PKC δ and βII Regulate Angiotensin II Mediated Fibrosis through p38: A Mechanism of RV Fibrosis in Pulmonary Hypertension

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ABSTRACT

Aims: Pulmonary hypertension (PH) eventually leads to RV fibrosis and dysfunction that is associated with increased morbidity and mortality. While angiotensin II plays an important role in RV remodelling associated with hypoxic PH, the molecular mechanisms underlying RV fibrosis in PH largely remain unresolved. We hypothesized that the PKC-p38 signaling is involved in RV collagen accumulation in PH and in response to angiotensin II stimulation.

Methods & Results: Adult male Sprague-Dawley rats were exposed to 3 weeks of normoxia or hypoxia (10% FiO₂) as a model of PH. Hypoxic rats developed RV hypertrophy and fibrosis associated with an increase in PKC βII and δ protein expression and p38 dephosphorylation in freshly isolated RV cardiac fibroblasts. Further mechanistic studies were performed in cultured primary cardiac fibroblasts stimulated with angiotensin II, a key activator of ventricular fibrosis in PH. Angiotensin II induced a reduction in p38 phosphorylation which was attenuated following chemical inhibition of PKC βII and δ. Molecular and chemical inhibition of PKC βII and δ abrogated angiotensin II-induced cardiac fibroblast proliferation and collagen deposition in vitro. The effects of PKC inhibition on proliferation and fibrosis were reversed by chemical inhibition of p38. Conversely, constitutive activation of p38 attenuated angiotensin II-induced increase of cardiac fibroblast proliferation and collagen accumulation.

Conclusion: PKC βII- and δ-dependent inactivation of p38 regulates cardiac fibroblast proliferation and collagen deposition in response to angiotensin II, which suggests that the PKC-p38 signaling in cardiac fibroblasts may be involved and important in the pathophysiology of RV fibrosis in PH.
INTRODUCTION

Right ventricular (RV) failure is the main cause of death in patients with pulmonary hypertension (PH) (7). PH causes severe and prolonged RV pressure overload resulting in compensatory RV hypertrophy as an adaptive response. However, a persistent increase in RV afterload results in RV fibrosis and dysfunction that is maladaptive and observed in heart failure (2). Cardiac fibroblasts play a key role in cardiac remodelling and fibrosis through proliferation and matrix generation and degradation in addition to other functions (37). While many studies have evaluated the signaling mechanisms important in RV hypertrophy with a focus on cardiac myocytes, the molecular mechanisms underpinning RV fibrosis in settings of PH remain unclear.

While several factors affect the phenotype and function of cardiac fibroblasts including TGF-β (29) and insulin-like growth factor (3), angiotensin II, together with many components of the renin-angiotensin-aldosterone system produced locally in the heart, appears to be one of the most important factors regulating cardiac fibrosis and remodelling (42). In left ventricular hypertrophy and dysfunction, inhibitors of angiotensin signalling are associated with significantly improved outcomes in humans and thus are established therapeutic agents (36). Recent studies indicate that angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers influence RV mass and volume (40). Moreover in the hypoxia associated PH model in rats, activity of angiotensin converting enzyme is significantly increased in the RV, while it was decreased in the left ventricle and septum (32). These data suggest that angiotensin II may also play a key role in RV remodelling associated with hypoxic PH.

Protein kinases C (PKCs) are serine–threonine protein kinases that have been shown to play an important role in cardiac diseases including heart failure and cardiac fibrosis (reviewed in (9)). Several PKC isoforms have been shown to be involved in signalling underlying the profibrotic effects of angiotensin II (9). We and others have shown that in the presence of increased RV afterload and fibrosis, PKC isoform expression is altered in tissue lysates from RV, but not the left ventricle (LV) (4, 8, 39). However, the identity of specific PKC isoforms mediating RV fibrosis, and the underlying regulatory mechanisms, in cardiac fibroblasts remains unclear.
p38 mitogen-activated protein kinases (MAPK) are one of the downstream targets of PKC isozymes and believed to play a role in cardiac ventricular fibrosis depending on the experimental model and underlying cause. For example, activation of p38 was shown to be associated with increased fibrosis related to inflammation in hypertension and myocardial infarction (24, 26, 31), whereas in LV pressure overload attenuation of p38 activity was shown to increase hypertrophy and fibrosis (5). Hence, a role for p38, and its upstream effectors, in cardiac fibrosis in settings of PH has yet to be elucidated.

In this study, we sought to identify the signaling mechanism related with angiotensin II involved in RV fibrosis using hypoxic PH as our model of increased RV afterload. We hypothesized that PKC-p38 signaling is involved in RV collagen accumulation in PH and in response to angiotensin II stimulation.
MATERIALS AND METHODS

**Materials.** All materials were obtained from Sigma (Sigma, St. Louis, MO), unless otherwise noted. Collagenase II was obtained from Worthington (Lakewood, NJ). DMEM was obtained from Invitrogen (Carlsbad, CA). The vectors encoding dominant negative cDNA for PKCβII (pHACE-PKCβII^K371R) and δ (pHACE-PKCδ^K376R), wild-type MKK6 (pCDNA3-FLAG-MKK6wt) and dominant negative p38, mutated at the T^180/Y^182 phosphorylation site (pCMV5-p38agf) were purchased from Addgene (Cambridge, MA). cDNA for phosphorylated green fluorescent protein (pGFP-C1) was obtained from Clontech (Mountain View, CA) and wild-type p38 (pCMV-FLAG-p38wt) cDNA was a kind gift from Dr. Roger Davis (Howard Hughes Medical School/University of Massachusetts Medical School) (33). Sircol collagen assay was purchased from Accurate (Westbury, NY). Antibodies against phosphorylated p38 (T^180/Y^182) and total p38 were purchased from Cell Signalling (Beverly, MA). Actin, procollagen, AT1R, PKCβII and PKCδ antibodies and PKCβII chemical inhibitor, LY333531, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 reagent was purchased from Life Technologies (Grand Island, NY).

**Echocardiographic Measurements** Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were placed in either normoxic or normobaric hypoxic (10% FiO2) environment. Normobaric hypoxia was achieved by placing the rats in Biospherix A-chamber with Pro-Ox oxygen controller (Biospheix Ltd., Lacona, NY). Following 3 weeks of exposure to normoxia or hypoxia, transthoracic echocardiography was performed on animals anesthetized with continuous isoflurane inhalation (2-5 %), with a single-element transducer (Vevo 2100; VisualSonics, Toronto, ON, Canada). Adequacy of anaesthesia was assessed by pedal reflex. Two-dimensional (2D), Doppler and M-mode recordings were obtained to measure LV fractional shortening, LV and RV dimensions. The pulsed-wave Doppler recording at the right ventricular outflow tract (RVOT) was used to measure pulmonary acceleration time (PAT). Tricuspid annular plane systolic excursion (TAPSE) was measured using M-mode across the tricuspid valve annulus at the RV free wall. TAPSE was determined by measuring the excursion of the tricuspid annulus from its highest position to the peak descent during ventricular systole.
Tissue Doppler was used to measure the early diastolic velocity of the septum (at mitral annulus) and RV lateral wall (at tricuspid annulus). After echocardiographic measurements, rats were euthanized under isoflurane and the heart and lungs were collected. The RV, LV, and interventricular septum (IVS) were dissected, frozen and homogenized for Western blotting or cardiac fibroblasts from RV, LV and IVS were isolated (outlined in “Cardiac Fibroblast Isolation”).

All animal experimental protocols were approved by the Institutional Animal Care and Use Committees of the Providence Veterans Affairs Medical Center and comply with the Health Research Extension Act, US Public Health Service and US National Institutes of Health policy (protocol # 2011-001).

**Cardiac Fibroblast Isolation.** Cardiac fibroblasts were isolated as previously described (45) from normoxic or hypoxic rats, or from healthy adult male Sprague Dawley rats that were anesthetized with continuous isoflurane inhalation (2-5 %, assessed by pedal reflex). In brief, hearts were rapidly excised, retrogradely perfused for 2 min in Krebs-Henseleit bicarbonate (KHB) buffer at 37°C, and then switched to enzyme buffer 1 (KHB buffer containing 0.3 mg/ml collagenase II, 0.3 mg/ml hyaluronidase, and 50 μM CaCl₂). After perfusion, ventricular tissue was cut and further digested at 37°C in enzyme buffer 1 supplemented with increased CaCl₂ (500 μM), trypsin IX (0.6 mg/ml), and deoxyribonuclease (0.6 mg/ml). Cell suspensions were filtered into DMEM supplemented with 10% FBS, F12 and penicillin and streptomycin (complete medium), and centrifuged at 20 x g for 2 min followed by removal of the pelleted myocytes and centrifugation of the supernatant at 800 x g for 5 min. The resulting fibroblast pellet was resuspended in complete medium and plated into four 10 cm dishes. In the studies evaluating expression profile of PKC and activation status of p38 in rats with or without PH, fibroblasts isolated from the right ventricles were collected for Western blot analysis after they adhered to 10 cm culture dishes (in 2 hour). For the *in vitro* proliferation studies, cells were isolated from healthy rats and cultured in 6-well dishes for 2-3 days before they were trypsinized and used for the experiments. All experiments were performed on P1 (Passage 1) cells.

**Cardiac Fibroblast Inhibitor Studies.** Subconfluent P1 cells were exposed to serum-free DMEM, supplemented with 10 μg/ml insulin, 5.5 μg/ml
transferrin and 5 ng/ml sodium selenite (ITS) and penicillin and streptomycin. After 16 h, cells were pre-treated with SB203580 (p38 inhibitor) (100 nM) followed by treatment with LY333531 (PKC βII inhibitor, 50 nM) or rottlerin (PKC δ inhibitor, 3 μM) for additional 30 min. Cardiac fibroblasts were then exposed to angiotensin II (1 μM) for 15 mins to 48 h, followed by proliferation assay, collagen assay and Western blotting.

**Cardiac Fibroblast Transfections.** Cardiac fibroblasts were transiently transfected with cDNA (4 μg/well from 6 well plate) encoding dominant negative PKC βII (PKC βII^K371R) or PKC δ (PKC δ^K376R), wild-type p38 (p38 wt), dominant negative p38 (p38agf) or MKK6 (MKK6wt) or GFP, using Lipofectamine 2000 reagent according to the manufacturer’s instructions. At 24 h post-transfection, cardiac fibroblasts were quiesced for 24 h, and then the cells were used for experiments as described.

**Proliferation Assay.** Cardiac fibroblast proliferation was assessed by both cell counting and incorporation of ³H-thymidine into cells. Following pretreatment with inhibitors, cardiac fibroblasts were exposed to angiotensin II (1 μM) and ³H-thymidine (0.025 μCi) for 48 h, then rinsed with ice-cold PBS three times and incubated with 5% trichloroacetic acid (TCA) for 20 min on ice. After washing twice, cells were solubilized in 0.5 N NaOH and an aliquot of TCA-insoluble material was neutralized in 0.5 N HCl. Radioactivity was measured by liquid scintillation counter (LSM 6500, Beckman Instruments). Cell proliferation was assessed as counts per minute and normalized to vehicle conditions.

**Western Blot Analysis.** RV, LV and IVS tissue were homogenized at 4°C in homogenization buffer (20 mM HEPES, 250 mM sucrose, 100 mM NaCl, 0.2 mM EDTA, 200 μM PMSF, 0.5 mM DTT, 1 μM leupeptin, 1 μM aprotinin and phosphatase inhibitor cocktail III). Homogenates were then centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were used for total protein analysis.

Cardiac fibroblasts from the in vitro studies were collected in radioimmunoprecipitation assay (RIPA) buffer and incubated on ice for 10 min prior to centrifugation for 10 min at 15,000 x g. The supernatant was subjected to protein determination (Bradford).
Proteins (50 μg/lane) were resolved on 7.5% (procollagen) and 10% (p38 and PKCs) separating gels using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes and immunoblot analysis was performed using appropriate antibodies dilution of 1:1,000 for all antibodies with the exception of procollagen (1:200). Equal loading was confirmed by probing for vinculin (1:3,000) or actin (1:1,000) with similar results.

Quantitative densitometry was performed using the public domain ImageJ program. Phosphorylated proteins and total proteins were calculated as a ratio of vinculin or actin expression. Data is presented as a ratio of phosphorylated protein to total protein expression. Expression data were then normalized to experimental conditions as indicated (i.e., versus vehicle or normoxia).

Collagen Content Measurement. Collagen content measurements were made using Sircol collagen dye binding assay, following the manufacturer’s instructions. Cardiac fibroblast lysates were sonicated three times for 5 sec and centrifuged at 3,000 x g for 5 min. The resulting supernatant was analysed for collagen content. Sircol dye reagent was mixed to equal amounts of protein from crude homogenate and cells for 30 min with agitation. The collagen-dye complex was centrifuged at 12,000 x g for 10 min and the resulting pellet was washed in ice-cold Acid-Salt Wash Reagent (acetic acid, sodium chloride and surfactants). Samples were then centrifuged at 12,000 x g for 10 min, the pellet was dissolved by adding alkali agent (0.5 M sodium hydroxide) and incubating at room temperature for 5 min. Collagen content was measured using spectrophotometer at 555 nm. Data were normalized to vehicle or normoxic conditions.

Statistics. All data are presented as mean ± SEM for indicated number of rats studied (n). Statistical differences were assessed using unpaired, two-tailed Student's t-test or ANOVA for comparison of individual means. P ≤ 0.05 is considered as significant.
RESULTS

Right ventricular (RV) dysfunction and fibrosis in hypoxic PH

Rats exposed to chronic hypoxia displayed significantly reduced RV systolic function and elevated pulmonary arterial pressure compared to normoxic animals, indicated by TAPSE and PAT, respectively (Table 1). Furthermore, animals with PH had a lower RV lateral wall e’ consistent with presence of RV diastolic dysfunction (Table 1). Hypoxic PH significantly increased RV hypertrophy, assessed by elevated RV free wall thickness (Table 1) and RV mass (RV/BW and RV/LVS) (Figure 1a).

Also, we noted a significant increase in procollagen I expression in the RV, but not LV, from hypoxic PH rats compared with normoxic controls (Figure 1b). Hence, the model recapitulated the maladaptive changes associated with PH, namely RV fibrosis and dysfunction. We further observed an increase in angiotensin II receptor (AT1R) expression in the RV from rats with hypoxic PH compared with normoxic controls (Figure 1c), suggesting increased angiotensin II signaling in settings of PH.

Effect of PH on the expression profile of PKC and activation status of p38 in ventricular cardiac fibroblasts

In order to study the expression profile of PKC in response to PH, cardiac fibroblasts from the RV of rats exposed to chronic hypoxia were isolated and subjected to Western blot analysis for PKC isoforms α, βII, δ and ε. Hypoxic PH resulted in elevated protein expression of PKC βII and δ in RV cardiac fibroblasts (Figure 2a). Since p38 activation is an important signaling pathway downstream of PKC, we next studied the effect of hypoxic PH on p38 activation in isolated RV cardiac fibroblasts. We observed a significant decrease in p38 activity, assessed by phosphorylation at residues T180/Y182, in the RV fibroblasts (Figure 2b) whilst no change in p38 phosphorylation status was noted in fibroblasts isolated from the LV or septum (Figure 2c & d). In contrast, hypoxic PH had no effect on total ERK expression (data not shown) or ERK phosphorylation status, assessed as ratio of phosphorylated ERK1/2 to total ERK1/2, in isolated RV cardiac fibroblasts (Figure 2e).

Effect of angiotensin II on p38 phosphorylation and PKC expression in isolated cardiac fibroblasts

We performed further mechanistic studies in cultured primary cardiac fibroblasts stimulated with angiotensin II, a key activator of ventricular fibrosis. We
did not observe significant changes in the level of p38 phosphorylation following acute exposure to angiotensin II (15 min to 2 h), (Figure 3a). However, upon longer term exposure to angiotensin II (48 h), we observed a significant decrease in p38 phosphorylation (Figure 3a) and a significant increase in expression of PKCβII and δ (Figure 3b) similar to our in vivo observations. Next, we characterized the effect of angiotensin II on proliferation and collagen synthesis in isolated cardiac fibroblasts. Cell proliferation, as measured by both cell count and thymidine incorporation, was significantly elevated from 6 h post-treatment to 48 h (Figure 3c). Accumulation of collagen was also significantly increased at both 24 and 48 h following exposure to angiotensin II (Figure 3d). Thus, the angiotensin II-induced collagen accumulation, increase in PKCβII and δ expression and p38 dephosphorylation in isolated cardiac fibroblasts mimic the changes seen in the RV in hypoxic PH.

Effect of PKCβII and δ inhibition on angiotensin II-induced cell proliferation and collagen deposition

Since PH in rats caused an increase in expression of PKCβII and δ (Figure 2a) in cardiac fibroblasts in vivo, we next sought to study whether inhibition of these PKC isoforms would play a role in the elevated proliferation and collagen accumulation observed following exposure to angiotensin II (Figure 3). Molecular inhibition of PKCβII and δ was induced by overexpression of the dominant negative plasmid cDNAs encoding PKCβII^K371R or PKCδ^K376R (Figure 4a and b) or by chemical inhibition of PKCβII and δ using LY333531 and rottlerin (Figure 4c). Transfection efficiency, as assessed by percentage of GFP-positive cells compared to total number of cardiac fibroblasts, was 42.5 ± 9.9 %. As noted for untransfected cardiac fibroblasts (Figure 3c), GFP-transfected control cells exposed to angiotensin II for 48 h displayed a significant increase in cell number (Figure 4b) and collagen accumulation (Figure 4c). Cardiac fibroblasts transiently transfected with either PKCβII^K371R or PKCδ^K376R displayed no difference in proliferation or collagen deposition (Figure 4b&c), in the presence or absence of angiotensin II. Likewise, preincubation of cardiac fibroblasts with either LY333531 or rottlerin, the PKCβII and PKCδ inhibitors, blocked angiotensin II-induced increase in cell proliferation (Figure 4c). Thus, inhibition of PKCβII or δ in cardiac fibroblasts blocks the
elevated proliferation and collagen accumulation observed following exposure to angiotensin II.

**Effect of p38 activation on angiotensin II-induced cell proliferation and collagen deposition**

We next sought to understand the mechanism through which inhibition of PKC βII or δ blocked cell proliferation and collagen deposits in cardiac fibroblasts exposed to angiotensin II. We first studied whether inhibition of PKC βII or δ affected angiotensin II-induced dephosphorylation of p38, as observed in Figure 3b. p38 dephosphorylation, following angiotensin II treatment, was attenuated by chemical inhibition of PKC βII and δ using LY333531 and rottlerin respectively, while no significant change in total p38 expression was observed in these experiments (Figure 5). As p38 activity was down-regulated in RV cardiac fibroblasts isolated from rats with PH (Figure 2b) as well as angiotensin II-treated cardiac fibroblasts in vitro (Figure 3b), our next studies assessed the effect of altering p38 activity on isolated cardiac fibroblasts *in vitro*.

Upregulation of p38 activity in cardiac fibroblasts was achieved via overexpression of the p38 protein or by overexpressing the upstream activator MKK6 using wild-type plasmid cDNA (Figure 6). Activation of p38 was determined with Western blot analysis of transfected cardiac fibroblasts. As expected, transient p38<sup>wt</sup> cDNA transfection increased p38 phosphorylation and total protein expression, whereas MKK6<sup>wt</sup> cDNA transfection led to an increase in phosphorylation of endogenously expressed p38, but did not increase total p38 protein expression (Figure 6a). In both cases, angiotensin II-induced cardiac fibroblasts proliferation (Figure 6b) and collagen accumulation (Figure 6c) were prevented by upregulation of p38 expression (p38<sup>wt</sup>) and/or phosphorylation (MKK6<sup>wt</sup>). Therefore, activation of p38 attenuates angiotensin II-induced proliferation and collagen accumulation.

As p38 has been previously observed to function downstream of PKC (1, 23), we next sought to understand whether the effect of PKC βII or δ inhibition, on collagen accumulation, was dependent on p38 activity. p38 was inhibited in cardiac fibroblasts via pre-incubation with the chemical inhibitor SB203580, prior to treatment with PKC βII or δ inhibition (Figure 7a and b). Under baseline conditions, exposure to SB203580 exhibited no effect on vehicle or angiotensin II-induced cell count and collagen accumulation (Figure 7a and b). Similarly, inhibition of p38,
following transient transfection of dominant negative p38 (p38\textsuperscript{agf}), exerted no significant effect on proliferation of cardiac fibroblasts in absence of angiotensin II. Cell count (normalized to GFP) was 1 ± 0.05 a.u. for GFP and 1.11 ± 0.04 a.u. for p38\textsuperscript{agf}, despite a significant 4-fold increase in p38 expression in p38\textsuperscript{agf}-overexpressing cardiac fibroblasts. As previously observed (Figure 4), inhibition of PKC\(\beta\)\(II\) or \(\delta\) attenuated angiotensin II-induced increases in proliferation and collagen accumulation (Figure 7a and b). Following p38 inhibition, these effects of LY333531 and rottlerin, on proliferation and collagen levels were abrogated (Figure 7a and b).

In summary, our data suggest that PKC\(\beta\)\(II\) and \(\delta\) mediate cardiac fibroblast proliferation and collagen accumulation through the inhibition of a p38 pathway that may result in RV fibrosis and dysfunction in settings of PH.
DISCUSSION

PH is associated with significant morbidity and mortality related to RV failure. Maladaptive changes in the RV, in response to PH, are associated with RV fibrosis and dysfunction (2). We show that in a well-established PH model, exhibiting RV fibrosis and dysfunction, increases in PKC βII and δ occur concomitant with RV-specific inhibition of p38 in cardiac fibroblasts. Using isolated cardiac fibroblasts, we demonstrate that angiotensin II-induced collagen accumulation is dependent on PKC βII and δ activity. To the best of our knowledge, this is the first study to show that PKC βII and δ-mediated cardiac fibroblast proliferation and collagen content is blocked following p38 inhibition, whilst overexpression or activation of p38 prevents angiotensin II-induced collagen production and proliferation in the cardiac fibroblast. We propose that down-regulation of p38 activity, in cardiac fibroblasts, is responsible for the characteristic RV fibrosis seen in PH. Thus, regulating the activity of p38 may represent a therapeutic tool for RV failure in PH.

We focused our mechanistic investigation on angiotensin II, an important pro-fibrotic mediator in the heart (30). It has been shown that increase in membrane bound angiotensin-converting enzyme (ACE) and higher ACE activity is present in the RV of hypoxic rats (32). In contrast, no changes are seen in the left ventricle and septum. Furthermore, higher ACE activity was noted in areas with myocardial fibrosis in the RV (32). Similarly, we demonstrate increased expression of the angiotensin II receptor AT1-R, a key component of the angiotensin II signalling mechanism. Thus, higher local levels of angiotensin II may be present in the RV in settings of hypoxic PH resulting in increased fibroblast proliferation and fibrosis. Angiotensin II results in an increase in cytosolic calcium and diacylglycerol (DAG) (13) that can increase the activity of PKC isoforms (38). Thus the PH-induced increases in PKC βII and δ expression in the RV, observed in the present study is consistent with known downstream signaling related to angiotensin II. We further demonstrate that abrogation of angiotensin II-mediated cardiac fibroblast proliferation and collagen synthesis occurs via inhibition of PKC δ and PKC βII activity. Consistent with our studies, inhibition of PKC δ attenuates collagen I and III synthesis in vitro and in vivo, attributed to a rottlerin-sensitive gene promoter segment in collagen (19, 20). Likewise, increased activation of PKC βII, via IL-7 exposure or inhibition of 14-3-3 activity, correlates with enhanced collagen I and II
production and cardiac fibrosis (15, 27). Thus, in settings of PH, PKC βII and PKC δ in RV cardiac fibroblasts are likely to be key regulators promoting ventricular fibrosis. While we focused on angiotensin II, other profibrotic mediators such as endothelin-1 and TGF-β have been shown to signal via PKCs as well. Further investigation is needed in how these mediators interact in signaling through these pathways resulting in RV fibrosis. Also, while the evidence suggests increased Angiotensin II signaling in the RV in hypoxic PH, further experiments are needed to definitively link the response of RV cardiac fibroblasts to increased Angiotensin II in vivo in settings of PH, such as using mice with targeted deletion of AT1-R in cardiac fibroblasts.

Studies investigating the role of p38 in cardiac fibroblasts and ventricular fibrosis have shown conflicting results. We demonstrate that a reduction in p38 activity results in cardiac fibroblast proliferation, collagen deposition and is associated with RV fibrosis and dysfunction. Our findings are consistent with those of Braz et al, who demonstrated an anti-fibrotic effect of p38 activation in unstressed and angiotensin II-exposed hearts using transgenic mouse models expressing dominant negative p38, MKK3 or MKK6 kinases (5). In contrast, short-term angiotensin II (5 min - 24 hr) exposure has been shown to activate the Ras/p38/CREB pathway in isolated rat cardiac fibroblasts (25, 41). TGF-β-mediated ECM deposition is associated with increased p38 activity in dermal and lung fibroblasts and chemical inhibition of p38 decreases stretch-induced collagen I transcripts in lung fibroblasts (22, 34). Whether these discrepancies may be related to the nature and duration of stimulus provided, or heterogeneity of fibroblasts from different tissue, remains to be resolved.

There are several possible mechanisms that may explain the relationship between reduced p38 activity and increased fibrosis which merit further investigation in settings of cardiac fibrosis. In cardiac fibroblasts, calcineurin/NFAT signalling, which is inhibited by p38 in other cell types, suppresses expression of the fibrosis markers collagen and fibronectin (5, 17, 44). In addition, p38 activation results in G1-phase arrest of the cell cycle, thus decreased proliferation, due to increased p21 levels and phosphorylation of GADD153 (11, 43).

We demonstrate that p38 inhibition abrogates the reduced collagen accumulation noted following PKC βII and δ inhibition, whilst p38 activation
correlates with inhibition of PKC βII and δ in cardiac fibroblasts. We further show that angiotensin II-induced dephosphorylation of p38 is attenuated by PKC βII and δ inhibition. Other studies have observed a similar relationship between PKC and p38 (6, 12). In contrast, some studies suggest increased p38 activity is associated with PKC activation (14, 18, 21). A potential explanation for these discrepancies may be related to differential p38-mediated signaling dependent on the cellular stress or mitogenic stimuli applied (11). While reduced p38 phosphorylation was necessary to increase cardiac fibroblast proliferation in response to angiotensin II, we did not find the p38 inhibition was sufficient to increase cardiac fibroblast proliferation in absence of angiotensin II. These data suggest that other parallel signaling by angiotensin II that may interact with p38 signaling plays a role in cardiac fibroblast proliferation and collagen production as well. Also, while we show that inhibition of PKC βII and δ prevents a decrease in p38 phosphorylation, the underlying mechanism remains to be elucidated. One possible mechanistic pathway may include MAPK phosphatases that can dephosphorylate p38, however, there are currently more than 15 members of the MAPK phosphatase family that need to be systematically evaluated for candidate phosphatase(s) in future studies (10, 16).

p38 inhibitors are under evaluation as therapeutic tools to attenuate cardiac remodeling and heart failure (28, 35). However, our study and another report (5) suggest that a decrease in p38 activity may be associated with increased cardiac fibrosis in settings of increased afterload. Furthermore, p38 inhibition exhibited no effect on angiotensin II-induced cardiac fibroblast proliferation or collagen deposition. Hence, these data suggest that more investigation is needed prior to the use of p38 inhibitors as therapeutic agents in heart failure.

In conclusion, the data presented show an association between increased RV collagen and p38 inactivation. In PH, p38 inactivation was specific to RV cardiac fibroblasts. Furthermore, inhibition of PKC βII or δ attenuated angiotensin II-induced cardiac fibroblast proliferation and collagen production in a p38-dependent manner. We therefore propose that PKC βII and δ-dependent inactivation of p38 is involved in increasing in RV collagen content; a mechanism that may be important in the pathophysiology of RV fibrosis in PH.
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REFERENCES


**Table 1:** Pulmonary hypertension causes right ventricular dysfunction associated with the disease.

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<th>Normoxia</th>
<th>Hypoxia</th>
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<tr>
<td>TAPSE (mm)</td>
<td>2.41 ± 0.24</td>
<td>1.68 ± 0.09*</td>
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<td>Ejection Fraction (%)</td>
<td>81.73 ± 3.41</td>
<td>72.60 ± 3.81</td>
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<tr>
<td>TV LW e' (mm/s)</td>
<td>-72.09 ± 14.48</td>
<td>-34.67 ± 4.31*</td>
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<tr>
<td>LV Septal Wall e' (mm/s)</td>
<td>-55.47 ± 12.76</td>
<td>-25.09 ± 1.23*</td>
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<tr>
<td>PAT (ms)</td>
<td>32.84 ± 2.43</td>
<td>25.11 ± 1.93*</td>
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<tr>
<td><strong>RV Diastolic Dimension (mm)</strong></td>
<td>1.90 ± 0.2</td>
<td>2.45 ± 0.22</td>
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<tr>
<td><strong>RV Free Wall Thickness (mm)</strong></td>
<td>0.62 ± 0.04</td>
<td>1.18 ± 0.15*</td>
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Echocardiogram data were obtained at experimental end-point and are presented as mean ± SEM. n=6, *p<0.05 vs normoxia. TAPSE: Tricuspid Annular Plane Systolic Excursion, TV LW e': Early diastolic tissue Doppler Velocity of RV lateral wall at tricuspid valve annulus, PAT: Pulmonary Acceleration time.
FIGURE LEGENDS

Figure 1: Pulmonary hypertension causes hypertrophy, collagen accumulation and increased angiotensin II receptor expression in the right ventricle.
(a) Weights of right and left ventricles (RV, LV) and interventricular septum (IVS) (normalized to body weight, BW) in rats exposed to normoxic and hypoxic (10% FiO₂) conditions (n=6) for 3 weeks. RV weight was also expressed as a ratio to LV plus IVS weight. (b) and (c) Western blot analysis of equal protein (50 μg/lane) from tissue homogenates of RV, LV and IVS from rats (n=6) exposed to 3 weeks of normoxia or hypoxia (10% FiO₂) that were probed with an antibody specific to procollagen or angiotensin II receptor type 1 (AT1R). Data are expressed as ratio to vinculin and normalized to respective normoxic controls. *p<0.05 vs normoxia.

Figure 2: Differential PKC isoform expression and MAPK phosphorylation status in cardiac fibroblasts from rats with pulmonary hypertension.
Western blot analysis of equal protein (50 μg/lane) from isolated right ventricular (a,b, and e), left ventricular (c) or intraventricular septum (d) cardiac fibroblast lysates of rats (n=4) exposed to 3 weeks of normoxia or hypoxia (10% FiO₂) that were probed with antibodies specific to either PKC α, βII, δ, ε (a) or phosphorylated p38 or ERK1/2 and total p38 or ERK1/2 (b-e). Data are calculated as a ratio of phosphorylated protein to total protein expressed and normalized to respective normoxic controls. *p<0.05 vs normoxia.

Figure 3: Angiotensin II-induced increase in cardiac fibroblast proliferation and collagen accumulation concomitant with enhanced PKC βII and δ expression and p38 dephosphorylation.
Cardiac fibroblasts from healthy control rats that were treated with angiotensin II (1 μM) or vehicle for indicated times (0 to 2, 24 or 48 h), were assessed for: (a) p38 phosphorylation (n=3), (b) PKCβII and δ expression (n=4), (c) cell proliferation, assessed by cell count (n=6) and thymidine incorporation (n=4) and (d) collagen
content (n=3). Data are normalized to vehicle and presented as mean ± SEM,
*p<0.05 vs vehicle, *p<0.05 vs 0 hr time point.

Figure 4: Molecular and chemical inhibition of PKC βII and δ abolishes angiotensin II-induced increases in cardiac fibroblast proliferation and collagen accumulation.
Cardiac fibroblasts from healthy control rats that were transfected with PKC βII<sup>K371R</sup> and PKC δ<sup>K376R</sup>, or GFP as control. Transfections were confirmed using Western blot analysis for PKC βII and δ (a). Transfected cardiac fibroblasts treated with angiotensin II (1 μM), or vehicle, for 48 h were measured for cell count (n= 7) (b) or collagen content (n=5) (b). Cardiac fibroblasts pre-incubated with LY333531 (50 nM, 30 min) and rottlerin (3 μM, 30 min) followed by treatment with angiotensin II (1 μM), or vehicle, for 48 h were measured by cell count (n=6) and thymidine incorporation (n=4) (c). Data are normalized to vehicle and presented as mean ± SEM. *p<0.05 vs vehicle, *p<0.05 vs GFP, angiotensin II treatment.

Figure 5: Chemical inhibition of PKC βII and δ inhibits angiotensin II-induced dephosphorylation of p38. Cardiac fibroblasts from healthy control rats that were pre-incubated with LY333531 (50 nM, 30 min) and rottlerin (3 μM, 30 min), followed by treatment with angiotensin II (1 μM) or vehicle for 48 h, were assessed for p38 phosphorylation status and total p38 expression. Data are calculated as a ratio of phosphorylated protein to total protein expressed, or total protein to vinculin, and normalized to respective normoxic controls. Grouped data from 4 separate experiments are averaged and presented as mean ± SEM. *p<0.05 vs vehicle/vehicle, *p<0.05 vs vehicle/angiotensin II treatment.

Figure 6: Molecular activation of p38, via overexpression of p38 or the upstream activator MKK6, abolishes angiotensin II-induced increases in cardiac fibroblast proliferation and collagen accumulation.
Cardiac fibroblasts from healthy control rats that were transfected with p38<sup>wt</sup> and MKK6<sup>wt</sup>, or GFP as control. Transfections were confirmed using Western blot analysis for p38 phosphorylation status and total p38 expression (a). Transfected cardiac fibroblasts treated with angiotensin II (1 μM), or vehicle, for 48 h were
measured for cell count (n= 7) (b) and collagen content (n=4) (c). Data are calculated as a ratio of phosphorylated protein to total protein expressed, or total protein to vinculin, and normalized to respective normoxic controls. Grouped data from 4 separate experiments are averaged and presented as mean ± SEM. *p<0.05 vs vehicle, *p<0.05 vs GFP, angiotensin II treatment.

**Figure 7: Inhibition of p38 prevents the effect of PKC βII and δ inhibition on angiotensin II-induced proliferation and collagen content.**
Cardiac fibroblasts from healthy control rats that were pre-incubated with SB203580 (100 nM, 30 min), followed by treatment with LY333531 (50 nM, 30 min) and rottlerin (3 μM, 30 min), and angiotensin II (1 μM), or vehicle, for 48 h were measured for cell count (n=9) (a) and collagen content (n=5) (b). Data are normalized to vehicle and presented as mean ± SEM. *p<0.05 vs vehicle/vehicle, *p<0.05 vs vehicle/angiotensin II treatment.
Figure 1

(a) Graph showing the comparison of RV/BW and LV/BW between Normoxia and Hypoxia for different regions: Right Ventricle, Left Ventricle, and Septum.

(b) Graph showing the comparison of Procollagen (a.u.) between Normoxia and Hypoxia for different regions: Right Ventricle, Left Ventricle, and Septum.

(c) Graph showing the comparison of IVS/BW between Normoxia and Hypoxia.

(d) Graph showing the comparison of RV/LV+ IVS between Normoxia and Hypoxia.

(e) Graph showing the comparison of AT1R/vinculin (normalized to normoxia) between Normoxia and Hypoxia.

(f) Graph showing the comparison of AT1-R/vinculin between Normoxia and Hypoxia.
Figure 2

a. Bar graph showing PKC isoform expression with N (normoxia) and H (hypoxia) conditions. The bar graph compares PKC isoforms α, β, δ, and ε (normalized to vinculin).

b. Bar graph depicting Phospho-p38/Total p38 expression with N (normoxia) and H (hypoxia) conditions. The graph shows a significant increase in Phospho-p38/Total p38 in hypoxia compared to normoxia.

c. Image showing Phospho-p38 expression in LV with N (normoxia) and H (hypoxia) conditions.

d. Image showing Phospho-p38 expression in IVS with N (normoxia) and H (hypoxia) conditions.

e. Image showing Phospho-ERK1/2 expression in RV with N (normoxia) and H (hypoxia) conditions.
Figure 3

(a) Phospho-p38/Total p38 (normalized to 0 hr) over time course (mins).

(b) PKC expression (normalized to vehicle).

(c) Thymidine incorporation (normalized to vehicle).

(d) Collagen content (normalized to vehicle).
Figure 4

a

b

Cell Count (Normalized to Vehicle)

Ang II

- + - + - +

GFP PKCδK376R PKCβllK371R

Vinculin

Collagen content (Normalized to Vehicle)

Ang II

- + - + - +

GFP PKCδK376R PKCβllK371R

PKCβ II

Vinculin

PKC δ

GFP PKCδK376R

# #

c

Cell Count (Normalized to Vehicle)

Ang II

Vehicle Rottlerin LY333531

Thymidine incorporation (normalized to Vehicle)

Ang II

Vehicle Rottlerin LY333531

* #

* #
Figure 5

| Rottlerin: | - | + | - | + | - | + |
| LY333531: | - | - | + | - | - | + |

**phospho-p38**

**p38**

**vinculin**

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**Phospho p38/Total p38 (normalized to Vehicle for AngII)**

- Vehicle - +
- Ang II - +
- Rottlerin - +
- LY333531 - +

**Total p38/vinculin (normalized to Vehicle for AngII)**

- Vehicle - +
- Ang II - +
- Rottlerin - +
- LY333531 - +

*Significance markers: * denotes p < 0.05 vs. Vehicle, # denotes p < 0.05 vs. Ang II.
Figure 6

(a) GFP p38\textsuperscript{wt} MKK6\textsuperscript{wt}

(b) Cell Count (Normalized to Vehicle)

(c) Collagen content (Normalized to Vehicle)
Figure 7

(a) Cell Count (Normalized to Vehicle)

(b) Collagen content (normalized to Vehicle)