ORIGINAL ARTICLE

Angiotensin II-induced cardiomyocyte hypertrophy *in vitro* is TAK1-dependent and Smad2/3-independent

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Cardiac hypertrophy occurs as an adaptation to hypertension but a sustained hypertrophic response can ultimately lead to heart failure. Angiotensin-II (Ang II) is released following hemodynamic overload and stimulates a cardiac hypertrophic response. AngII also increases expression of the regulatory cytokine, transforming growth factor- β 1 (TGF β 1), which is also implicated in the cardiac hypertrophic response and can stimulate activation of Smad2/3 as well as TGF β -activated kinase 1 (TAK1) signaling mediators. To better understand the downstream signaling events in cardiac hypertrophy, we therefore investigated activation of Smad2/3 and TAK1 signaling pathways in response to Ang II and TGF β 1 using primary neonatal rat cardiomyocytes to model cardiac hypertrophic responses. Small interfering RNA (siRNA) knockdown of Smad 2/3 or TAK1 protein or addition of the TGF β type I receptor inhibitor, SB431542, were used to investigate the role of downstream mediators of TGF β signaling in the hypertrophic response. Our data revealed that TGF β 1 stimulation leads to cardiomyocyte hypertrophic phenotypes that were indistinguishable from those occurring in response to Ang II. In addition, inhibition of the TGF β 1 type receptor abolished Ang II-induced hypertrophic changes. Furthermore, the hypertrophic response was also prevented following siRNA knockdown of TAK1 protein, but was unaffected by knockdown of Smad2/3 proteins. We conclude that Ang II-induced cardiomyocyte hypertrophy *in vitro* occurs in a TAK1-dependent, but Smad-independent, manner.

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INTRODUCTION

Hypertension causes an increased cardiovascular mechanical load that can deleteriously affect heart function. An early myocardial response is an increase in cardiac Angiotensin II (Ang II) protein levels, which is intimately associated with the pathogenesis of cardiac hypertrophy and heart failure.^{1,2} Furthermore, upregulation of Ang II stimulates transforming growth factor- β 1 (TGF β 1) expression in the myocardium, which also stimulates hypertrophic responses in the heart.^{1,3–5} Moreover, Ang II-induced cardiac hypertrophy has been shown to be TGF β 1-dependent *in vivo*, revealing the close interaction between Ang II and TGF β -mediated responses in this pathology,⁶ although the exact nature of this interaction remains unclear.

To promote TGF β signaling, TGF β 1 ligand first binds to the TGF β type II receptor (TGFBR2) at the cell surface stimulating it to activate the TGF β type I receptor (TGFBR1, also known as ALK5), which in turn phosphorylates downstream SMAD2 and SMAD3 proteins. These activated SMAD proteins translocate to the nucleus in complex with SMAD4, where they regulate the transcription of TGF β -target genes.⁷ In addition to the canonical SMAD-dependent signaling pathway, TGF β can activate other signaling molecules including TGF β -activated kinase 1 (TAK1, also known as MAP3K7). TAK1 is a central mediator in the inflammatory response.⁸ It is also rapidly phosphorylated in cardiomyocytes following exposure to TGF β 1, and

is activated in the mouse myocardium following pressure overload.⁹ Furthermore, overexpressed constitutively active TAK1 leads to cardiac hypertrophy and heart failure in a mouse model,⁹ and activated TAK1 is associated with the hypertrophic response in spared cardiomyocytes following myocardial infarction.¹⁰ We have previously shown a direct association between TAK1 and TGFBR2, which may facilitate ligand-dependent activation of TAK1 by ALK5, to stimulate a MAP kinase signaling cascade and cardiomyocyte hypertrophy.¹¹ As TGF β ligand is involved in activation of both the SMAD2/3 and the TAK1 signaling pathways, we were interested to determine the importance of each of these signaling cascades to the hypertrophic response.

To address this question we used a well-established *in vitro* model of cardiomyocyte hypertrophy. When cultured rat neonatal cardiomyocytes are treated with hypertrophic agents, such as Endothelin-1 or Ang II, they undergo a series of changes that model aspects of the hypertrophic response, including an increase in cell size, reorganization of contractile proteins and the re-expression of fetal cardiac genes such as brain natriuretic peptide.^{2,12} We have previously shown that endothelin-1 and Ang II generated a very similar hypertrophic response *in vitro*.¹³ Using this model we now show that Ang II-stimulated cardiomyocyte hypertrophy is dependent on TGF β receptor activity and requires the presence of TAK1, but not SMAD2/3.

METHODS

Antibodies and reagents

Primary antibodies were anti-Smad2 (#3122), anti-Smad3 (#9513), anti-phospho Smad2 (#3101) and anti-TAK1 (#4505) from New England Biolabs (Hitchin, UK). Secondary antibody was Goat anti-Rabbit IgG conjugated to horseradish peroxidase (#111-035-144; Jackson ImmunoResearch Laboratories, Newmarket, UK). Ang II (A9525) and SB431542 (S4317) were from Sigma (Poole, UK), and recombinant TGF β 1 (100-21R) was from PeproTech EC (London, UK).

Animals

Sprague–Dawley rats were obtained from Charles River Laboratories (Margate, UK). All animal experiments were complied with the United Kingdom Animal Scientific Procedures Act 1986 as well as with 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) and were approved by the ethical committee of Newcastle University.

Cell culture

Primary cultures of neonatal ventricular cardiomyocytes from 2-day-old Sprague–Dawley rats were prepared as previously described.¹³ Heart ventricles were digested with collagenase (0.4 mg ml⁻¹) and pancreatin (0.6 mg ml⁻¹) in 116 mM NaCl, 20 mM HEPES (pH 7.35), 0.8 mM NaH₂PO₄, 5.6 mM glucose, 5.4 mM KCl and 0.8 mM MgSO₄. The recovered cells were pre-plated for 30 min on 60 mm Primaria culture dishes in plating medium (70% Dulbecco's modified Eagle's medium, 15% M199, 15% fetal calf serum, 100 units per ml penicillin and streptomycin) and non-adherent primary neonatal cardiomyocytes (PNCM) were transferred to gelatin-coated 60-mm Primaria culture dishes for 18 h, before changing to serum-free maintenance media (80% Dulbecco's modified Eagle's medium, 20% M199, 100 units per ml penicillin

and streptomycin). Cardiomyocyte purity was routinely 85%, as judged by positive sarcomeric α -actinin staining.

Hypertrophy assays

Cardiomyocytes were stimulated with either 200 nM Ang II or 5 ng ml⁻¹ TGF β 1 for 4 h before analysis of changes in gene expression using real-time reverse-transcription PCR, and for 48 h before analysis of changes in cytoskeleton and cell morphology. For real-time reverse-transcription PCR, RNA was isolated using RNeasy kits (Qiagen, Crawley, UK) and converted to complementary DNA using a high-capacity reverse transcription kit (Applied Biosystems, Paisley, UK), according to manufacturers' protocols. Expression of the following hypertrophic and endogenous control genes were measured using the TaqMan 7900HT Fast real-time PCR system and assay probe sets (Applied Biosystems) for brain natriuretic peptide (Rn00580641_m1), hypoxanthine guanine phosphoribosyl transferase gene (Rn01527840_m1) and glyceraldehyde-3-phosphate dehydrogenase endogenous control VIC probe (4352338E). Data are presented as mean+s.e.m. of *Rq* values from three independent experiments. To examine changes in cell morphology and cytoskeleton, cells were fixed in 4% paraformaldehyde, stained with FITC-conjugated Phalloidin (Sigma) for 30 min and mounted in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK). Images were acquired using a Zeiss Axioplan II microscope and cell area was measured with Axiovision software (Zeiss, Welwyn Garden City, UK). Where indicated, SB431542 was used to inhibit Alk5 activity in a similar way to previously published protocols.^{14,15}

Small interfering RNA

PNCM cells were incubated for 18 h with small interfering RNA (siRNA) oligonucleotides, each consisting of a pool of 3–5 target-specific siRNAs, from Santa Cruz (Heidelberg, Germany). These were Smad2 (sc-77325), Smad3 (sc-77326), TAK1 (sc-36606) or non-targeting scrambled control oligonucleotides (sc-37007) and were used at a concentration of 30 nM to transfect cells using the

Figure 1 Ang II or TGF β 1 generate hypertrophic responses in cardiomyocytes *in vitro*. **(a)** Primary neonatal rat cardiomyocytes (PNCM) show rearrangement of the actin cytoskeleton, detected using FITC-phalloidin, following treatment with either 200 nM Ang II or 5 ng ml⁻¹ TGF β 1. Insets show a digital zoom of individual cells that reveal organization of F-actin fibers in more detail. **(b)** Average cell area of PNCM increases after treatment with Ang II or TGF β 1. **(c)** Real-time PCR shows brain natriuretic peptide (BNP) mRNA expression increases in PNCM after treatment with Ang II or TGF β 1 compared with untreated control cells. **P*<0.002 and ***P*<0.0001 vs. untreated control cells. A full color version of this figure is available at the *Hypertension Research* journal online.

manufacturer's instructions. Smad2 and Smad3 oligonucleotides were combined with a final oligonucleotide concentration of 60 nM in order to simultaneously reduce both proteins. Transfected cells were incubated for 48 h before the addition of hypertrophic agonists.

Immunoblotting

Cardiomyocyte cell pellets were resuspended in 50 μ l ice-cold NTT lysis buffer (100 mM NaCl, 50 mM Tris-HCl (pH 8), 1% Triton X-100, 10 mM NaF) in the presence of protease inhibitors (bestatin, leupeptin, pepstatin A, aprotinin, E64; Sigma) and separated using SDS-PAGE. Protein was transferred to Hybond-P PVDF membrane (GE Healthcare, Little Chalfont, UK) and blocked with PBS/0.3% Tween-20/5% skimmed milk before incubation with primary antibody. Washed membranes were incubated with horseradish peroxidase-conjugated secondary antibody and proteins were visualized using Super Signal chemiluminescent substrate (Perbio, Cramlington, UK).

Statistical analysis

Cell area data are presented as mean values, and error bars indicate the s.e.m. from 20 to 30 individual cell measurements. Data were analyzed using two tailed Student *t*-tests. Gene expression data were analyzed using the RQ Manager 1.2 software (Applied Biosystems), and *Rq* values from a minimum of three independent experiments were analyzed. Statistical significance was accepted at $P < 0.05$.

RESULTS

Ang II and TGF β 1 induce similar hypertrophic responses *in vitro*

Ang II stimulation of rat PNCM *in vitro* resulted in rearrangement of cortical cytoskeletal actin to form aligned stress fibers (Figure 1a). This was accompanied by a 2.3-fold increase in average cellular footprint

(Figure 1b) and a twofold increase in brain natriuretic peptide mRNA expression relative to endogenous control genes hypoxanthine guanine phosphoribosyl transferase and glyceraldehyde-3-phosphate dehydrogenase (Figure 1c). These phenotypes are similar to previously reported cardiomyocyte hypertrophic responses *in vitro*.^{13,16–18} TGF β 1 alone also stimulated comparable hypertrophic responses to those induced by Ang II treatment (Figures 1a–c), and in a similar way to a previous report.¹⁹

Functional TGF β 1 signaling is essential for the stimulation of cardiomyocyte hypertrophy by Ang II

Immunoblot experiments showed that TGF β 1 and AngII stimulated phosphorylation of Smad2 in PNCM cells (Figure 2a), in a similar way to previous reports.^{9,20,21} Inhibition of TGF β 1 signaling with 6 μ M SB431542 blocked phospho-Smad2 responses to Ang II and TGF β (Figure 2a) indicating that the Smad 2 phosphorylation response to Ang II requires TGF β receptor activity. Moreover, when PNCMs were treated with SB431542 inhibitor there was no detectable hypertrophic response to Ang II; cell shape, cytoskeletal actin organization and brain natriuretic peptide expression appeared unchanged and were indistinguishable from untreated control cells (Figures 2b–d). Thus, Ang II induction of hypertrophic responses *in vitro* is dependent on TGF β 1 signaling in a similar way to previously reported *in vivo*.⁶

Canonical TGF β 1 signaling through Smad proteins is not essential for cardiomyocyte hypertrophy induced by Ang II

TGF β 1 signalling involves both canonical signaling through the Smad2/3 pathway and non-canonical signaling through TAK1.

Figure 2 Ang II-induced hypertrophic responses *in vitro* depend on active TGF β 1 signaling. (a) Western blots show that AngII (200 nM) and TGF β (5 ng ml⁻¹) stimulation lead to phosphorylation of Smad2 in PNCM cells and this is abrogated in the presence of the ALK5 inhibitor SB431542. (b–d) Hypertrophic responses in PNCM following Ang II treatment are blocked in the presence of SB431542; these include reorganization of actin fibers (b), cell enlargement (c) and increased brain natriuretic peptide (BNP) mRNA expression measured using real-time PCR (d). * $P < 0.02$ vs. untreated control cells. A full color version of this figure is available at the *Hypertension Research* journal online.

Figure 3 Hypertrophic responses induced by Ang II and TGFβ1 are Smad2/3 independent. **(a)** Western Blots of PNCM cell extracts show knockdown of Smad2 and Smad3 proteins after treatment with Smad2/3 siRNA but not with scrambled (SCR) siRNA. **(b, c)** Treatment of PNCM with either 200 nM Ang II or 5 ng ml⁻¹ TGFβ1 promotes reorganization of actin fibers and increased cell size in both Smad2/3 siRNA-treated and SCR siRNA control cells. **(d)** Real-time PCR shows increased brain natriuretic peptide (BNP) mRNA synthesis after treatment with Ang II or TGFβ1 is not affected by Smad2/3 knockdown. **P* < 0.02 vs. SCR siRNA control cells. A full color version of this figure is available at the *Hypertension Research* journal online.

A number of studies have pointed to the importance of TAK1 signalling in the hypertrophic response.^{9,10,22} We therefore wished to investigate whether either Smad2/3 or TAK1 or both were required for hypertrophic responses in cardiomyocytes in culture. SiRNA oligonucleotide transfection was first used to knockdown the expression of Smad2/3, and scrambled oligos were used to control for non-specific effects of transfection. Downregulation of Smad2/3 proteins did not affect the ability of Ang II to stimulate PNCMs to undergo hypertrophic responses (Figures 3a–d). There was also a comparable hypertrophic response to TGFβ1 treatment, irrespective of Smad2/3 knockdown (Figures 3b–d), indicating that Smad2/3 proteins are not required for the induction of cardiomyocyte hypertrophy by Ang II or TGFβ1.

TAK1 is essential for cardiomyocyte hypertrophy induced by Ang II or TGFβ1

To evaluate the importance of TAK1 signaling in hypertrophy, TAK1 protein was reduced in cardiomyocytes by siRNA oligonucleotide transfection (Figure 4a) and hypertrophic phenotypes were assessed as before. Control cells that retained TAK1 expression displayed the classic hypertrophic changes in cell size and actin fiber rearrangement in response to both Ang II and TGFβ1 treatments (Figures 4b and c). However, these responses were abrogated in cells where TAK1 expression had been reduced by siRNA (Figures 4b–d), indicating that TAK1 is essential for cardiomyocyte hypertrophy induced *in vitro* by Ang II or TGFβ1.

DISCUSSION

We have shown that Ang II requires functional TGFβ1 signaling in order to initiate hypertrophic responses *in vitro* using a neonatal cardiomyocyte model. Moreover, TGFβ1 treatment alone induced hypertrophic phenotypes that were indistinguishable from those initiated by Ang II. Inhibiting TGFβ1 signaling with SB431542 prevented Ang II-induced hypertrophy, in agreement with *in vivo* evidence that TGFβ1 signaling is involved in the cardiac hypertrophic response.⁶ This intimate association between AngII and TGFβ1 signalling has also been shown in mouse models of hypertrophic cardiac myopathy where TGFβ1 is an essential mediator of hypertrophy and the Ang II type I receptor antagonist losartan prevented the hypertrophic response.²³

An important finding in our study is that neither Ang II nor TGFβ1 stimulated hypertrophy in cardiomyocytes in which TAK1 protein was downregulated by siRNA treatment. These data indicate that AngII and TGFβ1 promote a hypertrophic response, which is TAK1-dependent and is consistent with *in vivo* data showing the importance of TAK1 in cardiac hypertrophy.^{9,10,22} Our data also suggest that the previously reported TGFβ1-dependent induction of cardiac hypertrophy by Ang II *in vivo* would have required TAK1 activity, but not Smad2/3 signalling.⁶

We found that reduction of Smad2/3 proteins did not inhibit the hypertrophic response, suggesting cardiac hypertrophy is Smad2/3 independent. In fact, Smad signalling may have a role in protecting

Figure 4 Hypertrophic responses induced by both Ang II and TGFβ1 are TAK1-dependent. (a) Western Blots of PNCM cell extracts showing knockdown of TAK1 protein after transfection with TAK1 siRNA, but not with scrambled (SCR) siRNA. (b) Reduced TAK1 levels in PNCM cells abrogates reorganization of actin fibers and increases in cell size in response to 200nm Ang II or 5ngml⁻¹ TGFβ1 stimulation. (c) Quantitation of the inhibitory effects of TAK1 knockdown on PNCM cell-enlargement responses to agonist treatments; vs. SCR siRNA control cells. (d) Real-time PCR shows increased brain natriuretic peptide (BNP) mRNA synthesis after treatment with Ang II or TGFβ1 is abrogated by TAK1 knockdown. **P*<0.02 vs. SCR siRNA control cells. A full color version of this figure is available at the *Hypertension Research* journal online.

against cardiac hypertrophy as cardiac-specific depletion of the common Smad mediator, Smad4, leads to cardiac hypertrophy in a mouse model even in the absence of any external hypertrophic agonist.²⁴

We also observed that the treatment with the Alk5 inhibitor SB431542 abolished the hypertrophic response. It has also been shown that Alk5 inhibition *in vivo* ameliorates the hypertrophic response associated with myocardial infarction.²⁵ In contrast, myocyte-specific loss of Alk5 activity did not affect the cardiac hypertrophic response to aortic constriction, whereas myocyte-specific loss of Tgfr2 did lead to reduced TAK1 activity and reduced hypertrophy.²² This indicates the complexity of the functional interaction between TAK1 and the TGFβ type I and type II receptors. We have previously shown that Alk5 associates with TAK1 in mammalian cardiomyocyte cells, likely through a common interaction with Tgfr2,¹¹ but further work is required to better understand the complex interactions between these three proteins during the hypertrophic response.

In conclusion, we show that TAK1 is downstream from, and essential for, hypertrophic signaling initiated by Ang II or TGFβ1 *in vitro* and that hypertrophic responses occur independently of canonical TGFβ1 signaling through Smad proteins. Currently, TAK1 is being actively explored as an immunomodulatory drug target for inflammatory disorders²⁶ and our findings suggest it would also have benefits in protecting the heart from deleterious remodelling in response to hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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