Role of Protein Kinase Cδ in Endothelin-Induced Type I Collagen Expression in Cardiac Myofibroblasts Isolated from the Site of Myocardial Infarction

Vishnu Chintalgattu and Laxmanma C. Katwa

Department of Physiology, the Brody School of Medicine, East Carolina University, Greenville, North Carolina

Received April 23, 2004; accepted July 6, 2004

ABSTRACT

The role of endothelin-1 (ET) in tissue remodeling/fibrogenesis has been demonstrated in various in vitro and in vivo models. Our previous studies have revealed ET-induced expression of type I collagen in cardiac myofibroblasts (myoFb). Here we report that protein kinase Cδ (PKCδ) and mitogen-activated protein kinase/extracellular signal-regulated kinase-1/2 (MAPK/ERK1/2) play a role in ET-induced type I collagen expression using specific pharmacological inhibitors. The present study also reveals the expression of various isoforms of PKC including PKCα, PKCβII, PKCβIII, PKCγ, PKCδ, PKCe, PKCε, PKCη, and PKCζ in cardiac myoFb. Our results from mRNA and protein studies demonstrate that calphostin-C, a PKC inhibitor, decreased the ET-induced type I collagen expression suggesting a role for the PKC pathway. Further treatment with rottlerin, a PKCδ isoform-specific inhibitor, demonstrated attenuation of 80 to 90% of type I collagen expression induced by ET. However, Go6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo [3,4-c]carbazole], an inhibitor of Ca2⁺-dependent PKC isoforms (PKCα and PKCβII), showed little to no effect on ET-stimulated type I collagen expression. Furthermore, the MAPK inhibitor PD98059 (2’-amino-3’-methoxyflavone) attenuated ET-dependent activation of p44/42 MAPK (pERK1/2) and also down-regulated type I collagen expression. Similarly, rottlerin inhibited the activation of p44/42 MAPK (pERK) implicating the involvement of PKC and MAPK/ERK1/2 in ET-induced type I collagen expression. Our protein/DNA array and reverse transcription-polymerase chain reaction results from ET-treated samples showed a significant increase in Sp1 expression. PD98059 and rottlerin decreased ET-induced Sp1 expression, suggesting a possible interaction of Sp1 with PKCδ and MAPK in ET-induced type I collagen expression in cardiac myoFb.

Remodeling/repair is a common phenomenon following tissue injury. Collagens are major structural proteins associated with physiological and pathophysiological remodeling of injured tissues. Previous studies have implicated a role for various fibrogenic factors including endothelin-1 (ET-1) in tissue remodeling/fibrosis following injury (Guarda et al., 1993; Nguyen et al., 1998, 2001; Rossi et al., 1999; Thai et al., 1999; Bauersachs et al., 2000; Mulder et al., 2000; Fraccarollo et al., 2002; Pfeffer et al., 2000). The myofibroblasts (myoFb) found at the site of myocardial infarction (MI) are known to play a vital role in the accumulation of collagens during repair/remodeling (Campbell and Katwa, 1997; Katwa et al., 1997; Sun et al., 2000; Chintalgattu et al., 2003). Recent studies from our laboratory have shown ET-induced expression of type I collagen in cardiac myoFb (Katwa, 2003). The present study was designed to elucidate the involvement of PKC isoforms, MAPK, and transcriptional factors in ET-induced type I collagen expression in cardiac myoFb isolated from the site of MI.

The PKC family has at least 11 different isoforms of which the number and level of PKC isoform expression is tissue-specific, and their subcellular localization varies depending on the cell type (Mackay and Mochly-Rosen, 2001). Studies in other cell types, such as scleroderma fibroblasts and mesangial cells, have shown PKCδ involvement in TGF-β1 mediated collagen biosynthesis (Jimenez et al., 2001; Runyan et al., 2003). Additionally, studies have suggested that stabilization of elastin mRNA in lung fibroblasts by TGF-β1 requires Smads, PKCδ, and the MAP kinase p38 (Kucich et al., 1997; Katwa et al., 1997; Sun et al., 2000; Chintalgattu et al., 2003).

ABBREVIATIONS: ET-1, endothelin-1; myoFb, myofibroblasts; MI, myocardial infarction; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TGF-β1, transforming growth factor-β1; ERK1/2, extracellular signal-regulated kinase-1/2; RT-PCR, reverse transcription-polymerase chain reaction; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo [3,4-c]carbazole; PD98059, 2’-amino-3’-methoxyflavone; HRP, horseradish peroxidase; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; β2-MG, β2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Materials and Methods

Materials. Tissue culture and RT-PCR reagents were purchased from Life Technologies (Rockville, MD). PKC inhibitors (rottlerin and Go6976) along with MAPK and phospho-MAPK antibodies were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). PD98059 and calphostin-C were purchased from Calbiochem (San Diego, CA). PKC-PAN antibodies were obtained from PanVera Corp. (Madison, WI). PKCδ antibody and PKCδ-specific substrate were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-type I collagen and anti-mouse IgG-HRP were purchased from Sigma-Aldrich (St. Louis, MO). A protein DNA array kit and Sp1 reporter construct were purchased from Panomics (Redwood City, CA). Nuclear extraction kit was obtained from Active Motif (Carlsbad, CA). Other general laboratory grade chemicals were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Cell Culture and Treatments. Myofibroblasts were isolated from rat MI using the previously described method (Chintalgattu et al., 2003). In this study, we used early passages (<12) of myofb cultures to avoid possible morphological changes during sub-culturing. These cells were grown to 80% confluence in 10% DMEM in 100-mm plates. Prior to treatment, medium was replaced with serum-deprived medium (0.4% DMEM) for 12 h. Cells were pretreated in DMEM for 1 h with calphostin-C (10 μM), rotterlin (1 μM), PD98059 (100 μM), and Go6976 (10 nM) followed by ET (10 μM). Protein and RNA were isolated from treated cells after 3 and 24 h of incubation, respectively.

Protein Kinase Assay. The cells were grown in serum-deprived (0.4% fetal bovine serum) DMEM for 12 h and then treated with ET or ET + rottelin in serum-free medium for various time periods. The total protein was isolated from cells using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholate; 0.1% SDS) containing 1X protease inhibitor cocktail from Sigma-Aldrich. Immunoprecipitation was carried out using 100 μg of protein with 4 μg of PKCδ antibody and incubated 3 h at 4°C and then 50 μl of protein G-Sepharose overnight at 4°C. Kinase assay was carried out using a PKC kinase assay kit from Upstate (Waltham, MA) with 10 μl of immunocomplexes. After 10 min of incubation, samples were spotted on P81 phosphocellulose paper and washed with 0.75% phosphoric acid. The amount of incorporated radioactivity (γ-32P-ATP) into substrate was measured by scintillation counting. The kinase assay was also repeated with crude protein (50 μg) and PKCδ-specific substrate from Santa Cruz Biotechnology, Inc. using the above kinase assay kit.

 Luciferase Assay and Transient Transfection. Cells were grown in 6-well plates until 70% confluent and then serum-deprived medium was added for 12 h. In accordance with the manufacturer’s instructions (Panomics), 2 μg of Sp1 reporter vector or 2 μg of pSilencer Sp1 siRNA along with 0.5 μg of pSV-β-galactosidase vector as a control for transfection efficiency were transfected into myofb. After 18 h of transfection in Opti-MEM I (Invitrogen, Carlsbad, CA) with LipofectAMINE 2000 (Invitrogen), cells were treated with ET or ET + rotterlin for 24 h and measured for luciferase and β-galactosidase activity according to the manufacturer’s instructions (Promega, Madison, WI).

Western Blot and ELISA. After 24-h treatment, protein was isolated using lysis buffer (1X protease inhibitor cocktail, 10 mM Tris-HCl, pH 6.8; 150 mM NaCl; 1% Nonidet P-40). Protein samples were electrophoresed using a 4 to 12% SDS-polyacrylamide gel under reducing conditions as described previously (Chintalgattu et al., 2003). The proteins were transferred to polyvinylidene difluoride membranes and blocked with phosphate-buffered saline containing 5% nonfat milk powder for 1 h at room temperature. Membranes were incubated with the appropriate primary antisera. Type I collagen, MAPK, and pMAPK antiserum was used at 1:1000 dilutions. All PKC antibodies were used at 1:100 dilutions. HRP conjugated goat anti-rabbit or goat anti-mouse IgG was used as the secondary antiserum at 1:5000 dilutions. Protein bands were visualized using a chemiluminescence detection system (ECL; Amershams Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Extracellular type I collagen was analyzed using ELISA as described previously (Katwa, 2003).

Protein/DNA Array. Nuclear extracts were prepared using Active Motif nuclear extraction kit. The protein/DNA array was carried out according to the manufacturer’s instructions (Panomics). Briefly, 5 μg of nuclear extract was electrophoresed on a 2% agarose gel at 120 V. The protein DNA complex was isolated from the gel after 15 min. The isolated protein DNA complex was incubated with a protein/DNA array membrane overnight at 42°C. The membrane was washed with buffer and developed with streptavidin-HRP conjugate. Expression levels of various transcription factors were analyzed using densitometric analysis.

RT-PCR. Total RNA was isolated from 80% confluent cells using the Trizol method. Reverse transcription was performed on 2 μg of total RNA with 0.5 μg/ml oligo(dT)12-18 primer and MMLV reverse transcriptase. PCR amplification of specific type I collagen, Sp1, and β2-microglobulin (β2-MG) gene sequences was performed with the cDNA in a 25-μl reaction using a high fidelity PCR kit (Life Technologies). The following gene-specific oligonucleotide primers were used in the reaction: type I collagen: forward 5’-GCGAGG-GCAACAGTCTGATTCT-3’, reverse 5’-CCCAACTTCCGGTGTG- ACTC-3’; Sp1: forward 5’-GATCATCAGGGACTTGTCAAAG-3’, reverse 5’-CACAGTGACTGTGCCAGCAGAAAT-3’; β2-MG: forward 5’-ATGTCAGGTGTTTTAATCTGCAAGC-3’, reverse 5’-CTCCCCAATCTACAGTTCTCG-3’. The above genes were amplified for 32 cycles at 94°C for 15 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C using GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA). PCR products were fractionated on a 1.5% agarose gel containing ethidium bromide and visualized under UV light.

Quantitative Real-Time RT-PCR (qRT-PCR). Analysis of type I collagen and GAPDH mRNA expression by qRT-PCR was performed using a Cepheid Smart Cycler real-time PCR (Cepheid, Sunnyvale, CA). Type I collagen and GAPDH gene-specific primers and TaqMan probes were synthesized (Synthegen, LLC, Houston, TX) and are as follows: GAPDH primers: forward 5’-GTGGTGAAGTC TACTGTGAGATCC-3’, reverse 5’-GACAGTACTGTGATGCTAT GAG-3’, probe HEX: 5’-TACATCCGACACTTCTGAGAC-3’. The above described type I collagen RT-PCR primers were used in qRT-PCR. These primers were designed to span exon-intron junctions to prevent amplification of genomic DNA. TaqMan probes were labeled at the 5’-end with the reporter dye molecule TET and HEX (tetra-chloro-6-carboxyfluorescein; absorbance 522 nm, emission 538 nm) and hexachlorofluorescein; absorbance 538 nm, emission 552 nm) and at the 3’-end with the quencher dye molecule TAMRA (6-carboxytetramethylrhodamine; emission 582 nm). Thermocycle one
Role of PKCδ in ET-Induced Collagen Expression

Results

Presence of Protein Kinase C Isoforms and Role of Rottlerin in ET-Induced PKCδ Kinase Activity in Cardiac Myofibroblasts. The number and levels of PKC isoform expression vary in different tissues. Our Western blot experiments showed the expression of various PKC isoforms such as PKCα, PKCβ1, PKCβII, PKCγ, PKCδ, PKCε, PKCη, and PKCζ in cardiac myoFb (Fig. 1A). These isoforms may be involved in various cellular mechanisms including the collagen synthesis pathway. There was a significant increase in PKCδ expression in ET-treated myoFb (Fig. 1B) clearly suggesting the participation of PKCδ in ET-mediated signaling mechanisms.

Isolated protein from myoFb treated with ET alone or along with rottlerin for 24 h was subjected to immunoprecipitation with PKCδ-specific antibody and then immunocomplexes were used for in vitro kinase assay. Results from Fig. 1C clearly demonstrate that ET induced a 2-fold increase in PKCδ activity and attenuation of ET-induced PKCδ activity by rottlerin; this suggests a role for PKCδ in ET-induced collagen expression. Although the endpoint for most of our experiments was 24 h, we observed a time-dependent increase in PKCδ activity with ET-treated protein samples (data not shown). We found similar results when we measured the kinase activity with PKCδ-specific substrate with crude protein from ET and rottlerin treatments. We found that rottlerin (1 μM) was able to inhibit the ET-induced collagen expression (Fig. 2), therefore, we used 1 μM rottlerin in all experiments in this study.

The Role of PKCδ and MAPK in ET-Induced Type I Collagen Expression by Western Blot and ELISA. In this study, we used Western blot and ELISA to analyze intracellular and extracellular expression of type I collagen, respectively. To understand the interaction of the PKC and MAPK/ERK1/2 in ET-stimulated expression of type I collagen, myoFb were treated with calphostin-C (which inhibits most of the PKC isoforms) and PD98059 (a MAPK inhibitor) along with ET. The results showed a decreased expression of type I collagen suggesting a role for the PKC and MAPK in ET-induced expression of type I collagen. Protein as well as medium samples from Go6976 (an inhibitor of Ca2+-dependent PKCα and PKCβII isofrom) (Martiny-Baron et al., 1993) and ET-treated myoFb were analyzed using Western blot and ELISA. Results from ET + Go6976 treatment show little to no effect on type I collagen protein expression (Fig. 3, A and B). These findings clearly demonstrate the minimal role, if
any, of the Ca$^{2+}$-dependent PKCa and PKC$\beta$I isomers on ET-stimulated type I collagen expression.

Myofibroblasts treated with rottlerin (1 $\mu$M), a specific inhibitor of PKC$\delta$ (Gschwendt et al., 1994), attenuated 80 to 90% of ET-induced type I collagen protein expression (Fig. 3A). Media samples from ET + rottlerin-treated myoFb showed similar decreases in extracellular type I collagen expression levels (Fig. 3B). These results confirm the involvement of the PKC$\delta$ in ET-induced type I collagen expression.

**The Role of PKC$\delta$ and MAPK in ET-Induced Type I Collagen Gene Expression Using RT-PCR and Real-Time RT-PCR.** As shown in Fig. 4A, calphostin-C and PD98059 reduced ET-induced type I collagen RNA expression in myoFb, indicating the involvement of PKC and MAPK/ERK1/2 in ET-stimulated type I collagen expression. Myofibroblasts treated with Go6976 did not show significant effects on ET-induced type I collagen expression, suggesting Ca$^{2+}$-dependent PKC isomers may not be involved in ET-induced type I collagen expression. Myofibroblasts treated with rottlerin and ET showed a 90% reduction of type I collagen RNA expression, indicating a predominant role for PKC$\delta$ in ET-stimulated type I collagen expression.

The RT-PCR data were further confirmed by qRT-PCR (Fig. 4B). The real-time PCR data also demonstrated a similar decrease in relative copy number of type I collagen mRNA in ET + rottlerin-treated samples (Fig. 4B). The data shown above from RNA as well as protein studies (Fig. 3, A and B; Fig. 4, A and B) clearly demonstrate the interaction between PKC (specifically PKC$\delta$) and the MAPK pathways in ET-induced type I collagen expression.

**Effect of the MAPK Inhibitor PD98059 on Endothelin-Mediated ERK Phosphorylation.** To determine whether ET stimulates ERK1/2 activity, ERK1/2 phosphorylation was measured after 24 h of treatment with ET (Fig. 5). Our Western blot experiments revealed the up-regulation of pERK1/2 in ET-treated samples indicating possible interactions of ET with the MAP kinase cascade resulting in ERK1/2 activation. Thus having shown that ET induced ERK activation, we wanted to determine whether ERK phosphorylation was required for type I collagen protein production. If ERK phosphorylation is necessary for type I collagen expression, blocking the activation of ERK should attenuate the effects of ET in the up-regulation of type I collagen expression. We found that the MAPK inhibitor, PD98059, with the inhibition of ERK1/2 was able to suppress type I collagen protein expression at a concentration of 100 $\mu$M (Fig. 3, A and B; Fig. 4, A and B). Furthermore, PD98059 also abrogated the effects of ET on ERK1/2 activation at the same concentration (Fig. 5). These results further suggest a role for MAPK/ERK1/2 in ET-mediated type I collagen expression.

The PKC inhibitor rottlerin was used to further correlate the interaction between PKC$\delta$, MAPK, and type I collagen protein expression. We found that rottlerin (1 $\mu$M) was able to inhibit the activation of pMAPK/pERK following ET treatment (Fig. 5). Taken together, our results (Fig. 3, A and B; Fig. 4, A and B; Fig. 5) strongly suggest that PKC$\delta$ mediates ET induction of type I collagen through MAPK.

**ET-Induced Expression of Various Transcription Factors by Protein/DNA Array.** Array membranes were treated with nuclear extracts isolated from control (untreated) and ET-treated myoFb. ET treatment resulted in 10-fold increases in Sp1 expression, whereas Smad3/4, Stat1, Stat3, and Stat4 showed 2- to 5-fold increases. Stat5 and Stat6 as well as AP-1 and -2 showed a 1- to 2-fold increase in their expression levels (Fig. 6). These results clearly indicated a role for Sp1 in ET-dependent signaling mechanisms. Our RT-PCR results from myoFb treated with ET + PD98059 and ET + rottlerin showed a decrease in Sp1 expression, suggesting a role for PKC$\delta$ in ET-stimulated Sp1 RNA expression (Fig. 7). These results suggest a possible interaction of Sp1 with the PKC and MAPK in ET-induced type I collagen expression.

**Role of Rottlerin on ET-Induced Sp1 in Vivo Binding Activity.** To determine the influence of rottlerin on ET-induced Sp1-binding activity in vivo, cells were transfected with 2 $\mu$g of Sp1 reporter vector and pSilencer containing siRNA Sp1 (Banchio et al., 2003) or empty vectors (pSilencer or Sp1 reporter vector) along with pSV-β-galactosidase vector (control for transfection) for 18 h. Cells were then treated with ET or ET + rottlerin for 24 h. The ET treatment significantly increased Sp1 binding activity, whereas ET + rottlerin or ET + pSilencer Sp1 decreased the ET-induced Sp1 activity suggesting a possible role for Sp1 in ET-induced collagen expression (Fig. 8).

**Role of PKC/MAPK Inhibitors on Myofibroblast Cell Death (Apoptosis).** To analyze and rule out the nonspecific (toxicity) role of pharmacological agents (rottlerin, Go6976, and PD98059) on myoFb cell death (apoptosis), myoFb was treated with various pharmacological inhibitors of PKC$\delta$ and -α (rottlerin, 1–6 $\mu$M; Go6976, 10 nM) MAPK (PD98059, 100 $\mu$M) for 24 h and analyzed for flow cytometry as described under Materials and Methods. Results clearly demonstrated that more than 94% myoFb are viable after treatment with various concentrations of pharmacological agents (rottlerin, Go6976, and PD98059), suggesting no influence of these inhibitors on cell death (apoptosis) under these experimental conditions (Fig. 9).

**Discussion**

Abnormal accumulation of extracellular matrix leads to tissue fibrosis resulting in organ dysfunction. However, the
mechanisms underlying abnormal deposition of collagens are not completely understood. Previously, we have demonstrated the ET-induced collagen expression and its attenuation by bosentan (dual ET$_A$ and ET$_B$ receptor blocker). In this study, our major focus was to understand the involvement of downstream signaling mechanisms in ET-induced type I collagen expression in cardiac myofibroblasts isolated from the site of MI. Our results demonstrated that rottlerin (a PKC$_\delta$ isoform-specific blocker) and PD98059 (a MAPK pathway blocker) reduced the ET-induced collagen expression in myofibroblasts, suggesting the association of PKC$_\delta$ and MAPK in ET-stimulated collagen biosynthesis pathway. Rottlerin treatment resulted in decreasing the ET-induced Sp1-binding activity and Sp1 expression, indicating its possible involvement in ET-induced collagen expression cascade. Our results demonstrate the involvement of PKC$_\delta$, MAPK, and Sp1 in ET-induced collagen synthesis.

Clinical and experimental studies on the heart have identified a number of conditions such as pathological cardiac hypertrophy, heart failure, and ischemic preconditioning in which PKCs are activated (Dunmon et al., 1990; D’Angelo et al., 1997; Miyamae et al., 1998; Qiu et al., 1998; Bowling et al., 1999). Despite these known associations and the importance of PKC isoforms as being essential and ubiquitous signaling molecules, their contribution to clinical situations like abnormal deposition of extracellular matrix and tissue fibrosis in the heart is yet to be fully established. One study on osteoblastic cells suggests the involvement of PKC and ERK pathways in TGF-\(\beta_1\)-induced type I collagen expression (Palcy and Goltzman, 1999). Several previous reports have implicated the role of Sp1 and Sp3 in collagen transcription in various models (Li et al., 1995; Chen et al., 1998; Ihn and Tamaki, 2000; Ruiz et al., 2000; Czuwara-Ladykowska et al., 2001; Garcia-Ruiz et al., 2002). In the present study, we show a role for PKC, ERK1/2, and Sp1 in ET-induced type I collagen expression in cardiac myofibroblasts.

Results from our Western blot demonstrate the expression of PKC isoforms PKC$\alpha$, PKC$\beta_1$, PKC$\beta_2$, PKC$\gamma$, PKC$\delta$, PKC$\varepsilon$, PKC$\eta$, and PKC$\zeta$ by these cells. Mackay and Mochly-Rosen (2001) demonstrated the tissue-specific and differential cellular localization of PKC isoforms. The isoforms PKC$\alpha$, PKC$\gamma$, PKC$\delta$, PKC$\varepsilon$, PKC$\eta$, and PKC$\zeta$ are consistently expressed, whereas expression of PKC$\beta_1$ and PKC$\beta_2$ were inconsistently reported in neonatal and adult ventricular myocytes (Mackay and Mochly-Rosen, 2001). Previous studies have also demonstrated a role for PKC isoforms in various pathological conditions in the heart; however, the role of these isoforms in cardiac myofibroblasts is unclear. Since ET has been shown to play a key role in abnormal accumulation of type I collagen in these cells, we attempted to understand the role of PKC isoforms in this process using specific pharmacological inhibitors. Our in vitro kinase assay for PKC$\delta$ dem-

---

**Fig. 3.** A, effect of PKC inhibitor calphostin-C, rottlerin (PKC$\delta$ inhibitor), Go6976 (inhibitor for Ca$^{2+}$-dependent PKC), and PD98059 on ET-induced type I collagen protein expression in cardiac myofibroblasts (myoFb) by Western blot. Cells were treated with calphostin-C (10$^{-7}$ M), rottlerin (1 \muM), Go6976 (10 nM), and PD98059 (100 \muM) along with ET (10$^{-5}$ M) for 24 h and followed by isolation of protein as described under Materials and Methods. Upper panel shows a representative Western blot. Specificity of type I collagen antibody was confirmed using specific antigen. Lower panel in histogram is the densitometric ratio of type I collagen and \(\beta\)-actin from three Western blots done in duplicate. Significant differences were observed from control versus ET (*, \(p < 0.02\)), ET versus ET + calphostin-C (**, \(p < 0.05\)), ET versus ET + PD98059 (**, \(p < 0.05\)), and ET versus ET + rottlerin treatment (#, \(p < 0.003\)).

B, role of PKC (Go6976, Calphostin-C, and rottlerin) and MAPK inhibitors in ET-induced type I collagen expression in myoFb by ELISA. After 24 h, medium was collected from control (untreated), ET (10$^{-5}$ M), ET + Go6976 (10 nM), ET + calphostin-C (10$^{-7}$ M), ET + rottlerin (1 \muM), and ET + PD98059 (100 \muM)-treated myoFibroblasts, and type I collagen in the medium was analyzed using ELISA as described previously. Results presented in this figure were from four independent experiments in duplicate. Significant differences were observed from control versus ET (*, \(p < 0.02\)), ET versus ET + calphostin-C (**, \(p < 0.05\)), ET versus ET + PD98059 (**, \(p < 0.05\)), and ET versus ET + rottlerin treatment (#, \(p < 0.003\)).
onstrated that rottlerin resulted in attenuation of ET-induced PKCδ activity as well as ET-induced up-regulation of PKCδ expression in myoFb, suggesting a role for PKCδ in ET-induced collagen expression in myoFb. Similarly, Clerk et al. (1994) reported the ET-induced expression of various isoforms including PKCδ in neonatal ventricular myocytes.

In our study, experiments with isoform-specific inhibitors such as rottlerin, a PKCδ inhibitor, showed an 80 to 90% down-regulation of ET-induced type I collagen expression, suggesting a predominant role for the PKCδ isoform in the ET-induced collagen expression. Findings in dermal fibroblasts suggested the involvement of PKCδ in TGF-β1-mediated collagen expression (Jimenez et al., 2001). More recently, Runyan et al. (2003) demonstrated TGF-β1-stimulated collagen expression via PKCδ in human mesangial cells. Hua et al. (2001) suggested multiple PKC isoforms to be associated with ET-1-stimulated α1 (IV) expression in mesangial cells. Gschwendt et al. (1994) suggested that rottlerin not only specifically inhibit the PKCδ but also was able to differentiate, to some extent, between the various PKC isoforms. PKCδ required the lowest rottlerin (IC50, 3–6 μM) concentration for its inhibition, whereas other PKC isoforms required 5- to 10-fold higher concentrations, suggesting that at lower concentrations rottlerin exclusively inhibited PKCδ. Moreover, in this study we used a 1 μM concentration of rottlerin further diminishing the possibility of other PKC isoforms inhibition along with PKCδ by rottlerin. In addition, treatment with Ca2+-dependent inhibitor Go6976 (PKCδ and PKCβ inhibitor) along with ET showed little to no effect on ET-stimulated type I collagen expression. These data clearly outline the predominant role of PKCδ in ET-1-mediated collagen expression in cardiac myoFb.

In this study, results from RNA and protein experiments revealed decreased expression of collagen in myoFb treated with PD98059 (a specific inhibitor of MAPK) and ET, implicating the involvement of the MAPK/ERK1/2 pathway in ET-induced collagen gene expression in cardiac myoFb. Furthermore, rottlerin and ET-treated myoFb showed decreased expression of the pERK and type I collagen suggesting ET-induced collagen expression may be mediated by PKCδ and ERK pathway. Similarly, Rodriguez-Barbero et al. (2002) demonstrated the p38 MAPK-mediated TGF-β1-stimulated collagen expression in L6E9 myoblasts, whereas Hua et al. (2001) reported ERK1/2 involvement in ET-induced collagen expression in mesangial cells.

In the present study, protein/DNA array experiments demonstrated the ET-induced expression of various transcriptional factors. On the basis of previous studies and our array data, the transcriptional factors relevant to collagen expres-
Our results revealed ET-stimulated expression of Sp1, Smad, and Stat1–6 in cardiac myoFb. Among them, Sp1 levels were significantly increased when compared with controls in these cells. Findings from previous studies have also demonstrated the Sp1/Sp3 association with the collagen biosynthetic pathway (Chen et al., 1998; Ruiz et al., 2000; Czuwara-Ladykowska et al., 2001; Garcia-Ruiz et al., 2002). Our RT-PCR data also demonstrate ET-induced expression of Sp1, whereas rottlerin and PD98059 attenuated the

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Factor</th>
<th>Fold increase compared to control</th>
<th>S.No.</th>
<th>Factor</th>
<th>Fold increase compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sp1</td>
<td>10</td>
<td>7</td>
<td>Smad3/4</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Stat1</td>
<td>3</td>
<td>8</td>
<td>Ap-1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Stat3</td>
<td>2</td>
<td>9</td>
<td>Ap-2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Stat4</td>
<td>5</td>
<td>10</td>
<td>Ap-1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Stat5</td>
<td>1</td>
<td>11</td>
<td>Ap-2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Stat6</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. ET-stimulated expression of transcriptional factors (TFs) in cardiac myoFb. Nuclear extracts were isolated from ET-treated and -untreated myoFb. Five micrograms of nuclear extract treated with protein/DNA array membrane according to the method described under Materials and Methods (n = 2). The TF-related extracellular matrix modulation is listed in the table (bottom panel). Fold increase in the TF expression was calculated from the densitometric scanning ratio between ET and control array membrane-expressed TFs. Results presented in the table were from the average of two independent experiments. Among all TFs, Sp1 showed a significant increase in ET-treated nuclear extracts.

Fig. 7. ET-induced Sp1 RNA expression was abrogated by rottlerin (PKCδ inhibitor) and PD98059 (MAPK/ERK1/2 inhibitor). Total RNA was isolated after 3 h of treatment, and Sp1 expression levels were assessed by RT-PCR as described under Materials and Methods. Upper panel is a representative Western blot. Lower panel in histogram represents the densitometric ratio of Sp1 and β2-MG from four independent experiments in duplicate. *, p < 0.05; control versus treatment.

Fig. 8. Influence of rottlerin on ET-induced Sp1-binding activity. MyoFb were transfected with 2 μg of Sp1 luciferase reporter vector (Luc Sp1), 2 μg of Sp1 siRNA pSilencer, or control vectors (pSilencer or Sp1 reporter vector without Sp1 insert) along with 0.5 μg of pSV-β-galactosidase vector (gal) as a control for transfection for 18 h. Transfected myoFb were treated with ET with and without rottlerin for 24 h, and luciferase and β-galactosidase activity was measured using Promega kits. Results presented are average of three independent experiments. Control versus treatments: *, p < 0.002; **, p < 0.005.

Role of PKCδ in ET-Induced Collagen Expression
ET-induced expression of Sp1. Rottlerin and Sp1-siRNA reduced the ET-induced Sp1 in vivo binding activity. This suggests the involvement of Sp1 in ET-mediated type I collagen expression.

In conclusion, our data demonstrates the expression of various PKC isoforms and the involvement of PKC/H9254, ERK1/2, and Sp1 in ET-induced expression of type I collagen in cardiac myoFb. Our data strongly support the use of pharmacological intervention via PKC/H9254 inhibitors to reduce the ET-induced collagen expression in myoFb, which may serve as a potential therapeutic agent in preventing the abnormal accumulation of collagen/fibrosis seen in myocardial infarction.

Acknowledgments
We thank Gregory S. Harris and Dhanasekaran Ramasamy for reading this manuscript. We also thank Dr. Dennis E. Vance and Dr. Claudia Banchio for providing Sp1-pSilencer vector for this study.

References


Ihn H and Tamaki K (2000) Increased phosphorylation of transcription factor Sp1 in...


Address correspondence to: Dr. Laxmansa C. Katwa, Department of Physiology, 6N-99, Brody Medical Sciences Bldg., East Carolina University Brody School of Medicine, 600 Moye Blvd., Greenville, NC 27834. E-mail: katwal@mail.ecu.edu