Angiotensin II increases periostin expression via Ras/p38 MAPK/CREB and ERK1/2/TGF-\(\beta\)1 pathways in cardiac fibroblasts

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Aims Angiotensin II (AngII) is involved in extracellular matrix (ECM) accumulation contributing to heart failure. Periostin, a 90 kDa ECM protein, is a key regulator of cardiac fibrosis, and its expression is significantly higher in failing hearts. We determined the modulatory effect of AngII on periostin level and explored the possible signal transduction mechanism.

Methods and results AngII (400 ng/kg/min) or normal saline was infused subcutaneously for 28 days into rats; AngII antagonism was with losartan (10 mg/kg/day orally). AngII infusion induced cardiac fibrosis and increased periostin expression, which was attenuated by losartan. In cultured adult rat cardiac fibroblasts, AngII promoted the mRNA and protein expression of periostin. AngII provoked activation of cAMP response element-binding protein (CREB), and CREB small interfering RNA (siRNA) suppressed AngII-induced periostin expression. Inhibition of p38 mitogen-activated protein kinase (p38 MAPK) with SB202190 attenuated AngII-induced CREB activation and periostin expression. Transfection with Ras guanyl-releasing protein 1 siRNA or RasN17 dominant-negative plasmid prevented AngII-induced p38 MAPK phosphorylation and periostin expression. Transforming growth factor (TGF)-\(\beta\)1 antibody decreased the stimulatory effect of AngII on periostin expression. The extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor PD98059 attenuated AngII-induced TGF-\(\beta\)1 expression, Smad2/3 nuclear accumulation, and periostin expression.

Conclusion The activation of the Ras/p38 MAPK/CREB pathway is required for AngII-induced periostin expression. ERK1/2 also participates in AngII-induced periostin expression by regulating TGF-\(\beta\)1/Smad signalling.

Keywords Angiotensin II • Periostin • Signal transduction • Cardiac fibroblasts

1. Introduction

Cardiac remodelling after long-term pressure overload results in ventricular dysfunction and heart failure, which results in poor outcome and high mortality.\(^{1}\) One common characteristic that underlies nearly all forms of heart failure is the excessive deposition of extracellular matrix (ECM), which is often associated with decreased ventricular compliance.\(^{2}\) Recent reports suggest that the heart failure-associated alteration in cardiac ECM is associated with the activation of local renin–angiotensin system in patients with hypertension and in a mouse model of pressure overload because of transverse aortic constriction.\(^{3,4}\) Angiotensin-converting enzyme inhibitors or angiotensin II (AngII) receptor blockers are effective in ameliorating heart failure, including alleviating ECM deposition and preventing cardiac remodelling.\(^{5}\) Therefore, excessive ECM deposition is regulated, at least in part, through AngII.

Periostin, a 90 kDa novel ECM protein, is secreted primarily from osteoblasts and fibroblasts and is expressed in bone and—to a lesser extent—in lung, kidney, and heart valves in the adult mammal under normal conditions.\(^{6}\) However, periostin expression is significantly greater in human failing hearts\(^{6}\) and in the remodelled mouse heart after transverse aortic constriction.\(^{7}\) Overexpression of periostin in rat heart leads to cardiac dysfunction, with significantly increased fibrosis.\(^{8}\) However, periostin-knockout mice show less fibrosis after long-term pressure overload.\(^{9}\) Thus, periostin may play a major role in myocardial fibrosis and serve as a key regulator of cardiac remodelling and heart failure after pressure overload.

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The present study was designed to investigate the effect of AngII on periostin expression in rats exposed to a continuous infusion of AngII and in cultured adult rat cardiac fibroblasts (CFs). We also studied the detailed signalling mechanism by which AngII regulates periostin expression.

2. Methods

2.1 Materials
AngII, PD98059, SB202190, and SP600125 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Losartan was from Merck & Co. (Whitehouse Station, NJ, USA). Antibodies for periostin, phospho-, and total cAMP response element-binding protein (CREB), Ras, Ras guanyl-releasing protein 1 (RasGRP1), phospho-Thr, phospho-, and total p38 mitogen-activated protein kinase (MAPK), transforming growth factor (TGF)-β1, Smad2/3, discoidin domain receptor 2 (DDR2), and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RasN17 dominant-negative plasmid was kindly provided by Dr Da-Long Ma (Department of Molecular Immunology, Peking University Health Science Center).

2.2 In vivo rat model of continuous AngII infusion
All experimental procedures were approved by the Ethics Committee of Animal Research, Peking University Health Science Center, and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague–Dawley (SD) rats weighing 250–280 g were randomly divided into control, AngII, and losartan groups. An osmotic mini-pump (model 2004, Durect Corp., Cupertino, CA, USA) was subcutaneously embedded in rats under anaesthesia with sodium pentobarbital (50 mg/kg, ip). AngII (400 ng/kg/min) or normal saline was infused constantly for 28 days. In the losartan group, rats also received losartan (10 mg/kg/day orally) during AngII infusion. At day 28, haemodynamic parameters were recorded, and hearts were excised for further investigation.

2.3 Preparation of adult rat CFs
Primary cultured adult rat CFs were prepared as described. Briefly, ventricles from SD rats weighing 180–200 g were minced and digested in 450 U/mL collagenase II (Worthington Biochemical, Lakewood, NJ, USA). Cells were pelleted and suspended in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were plated on culture plates. Non-adherent cells were removed, and CFs were cultured in 450 U/mL collagenase II (Worthington Biochemical, Lakewood, NJ, USA). The identity of CFs was confirmed by immunostaining for vimentin.

2.4 Quantitative RT–PCR
Total RNA of myocardial tissues or CFs was isolated by use of the Trizol reagent (Invitrogen), and cDNA was generated from total RNA by use of the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). Quantitative real-time RT–PCR involved the use of gene-specific primers (available in Supplementary material online) and SYBR Green (Molecular Probes Inc., Eugene, OR, USA). All data were quantified by the use of the comparative cycle threshold method, normalized to β-actin.

2.5 Western blot analysis
After treatments, CFs were washed immediately twice with pre-cold phosphate-buffered saline (PBS) and harvested with cell scraper. Myocardial tissues or collected CFs were lysed in a buffer containing 50 mM Tris–HCl, pH 7.2, 0.1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 40 mM NaF, 2.175 mM sodium orthovanadate, 0.1% sodium dodecyl sulfate (SDS), 0.1% aprotinin, and 1 mM phenylmethylsulphonyl fluoride. The lysates were centrifuged at 10 000 × g for 10 min (4°C), and the supernatant was collected. Equal amounts of proteins (50 μg) underwent SDS-polyacrylamide gel electrophoresis (PAGE) and were transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies and then probed with horseradish peroxygenase-conjugated secondary antibodies. Blots were visualized with the use of an enhanced chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ, USA). The densities of bands were quantified by use of the LI-COR 8100 image analysis system (Leica, Mannheim, Germany).

2.6 Electrophoretic mobility shift assay
Nuclear protein fractions were separated as described elsewhere. Detection of DNA–protein interactions involved the non-isotopic method of the LightShift™ chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Rockford, IL, USA). Briefly, nuclear extract protein was incubated with biotin-labelled DNA probes containing the consensus CRE sequence 5′-AGAGATTGCCTGACGCC 3′ and RasGRP1 siRNA sequence 5′-GACCCGGAUGUAAACCUUAUUAGUUtt-3′ and RasGRP1 siRNA sequence 5′-CATGAAUUCUGCAUCUGUUtt-3′ were synthesized by GeneChem (Shanghai). Plasmid transfection was performed as described previously.

2.7 Immunoprecipitation
CFs were collected and lysed in radioimmunoprecipitation assay lysis buffer on ice for 60 min. The lysates were clarified by centrifugation at 10 000 × g for 10 min at 4°C. The supernatants were precleared with control IgG and then incubated with anti-phospho-Thr antibody and protein A-agarose. The immunoprecipitates were collected, washed, and re-suspended in sample buffer and analysed with anti-RasGRP1 antibody by western blot analysis as described previously.

2.8 Ras activity assay
CFs were homogenized, and the glutathione S-transferase fusion protein corresponding to human Ras-binding domain (RBD; residues 1–149) of Raf-1 (Millipore, Temecula, CA, USA) was used as an affinity reagent to precipitate Ras-GTP from cell lysates. Immunoprecipitates were then resolved by SDS–PAGE and immunoblotted with anti-Ras antibody. Ras activation was quantified by comparing blots for activated Ras (Ras-GTP) and total Ras before Raf-1 RBD pull-down.

2.9 Small interfering RNA and plasmid transfection
CFs were cultured to 80% confluence and transfected with small interfering RNAs (siRNAs) of interest by use of Lipofectamine 2000 (Invitrogen). siRNA targeting CREB and non-specific control siRNA were from Santa Cruz Biotechnology. The potent rat extracellular signal-regulated kinase 1/2 (ERK1/2) siRNA sequence 5′-GACCCGGAUGUAAACCUUAUUAGUUtt-3′ and RasGRP1 siRNA sequence 5′-GACCGGAUGUAAACCUUAUUAGUUtt-3′ were synthesized by GeneChem (Shanghai). Plasmid transfection was performed as described previously.

2.10 Masson’s trichrome staining
Ventricles were fixed in 10% phosphate-buffered formalin and embedded in paraffin. De-paraffined sections (6 μm thickness) were stained with Masson’s trichrome. Microscopy images were analysed by use of Leica QWin image analysis software. Connective tissue was expressed as percentage of myocardial tissue area.
2.11 Immunohistochemistry

CFs were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS. Frozen sections were fixed with pre-cooled acetone. CFs were stained with anti-Smad2/3 antibody, and sections were stained with the antibodies anti-periostin and anti-cardiac troponin I (Covance, Emeryville, CA, USA) overnight at 4 °C and then secondary antibodies for 2 h at 37 °C. Nuclei were stained with 4, 6-diamidino-2-phenylindole (Sigma-Aldrich). Fluorescence images were captured by use of the Leica TCS SP5 confocal system. Periostin-positive areas were quantified by use of Leica QWin image analysis software. Periostin fluorescence density was expressed as periostin-positive area corrected for cardiac troponin I-positive area.

2.12 Statistical analysis

Data are expressed as means ± SEM. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni’s tests. P < 0.05 was considered statistically significant.

3. Results

3.1 AngII increases periostin expression in vivo

Compared with the control group, the rat model of continuous AngII infusion showed a greater degree of cardiac fibrosis (Figure 1A). Quantitative RT–PCR and western blot analysis revealed that AngII infusion increased the mRNA and protein expression of periostin (Figure 1B and C). Immunohistochemical analysis of periostin protein in ventricles showed no periostin expression in the control group but abundant accumulation of periostin within the interstitial space with AngII (Figure 1D). Losartan attenuated AngII-induced interstitial fibrosis and periostin expression. Systolic blood pressure, diastolic blood pressure, dP/dt max, and dP/dt min were greater with AngII but were all suppressed by losartan (see Supplementary material online, Table S1). AngII infusion resulted in cardiac hypertrophy, as revealed by the increase in the ratio of ventricular to body weight and cardiac myocyte cross-section area, which was greatly reduced with losartan treatment (see Supplementary material online, Figure S1 and S2).

3.2 AngII increases periostin expression in adult rat CFs

To determine the effect of AngII on periostin expression in vitro, CFs were incubated with 10⁻⁸–10⁻⁵ mol/L AngII for the indicated time. AngII induced the mRNA and protein expression of periostin in a dose-dependent manner (Figure 2A and B). CFs were then stimulated with 10⁻⁶ mol/L AngII for the indicated times. AngII had a
time-dependent stimulatory effect on periostin expression (Figure 2C and D).

3.3 AngII induces periostin expression through p38 MAPK-dependent CREB activation

To determine whether AngII induced the activation of CREB (a critical transcription factor in mediating fibrotic response), CFs were incubated with $10^{-6}$ mol/L AngII for 10–120 min. AngII increased the phosphorylation of CREB at Ser-133, a key transcriptional activation site of CREB (Figure 3A). AngII treatment for 60, 90, and 120 min increased the binding activity of CREB to the CRE sequence (lane 4, 5, 6) and a specific competitor demonstrated the specificity of the DNA–protein complex (Figure 3B, lane 7). To ascertain the role of CREB in AngII-stimulated periostin expression, CFs were transfected with control or CREB siRNA. AngII-induced protein expression of periostin was reduced by 71.5% when pretreated with CREB siRNA ($P < 0.05$; Figure 3C).

Next, we explored whether MAPK proteins were involved in AngII-induced CREB activation. Enhanced CREB phosphorylation and binding activity were significantly blocked by p38 MAPK inhibition with SB202190, but not with PD98059 (an ERK1/2 upstream kinase inhibitor) or SP600125 (a specific c-Jun N-terminal kinase inhibitor) pre-treatment. Losartan abolished the stimulatory effect of AngII on CREB activation (Figure 3D and E). To further confirm whether ERK1/2 was necessary for AngII-elicited CREB activation, we knocked down ERK1/2 protein expression by siRNA (see Supplementary material online, Figure S3). Targeted knock-down of ERK1/2 did not affect AngII-induced CREB phosphorylation (Figure 3F).

3.4 The RasGRP1/Ras pathway is involved in AngII-mediated p38 MAPK phosphorylation

Ras has a positive regulatory role in mediating MAPK activation and the fibrotic response to an array of stimuli. We found that AngII increased the Ras-GTP level by 61.0% ($P < 0.01$) at 5 min and 80.2% ($P < 0.01$) at 15 min (Figure 4A). RasGRP1 is known as a regulator of Ras activity by causing the release of GDP. Therefore, we measured phosphorylation of Thr sites critically controlling activation of RasGRP1 by immunoprecipitation. AngII treatment significantly increased phospho-Thr-RasGRP1 level (Figure 4B), and RasGRP1 siRNA attenuated the AngII-induced Ras-GTP level (Figure 4C and D). To assess the impact of RasGRP1 and Ras on AngII-induced p38 MAPK activation, we examined phospho-Tyr-p38 MAPK level. AngII-induced phospho-p38 MAPK level was reduced in cells pre-treated with RasGRP1 siRNA or RasN17 (Figure 4E and F).

3.5 RasGRP1/Ras, p38MAPK, and ERK1/2 are involved in AngII-induced periostin expression

AngII-induced periostin expression was reduced by 72.5% ($P < 0.05$) and 64.1% ($P < 0.05$) when transfected with RasGRP1 siRNA and
Figure 3  AngII induces periostin expression through p38 MAPK-dependent CREB activation. (A) AngII induced CREB phosphorylation at Ser-133. Quiescent CFs were treated with 10⁻⁶ mol/L AngII for 10–120 min. Subcellular cytosolic fractions were examined by western blot analysis for CREB phosphorylation at Ser-133. Blots were reprobed with CREB to confirm equal loading. (B) AngII enhanced DNA binding activity of CREB by EMSA with nuclear extracts of CFs and DNA probes containing consensus CRE sequence. Lane 1, control; lane 2–6, AngII treatment for the indicated times; lane 7, with 100-fold molar excess of unlabeled oligonucleotide. (C) CREB siRNA attenuated AngII-induced periostin protein expression. CFs were transfected with CREB or control siRNA (50 nmol/L) for 48 h and serum deprived for 24 h, then incubated with 10⁻⁶ mol/L AngII for 48 h. Western blot analysis was performed to detect levels of periostin and actin. (D) SB202190 inhibited AngII-induced CREB phosphorylation. Quiescent CFs were pretreated with losartan, PD98059, SB202190, or SP600125 (all 10 µmol/L) for 30 min, then stimulated with 10⁻⁶ mol/L AngII for 120 min. Western blot analysis was performed to detect the levels of phospho-CREB and CREB. (E) SB202190 abolished AngII-enhanced DNA binding activity of CREB as determined by EMSA. (F) ERK1/2 siRNA did not affect AngII-induced CREB phosphorylation. CFs were transfected with ERK1/2 or control siRNA (100 nmol/L) for 48 h and serum deprived for 24 h, then stimulated with 10⁻⁶ mol/L AngII for 120 min. Western blot analysis was performed to detect the levels of phospho-CREB and CREB. Data in (A), (C), (D), and (F) represent means ± SEM of three independent experiments. *P < 0.05, **P < 0.01 vs. control. #P < 0.05, ##P < 0.01 vs. AngII.
Figure 4 The RasGRP1/Ras pathway is involved in AngII-mediated p38 MAPK phosphorylation. (A) AngII increased Ras-GTP level. Quiescent CFs were treated with 10^{-6} mol/L AngII for 5–60 min. Cell lysates were immunoprecipitated (IP) with Raf-1 RBD, and then immunoblotted (IB) with anti-H-Ras antibody. The blots for Ras-GTP were compared with those for total Ras before Raf-1 RBD pull-down. (B) AngII induced Thr phosphorylation of RasGRP1. Quiescent CFs were treated with 10^{-6} mol/L AngII for 5–60 min. Cell lysates were IP with anti-phospho-Thr antibody, then IB with anti-RasGRP1 antibody. The blots for phospho-Thr-RasGRP1 were compared with those for total RasGRP1 before pull-down. (C) RasGRP1 siRNA knocked down RasGRP1 protein expression. CFs were transfected with RasGRP1 or control siRNA (100 nmol/L) for 48 h, and RasGRP1 protein level was analysed by western blot analysis with actin as an internal control. (D) RasGRP1 siRNA reduced AngII-elevated Ras-GTP level. CFs were transfected with RasGRP1 or control siRNA (100 nmol/L) for 48 h and serum deprived for 24 h, then stimulated with 10^{-6} mol/L AngII for 15 min. The levels of Ras-GTP and Ras proteins were analysed by IP and western blot analysis, respectively. (E) RasGRP1 siRNA attenuated p38 MAPK phosphorylation induced by AngII. CFs were transfected with RasGRP1 or control siRNA (100 nmol/L) for 48 h and serum deprived for 24 h, then incubated with 10^{-6} mol/L AngII for 30 min. Subcellular cytosolic fractions were examined by western blot analysis for phospho-p38 MAPK. The plots were reprobed with p38 MAPK to confirm equal loading. (F) RasN17 reduced the AngII-enhanced p38 MAPK phosphorylation. CFs were transfected with RasN17 for 24 h and serum deprived for 24 h, then stimulated with 10^{-6} mol/L AngII for 30 min. Western blot analysis was performed to detect levels of phospho- and total p38 MAPK. Data represent means ± SEM of three independent experiments. *p < 0.05, **p < 0.01 vs. control, #p < 0.05, ##p < 0.01 vs. AngII.
RasN17, respectively (Figure 5A and B). We further explored a role of MAPK in AngII-induced periostin expression. SB202190 and PD98059 (but not SP600125) partially but significantly inhibited the AngII-induced up-regulation of periostin expression. Losartan abolished the stimulatory effect of AngII on periostin expression (Figure 5C).

3.6 ERK1/2 participates in AngII-induced periostin expression by regulating TGF-β1/Smad signalling

Because we found that ERK1/2 contributes to AngII-induced periostin expression in a CREB-independent manner, we explored the downstream signals involved in this process. AngII increased TGF-β1 protein expression in a time-dependent manner (Figure 6A) and induced nuclear accumulation of Smad2/3, a major intracellular mediator of TGF-β1 signalling in CFs (see Supplementary material online, Figure S4). Incubation with anti-TGF-β1 antibody inhibited periostin protein expression induced by AngII (P < 0.05) (Figure 6B). The significant increase in TGF-β1 expression and Smad2/3 nuclear accumulation was attenuated by pre-treatment with PD98059 (Figure 6C and D) but not SB202190 or SP600125 (see Supplementary material online, Figure S5).

4. Discussion

In the present study, we demonstrated that AngII increased periostin expression in a rat model of continuous AngII infusion and in cultured adult rat CFs. We also found that activation of CREB and its upstream signals RasGRP1, Ras, and p38 MAPK were required for AngII-induced periostin expression. ERK1/2, by regulating TGF-β1/Smad signalling, also participates in AngII-induced periostin expression.

CFs, the predominant secretory cells producing ECM proteins, are an important target of AngII and are a key mediator of cardiac fibrosis. In our in vivo rat model of continuous AngII infusion showing extensive interstitial fibrosis, AngII had a stimulatory effect on periostin expression. Immunofluorescence analysis revealed abundant accumulation of periostin within the interstitial space. In cultured adult rat CFs, AngII induced the mRNA and protein levels of periostin, which is in agreement with the Iekushi et al. results in neonatal rat CFs. Some evidence suggests that periostin binds multiple ECM proteins such as fibronectin, collagen, and heparin, thus affecting collagen synthesis and maturation through a complex series of architectural interactions, and thereby facilitating ECM deposition and cardiac remodelling.

A series of studies have indicated that CREB responds to different stimuli and mediates the fibrotic response in a plethora of cell types; examples are proliferation of lysosphosphatidic acid-induced Rat-2 fibroblasts and extracellular cysteine/cystine redox potential-
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stimulated lung fibroblasts, nicotine-induced fibronectin expression, insulin-like growth factor-1-stimulated fibronectin expression, and serum depletion-induced collagen I and III expression in CFs. However, no reports describe the role of CREB activation in AngII-induced periostin expression. We showed that AngII induced the activation of CREB in adult rat CFs, which was associated with increased phosphorylation at Ser-133 and DNA binding activity. CREB siRNA inhibited the AngII induction of periostin expression, which suggests that CREB plays an important role in mediating AngII-induced periostin expression.

A diverse array of stimuli activates MAPK signal pathways to result in CREB activation, such as ERK1/2 in high glucose-stimulated pancreatic β cells and retinoic acid-stimulated neuronal cells, and p38 MAPK in tumour necrosis factor-α-stimulated endothelial cells and adenosine-stimulated macrophage cells. However, the exact signals responsible for CREB activation in this process are still poorly understood. We found that the p38 MAPK inhibitor SB202190 blocked the AngII-induced phosphorylation and DNA binding activity of CREB, but JNK inhibition by SP600125 and ERK1/2 inhibition by PD98059 or siRNA did not attenuate AngII-induced CREB activation, which indicates that p38 MAPK but not ERK1/2 or JNK was critical for AngII-induced CREB activation. p38 MAPK inhibition clearly prevented AngII-induced periostin expression, which confirms that activation of CREB and its upstream signal p38 MAPK are required for AngII-induced periostin expression.

The guanine nucleotide-binding protein Ras has been heavily implicated in controlling an array of signalling networks in cellular proliferation, differentiation, adhesion, and migration. RasGRP1 activates Ras by removing GDP from inactive RasGDP and allowing binding of GTP to Ras, thus forming active RasGTP, and the activity of RasGRP1 is regulated by phosphorylation at its Thr sites. Here, we found a significant increase in phospho-Thr-RasGRP1 level and
Ras activation in response to AngII. The decrease in Ras activation was consistent with the knock-down of RasGRP1, which suggests that RasGRP1 is a critical mediator in AngII-induced Ras activation in CFs. Although many previous studies have investigated Ras and ERK1/2 activation in the intracellular signalling of various stimuli, only a few have focused on the role of Ras in AngII-induced p38 MAPK activation; examples are methylglyoxal stimulation in mesangial cells, interleukin-1 stimulation in thymoma cells, and mechanical stress stimulation in smooth muscle cells. The current study demonstrated a significant increase in p38 MAPK phosphorylation induced by AngII, and inhibition of RasGRP1 or Ras blocked this activation. Thus, AngII phosphorylates RasGRP1 to activate the Ras/p38 MAPK pathway in this process. Recently, a series of studies have implicated the crucial role of Ras in the fibrotic response in various animal models and cell types, such as myocardial fibrosis in neurofibromatosis type 1 myocardial-specific knock-out mice, unilateral ureteral obstruction-induced tubulointerstitial fibrosis, thioacetamide-induced liver cirrhosis, and methylglyoxal-induced fibronectin gene expression in renal mesangial cells. We therefore focused on the possible role of RasGRP1 and Ras activation in AngII-induced perisostin expression and found AngII-increased perisostin expression attenuated when cells were transfected with RasGRP1 siRNA or RasN17. These data give more detailed evidence of a role for Ras activation in AngII-induced perisostin expression and provide an alternative mechanism for Ras activation in regulating fibrotic response.

An examination of ERK1/2 inhibition on perisostin expression provides additional insights into this process. A large body of evidence suggests that TGF-β1 promotes the synthesis of ECM components and that the development of cardiac fibrosis is controlled by a regulatory network involving AngII and TGF-β1. We found that AngII increased TGF-β1 expression, as was previously demonstrated in different pathological settings and cell types. Smad2/3 proteins are essential intracellular signal molecules downstream of TGF-β1 and participate in TGF-β1-induced cardiac fibrosis. We demonstrated clear induction of nuclear accumulation of Smad2/3 by AngII, which indicates the activation of TGF-β1 signalling. An anti-TGF-β1 antibody attenuated AngII-induced perisostin expression, which indicates the role of TGF-β1 in AngII-induced perisostin expression. ERK1/2 inhibition with PD98059 abolished AngII-induced TGF-β1 expression and Smad2/3 nuclear accumulation, which suggests a significant interaction between ERK1/2 and TGF-β1 signalling in AngII-induced perisostin expression in CFs.

In summary, we demonstrate that AngII enhances the expression of perisostin, a key regulator of cardiac fibrosis, in vivo in a rat model and in cultured rat CFs. CREB activation plays a major role in the AngII-induced perisostin expression. RasGRP1, Ras, and p38 MAPK are signalling molecules that mediate AngII-induced CREB activation and perisostin expression. ERK1/2 also participates in AngII-induced perisostin expression by regulating the TGF-β1/Smad pathway. These findings may improve our understanding of the molecular mechanisms involved in myocardial fibrosis and provide new insights into future therapeutic targets for cardiac remodelling.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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