubulin (Fig. 4A).

PTP is Involved in the OA-induced Disassembly of FA and Myofibrils

Increased PTP activity results in dephosphorylation of some specific FA proteins and is associated with FA disassembly [Maher, 1993]. We therefore determined whether PTP was involved in the decrease in phosphorylation levels of FAK and paxillin caused by OA. Sodium orthovanadate, a PTP inhibitor, prevented the OA-induced decrease in phosphorylation of FAK and paxillin in a dose-dependent manner. This treatment also prevented OA-induced downregulation of integrin β1D and α-actin (Fig. 5A, B). We then examined whether OA-induced myofibril disassembly was caused by PTP activation. The distribution of α-actinin was not affected by treatment with 50 μM sodium orthovanadate alone (Fig. 5C, c), when compared to the BSA-treated controls (Fig. 5C, a). Treatment with OA and sodium orthovanadate significantly prevented OA-induced myofibril disassembly (Fig. 5C, c–d); the percentages of cells showing myofibril disassembly decreased from 40% in OA groups to 10% in OA plus sodium orthovanadate groups. These data showed that activation of PTP was responsible for the tyrosine dephosphorylation of FAK and paxillin, which is required for OA-induced disassembly of FAs and myofibrils.

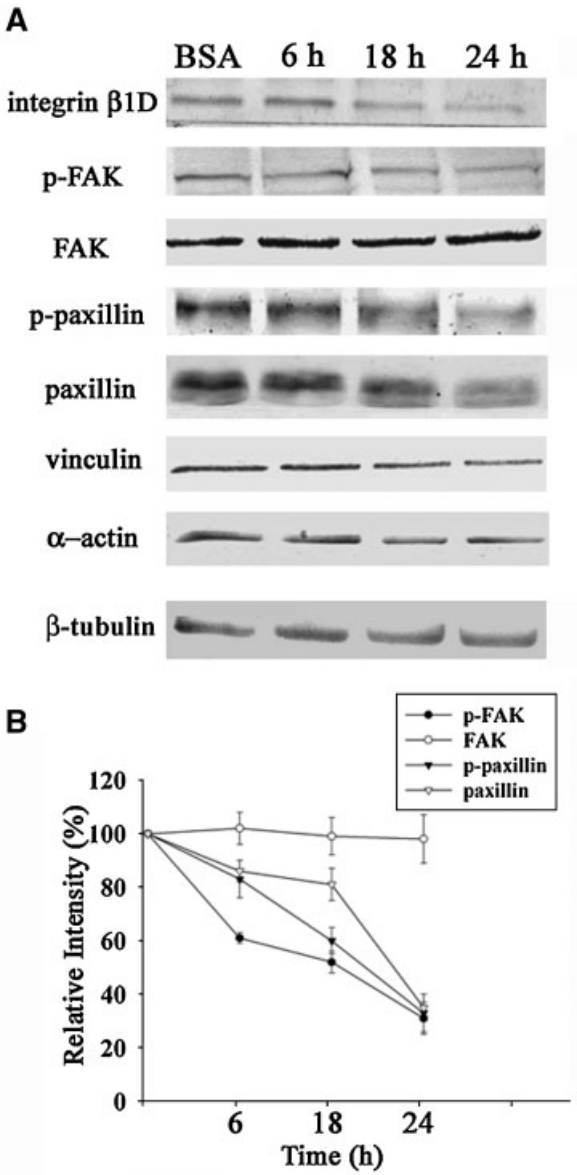

Fig. 4. Effect of OA on the expression of focal adhesion proteins. **A**: Western blots. Cells were treated with BSA alone for 24 h (BSA) or with 100 μg/ml of OA for 6, 18, or 24 h. The cell homogenates were subjected to electrophoresis and immunoblotting using antibodies against phosphorylated FA kinase (p-FAK), phosphorylated paxillin (p-paxillin), FAK, paxillin. β-Tubulin was used as an internal control. **B**: Densitometric data from three separate blots, presented as a percentage of the control value (mean ± SD).

OA Induces Upregulation of PTP-PEST

We then identified the PTP isoform responsible for the OA effects. PTP-PEST induced FA disassembly by dephosphorylating FAK and paxillin [Shen et al., 1998]. To investigate whether PTP-PEST was involved in OA-induced FA disassembly, we analyzed the

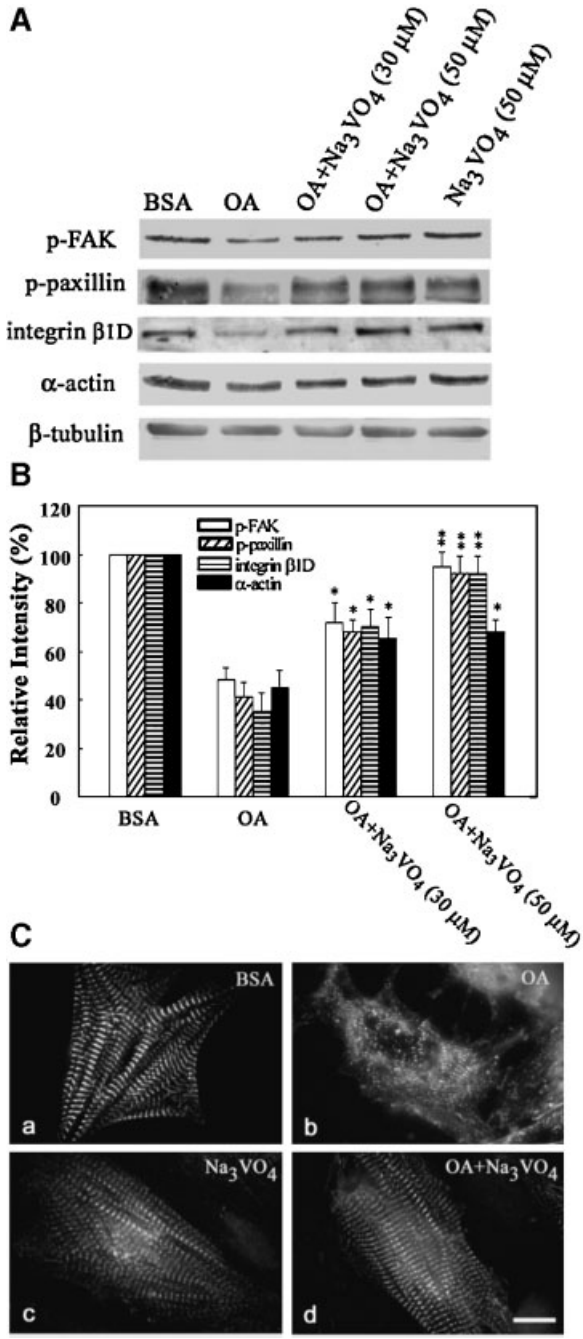

Fig. 5. Effects of sodium orthovanadate on OA-induced changes in FA proteins and myofibrillar structure. **A**: Cells were treated for 30 min with the indicated concentration of Na_3VO_4 prior to addition of OA for 24 h. The cell homogenates were subjected to electrophoresis and immunoblotting using antibodies against p-FAK, p-paxillin. β-Tubulin was used as an internal control. **B**: Densitometric data from three separate blots, presented as a percentage of the control value (mean ± SD). *, $P < 0.05$; **, $P < 0.01$, compared to the OA-treated control group. **C**: Cardiomyocytes treated for 30 min with 50 μM Na_3VO_4 prior to addition of OA for 24 h (d) or treated with BSA (a), OA (b), or Na_3VO_4 (c) alone for 24 h were labeled with antibody against α-actinin. Bar = 15 μm.

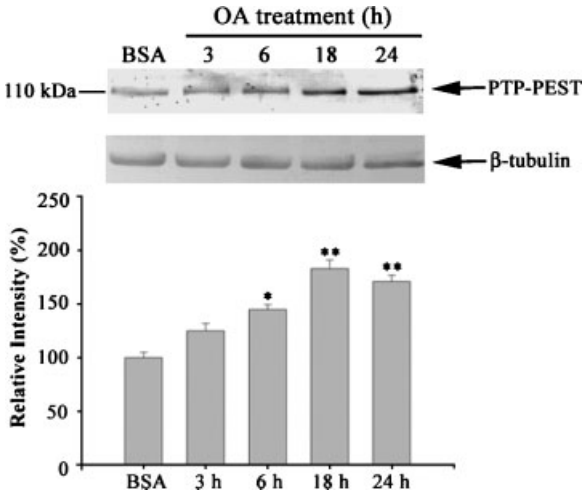

Fig. 6. OA-induced upregulation of PTP-PEST. Cells were treated for the indicated time with 100 μg/ml of OA, then the cell lysates were subjected to electrophoresis and immunoblotting using antibodies against PTP-PEST. β-Tubulin was used as an internal control. Densitometric data from three separate blots, presented as a percentage of the control value. *, $P < 0.05$; **, $P < 0.01$, compared to the BSA-treated control group.

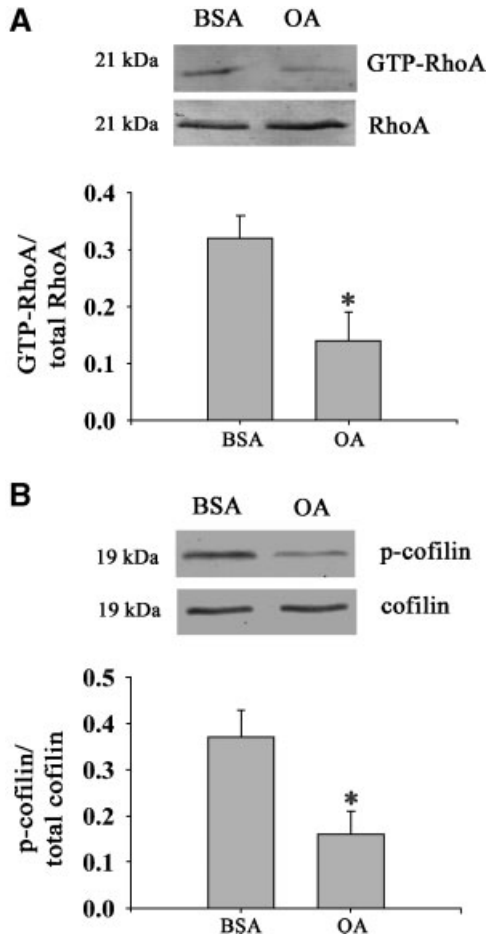

Fig. 7. Decrease in RhoA activity and cofilin phosphorylation in OA-treated cardiomyocytes. Cells were treated with OA for 6 h, and then activated (GTP-bound) RhoA was pulled down from cell lysates by Rhotekin-conjugated agarose beads and assayed for RhoA (**A**), phosphorylated cofilin (p-cofilin) and cofilin (**B**). The results of densitometric analysis are expressed as the ratios of GTP-RhoA/total RhoA or p-cofilin/total cofilin, respectively. *, $P < 0.05$, compared to the BSA-treated control group. $n = 3$.

expression of PTP-PEST after treatment. Western blot analysis showed that OA induced an increase in protein levels of PTP-PEST (Fig. 6). Protein levels of PTP-PEST significantly increased after 6 h of treatment, peaked at 18 h. These data suggest that PTP-PEST may regulate the disassembly of FAs caused by OA. In addition, pp60Src kinase plays a key role in integrin-mediated FA assembly [Laser et al., 2000]. Since OA induced FA disassembly, pp60Src kinase activity was also examined by Western blot analysis using a specific antibody against active Src kinase (Src family kinase phosphorylated on Tyr416). Levels of pp60Src kinase were not affected by OA treatment (data not shown), indicating that Src kinase was not involved in the response to OA treatment. Our previous study indicates that OA increases PKCε activity within 1 h of treatment [Huang et al., 2004]. However, in this study, pretreatment with PKCε inhibitor, eV1-2, did not abolish the OA-induced tyrosine dephosphorylation of FAK and paxillin, and the upregulation of PTP-PEST (data not shown).

OA Decreases RhoA Activity and Cofilin Phosphorylation

RhoA signaling participates in actin cytoskeleton rearrangement and FA formation [Maekawa et al., 1999]. We next examined the activation status of RhoA in OA-treated cardiomyocytes. After treatment with OA for 6 h, the levels of GTP-bound RhoA (active RhoA) were decreased in OA-treated cells, concomitantly with the decrease in phosphorylation levels of cofilin, compared with those of BSA control group (Fig. 7A, B).

DISCUSSION

Studies have shown that OA reduces conduction and contraction of cardiomyocytes [Hirschi et al., 1993; Zahabi and Deschepper, 2001; Huang et al., 2004]. In this study, we investi-

gated the underlying mechanism of the action of OA on myofibrils, with special attention to FAs. Our results showed that OA induced disassembly of costameres, FAs, and myofibrils. Moreover, the OA-induced dephosphorylation of FAK and paxillin and the myofibril disassembly could be completely blocked by the PTP inhibitor, sodium orothovandate. OA induced upregulation of PTP-PEST. Since PTP-PEST induces FA disassembly by dephosphorylating FAK and paxillin [Angers-Loustau et al., 1999], this suggests that PTP-PEST might be involved in the OA-induced disassembly of FAs and myofibrils. Moreover, OA reduced RhoA activity and decreased cofilin phosphorylation, indicating another potential mechanism involved RhoA-cofilin cascade. Increased cofilin activity might result in depolymerization of actin filaments and contribute to the OA-induced myofibril disassembly.

To elucidate the mechanism responsible for the aberrant myofibril integrity, FAs and costameres involved in myofibril organization were examined. OA induced breakdown of FAs and costameres prior to myofibril disorganization, as assessed by immunostaining for integrin β1D, vinculin, paxillin, and α-actinin. These results suggest that OA induces myofibril disassembly by first disrupting the integrity of FAs and costameres. Our data are in agreement with previous report which shows that disruption of FAs by addition of anti-integrin β1D antibodies to cultured neonatal cardiomyocytes inhibits myofibril assembly [Kim et al., 1999]. In mice, heart-specific ablation of β1 integrin results in severe cardiac abnormalities, including myocardial fibrosis, depressed contractility and relaxation, intolerance of transverse aortic constriction, and the development of cardiomyopathy [Shai et al., 2002]. In addition, costameres appeared to be more sensitive than FAs to OA treatment. A previous study showed that contraction activity can be modulated by costamere integrity and formation, since treatment of cardiomyocytes with a Ca^{2+} channel blocker to inhibit contractile activity results in the loss of costameres [Sharp et al., 1997]. Thus, the OA-mediated decrease in cardiac muscle contraction might correlate with the disappearance of costameres.

In the present study, OA caused rapid dephosphorylation of FAK Tyr397 without altering total FAK levels. Tyr397 phosphorylation of FAK plays an important role in FAK activation and FA assembly [Hamadi et al., 2005]. Transfection of neonatal rat ventricular myocytes with GFP-FRNK, which acts as a dominant-negative of FAK and lacks the kinase domain, including the Tyr397 autophosphorylation site, disrupts FA architecture by displacing FAK from FAs [Schaller and Parsons, 1995]. Recent study further demonstrates that heart-specific FAK-null mice exhibit disorganized myofibrils [Peng et al., 2006]. Thus, OA-induced tyrosine dephosphorylation of FAK might decrease its activity and induce disassembly of FA and myofibril. Src activation is required for FAK phosphorylation at Y576/Y577 [Calalb et al., 1995; Hanks et al., 2003]. However, we did not detect any change in the Tyr416 phosphorylation of Src on OA treatment.

Examination of FAK-mediated downstream and upstream signaling revealed that OA treatment caused a decrease in phosphorylated and total paxillin. Paxillin is a substrate for FAK and, once it is tyrosine phosphorylated, recruits other FA-associated proteins to FAs [Hagel et al., 2002]. Thus, the decrease in phosphotyrosine and protein levels of paxillin in OA-treated cardiomyocytes seen in the present study might represent a mechanism for the dissociation of complexes of vinculin and proteins containing SH2/SH3 domains. A previous study showed that dephosphorylation of paxillin by GFP-FRNK is accompanied by a reduction in total paxillin in cardiomyocytes [Heidkamp et al., 2002]. Thus, the OA-induced dephosphorylation of paxillin might account for the decrease in total paxillin levels in OA-treated cardiomyocytes. Although the mechanism for the OA-induced downregulation of integrin β1D and α-actin remains unclear, inhibition of contractile activity decreases integrin β1D and α-actin expression in cardiomyocytes [Sharp et al., 1997]. Thus OA-induced mtofibril diassembly might be associated with downregulation of integrin β1D and α-actin.

Interestingly, a PTP inhibitor completely prevented the OA-induced tyrosine dephosphorylation of FAK and paxillin. This result is consistent with those of a previous study showing that inhibition of PTP stimulates FA formation and cytoskeletal protein organization in human endothelial and Chinese hamster ovary cells [Defilippi et al., 1995]. Thus, based on this hypothesis, PTP inhibition might prevent the OA-induced FA disassembly and subsequent sarcomeric assembly. A previous study

also demonstrated that PKCε may participate in sarcomere assembly by increasing FAK phosphorylation in cardiomyocytes [Heidkamp et al., 2003]. The present study showed that dephosphorylation of FAK occurred in spite of the activation of PKCε by OA. It is possible that OA-upregulated expression of PTP-PEST overcomes the effect of PKCε on FAK phosphorylation.

Examination of PTP isoforms showed that PTP-PEST protein levels were elevated in OA-treated cardiomyocytes. Although the role of PTP-PEST in cardiomyocytes remains largely unknown, in fibroblasts, high PTP-PEST expression is correlated with FA disassembly and decreased cell motility through tyrosine dephosphorylation of paxillin and/or FAK [Angers-Loustau et al., 1999; Sastry et al., 2002]. Thus, our observations in OA-treated cardiomyocytes support a role for PTP-PEST in the negative regulation of integrin-mediated FA processes through dephosphorylation of paxillin and FAK.

Being an amphilic compound, OA is able to insert into the cell membrane [Katz and Messineo, 1981]. In OA-loaded cells, reduction in Ca^{2+} signaling, including calcium release channels on endoplasmic reticulum [Honen et al., 2003] and capacitative Ca^{2+} entry, are noted in several cell types [Gamberucci et al., 1997; Esenabhalu et al., 2003]. The OA-impaired Ca^{2+} mobilization is reversed by treatment with BSA [Kuroda et al., 2001]. In rat aortic smooth muscle cells, reduction in cytoplasmic Ca^{2+} levels increases PTP activity [Zhuang et al., 2005]. In this study, OA treatment increased PTP expression levels which might result from a decrease in low cytoplasmic Ca^{2+} levels. Therefore, the cessation of the flux of OA into the cardiomyocytes by changing the OA-medium to BSA-medium might remove OA bound to cell membrane, relieve the cells from inhibition of Ca^{2+} signaling, and enable the cells responsive to various Ca^{2+} mobilization stimuli, all favoring the increase in cytoplasmic Ca^{2+} concentrations and downregulation of PTP activity. This assumption is supported by our observation that removal of OA greatly prevented OA-induced dephosphorylation of FAK and paxillin, indicative of a decrease in PTP activity (data not shown). This mechanism might explain why 50% of the OA-affected cardiomyocytes exhibited normal myofibrillar patterns after OA removal.

RhoA activation is required for assembly of actin cytoskeleton and FAs. In this study, we detected that OA reduced RhoA activity, which might result in disorganization of myofibrils, FAs, and costameres. This observation is consistent with our previous study, showing that inhibition of RhoA activity by C3 exoenzyme leads to the loss of costameres and disassembly of myofibrils in cardiomyocytes [Wang et al., 1997]. Moreover, disruption of RhoA signaling by siRNA treatment results in lack of heart tube fusion in early chick embryo [Kaarbo et al., 2003]. RhoA acts on the actin cytoskeleton through downstream effector proteins, such as cofilin. Phosphorylation of cofilin by RhoA/Rho kinase/LIM-kinase decreases its actin-severing ability, which promotes actin assembly. Endothelin induces RhoA signaling, cofilin phosphorylation, and sarcomeric assembly in cardiomyocytes [Heidkamp et al., 2003]. In this study, OA induced a reduction in cofilin phosphorylation, leading to F-actin depolymerization and sarcomere disassembly. In addition, RhoA also mediates FA assembly by phosphorylation of FAK and paxillin [Clark et al., 1998]. The possibility can not exclude that low RhoA activity induced by OA might decrease the phosphorylation of FAK and paxillin, contributing to the disassembly of FA and myofibril. Recent study in fibroblasts indicates that PTP-PEST can modulate RhoA activity by both direct and indirect mechanisms in fibroblasts [Sastry et al., 2002]. Further studies are aimed at determining the presence of a cross talk between PTP-PEST and RhoA in response to OA.

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