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Angiotensin-II type 1 receptor and NOX2 mediate TCF/LEF and CREB dependent WISP1 induction and cardiomyocyte hypertrophy

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Abstract

Angiotensin-II (Ang-II) plays a key role in myocardial hypertrophy, remodeling and failure. We investigated whether Ang-II-induced cardiomyocyte hypertrophy is dependent on WNT1 inducible signaling pathway protein 1 (WISP1), a pro growth factor. Ang-II induced hypertrophy and WISP1 expression in neonatal rat cardiomyocytes (NRCM), effects that were significantly inhibited by pre-treatment with the AT1 antagonist losartan and by WISP1 knockdown. Further, Ang-II induced WISP1 was superoxide-dependent, and inhibited by DPI, an inhibitor of NADPH oxidases, and by knockdown of NOX2. AT1 physically associated with NOX2 both *in vitro* and *in vivo*, and Ang-II increased this interaction *in vivo*. Ang-II induced WISP1 expression via superoxide/Akt/GSK3β/β-catenin/TCF/LEF and by Akt-dependent CREB activation. Further, Ang-II also activated CREB via superoxide-mediated p38MAPK and ERK activation. Continuous infusion of Ang-II for 7 days induced myocardial hypertrophy in rats, and was associated with increased Akt, phospho-Akt, p-p38 MAPK, p-ERK1/2, and WISP1 expression. These results demonstrate that Ang-II induced cardiomyocyte hypertrophy is mediated through AT1, NOX2 and the induction of WISP1, and may involve the direct interaction of AT1 with NOX2. Thus targeting both WISP1 and NOX2 may have a therapeutic potential in improving cardiomyocyte survival and growth following myocardial injury and remodeling.

1. Introduction

Cardiomyocytes constitute the largest cell type by volume in the normal heart, and are second only to fibroblasts in numbers. Adult cardiomyocytes are terminally differentiated, and have limited regenerative capacity. Therefore, they undergo death following injury or inflammation, with remaining surviving cardiomyocytes compensating for the loss by

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undergoing growth or hypertrophy. While hypertrophy can be physiological (*e.g*., exercise-induced), pathological hypertrophy can be seen as a sequel to aortic valve stenosis, ischemic heart disease, and chronic hypertension [1], leading ultimately to myocardial failure.

Increased levels of angiotensin (Ang)-II, a well characterized and a critical component of the Renin-Angiotensin-Aldosterone System (RAAS), is associated with progressive myocardial dysfunction, adverse remodeling, and myocardial failure [2–4]. Ang-II has been shown to induce cardiomyocyte hypertrophy both *in vivo* and *in vitro*, predominantly via the Ang-II type 1 receptor (AT1). In smooth muscle cells (SMC), cardiomyocytes and various other cell types, binding of Ang-II to AT1 induces NADPH oxidase (NOX)-dependent superoxide generation and activates multiple downstream signal transduction pathways [5,6]. Of note, Ang-II/AT1 mediated ROI generation is implicated in left-ventricular hypertrophy *in vivo*[7], and inhibition of ROI attenuates Ang II-mediated cardiomyocyte hypertrophy *in vitro* [8], indicating the critical role of NOX-dependent ROI generation in Ang-II-mediated cardiomyocyte hypertrophy.

NOX-dependent generation of superoxide and hydrogen peroxide have been shown to play a role in the activation of oxidative stress-responsive transcription factors and the induction of various cytokines, chemokines and growth factors that contribute to inflammation, injury, contractile depression, hypertrophy and failure [9,10]. While the role of NOX has been extensively studied in neutrophils (phagocyte NOX or NOX2), cardiomyocytes also express several members of the NOX family, including NOX2 and NOX4 [9,10]. In Ang-II-treated neonatal rat cardiomyocytes, the membrane-associated NOX2, previously known as $gp91^{phox}$, is the predominant NOX isoform [8] that generates superoxide via electron transfer from NADPH to molecular oxygen, and its subsequent conversion to hydrogen peroxide. While Ang-II activated superoxide generation is AT1 and NOX2 dependent [8], it is however not known whether AT1 physically associates with NOX2, and whether their direct interaction induces superoxide generation in cardiomyocytes.

Ang-II activates various pro-growth signaling molecules in cardiomyocytes, including the well-characterized PI3K and Akt. Recently we demonstrated overexpression of WISP1, a CCN (cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed) family member, in post-infarct myocardium *in vivo*[11]. *In vitro* WISP1 exerts pro-growth effects inducing cardiomyocyte hypertrophy[11]. In these cells, WISP1 induced PI3K activation and stimulated Akt phosphorylation and its activity. Further, while mediating TNF-α induced cardiac fibroblast proliferation, WISP1 antagonizes TNF-α-induced cardiomyocyte death[12], demonstrating its pro-mitogenic, anti-apoptotic and pro-growth effects. Since Ang-II and WISP1 share similar downstream signal transduction pathways and since both induce cardiomyocyte growth, we investigated (i) whether Ang-II-induced cardiomyocyte hypertrophy *in vitro* is WISP1 dependent, and the underlying signal transduction pathways involved; (ii) the effects of continuous infusion of Ang-II on myocardial hypertrophy and WISP1 upregulation *in vivo*.

2. Materials and methods

2. 1. Animals

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (DRR/National Institutes of Health, 1996), and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Science Center at San Antonio. The isolation of neonatal rat cardiomyocytes (NRCM) was previously described[13]. For the *in vivo* studies, normotensive male Sprague Dawley rats (~3 m of age, ~108 g; Charles River Laboratories International, Inc., Wilmington, MA) were used. Animals were allowed 7 days to

acclimatize, and then trained for systolic blood pressure (SBP) measurement using a tail-cuff method without anesthesia (CODA Noninvasive Blood Pressure System, Kent Scientific, Torrington, CT). A subset of animals was infused with 700 μg/kg/day of Ang-II for one week via subcutaneously (midscapular region) implanted Alzet miniosmotic pumps (n=6). Control animals were implanted with sterile saline-filled pumps (n=6). After blood pressure measurements, body weights were recorded, and the animals sacrificed. The hearts were rapidly excised, rinsed in ice-cold physiological saline, and weighed. The right ventricle and atria were trimmed away, and the left ventricle (LV) was weighed. LV was cut into three pieces and two were snap frozen in liquid N_2 for not more than 3 days before analyzing for WISP1 mRNA and protein expression. Hypertrophy was analyzed by the ratio of left ventricular weight to body weight.

2. 2. Adeno and lenti viral transduction

NRCM were infected at ambient temperature with adenoviruses (Supplementary file) in PBS at the indicated multiplicities of infection (MOI). After 2 h, the adenovirus was replaced with culture media supplemented with 0.5% BSA. Assays were carried out 24 h later. Lentival infection was carried out for 48 h. The transfection efficiency with the adenovirus (*e.g.*, Ad.GFP) and lentiviral particles (copepod or copGFP) was near 100%, and infection with the viral vectors had no significant effect on cardiomyocyte shape, adherence, or viability.

2. 3. Measurement of O_2^- with lucigenin

Superoxide (O_2^-) production was quantified essentially as described [14] using a lucigenin-enhanced chemiluminescence assay. In brief, NRCM (1×10^3) were rinsed twice in PBS and suspended in Krebs/HEPES buffer (in mM: 115 NaCl, 20 HEPES, 1.17 K_2HPO_4, 1.17 $MgSO_4$, 4.3 KCl, 1.3 $CaCl_2$, 25 $NaHCO_3$, and 11.7 glucose, pH 7.4) containing NADPH (10^{-4} M), and incubated in a 37°C water bath for 20 min. Lucigenin (5×10^{-6} M) and ANG II (10^{-7} M) or ANG II + Tiron, Ang-II + DPI, or Ang-II+TEMPOL were added. After equilibrating for 30 min at 37°C, the tubes were placed in a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). Luminescence was measured for 10 s with a delay of 5 s. After subtracting background luminescence, results are expressed as RLU/second/1000 cells. Studies were also performed using NRCM infected with Ad.CuZnSOD (100 moi for 24 h) or lentivirus expressing NOX2-specific shRNA (48 h) prior to Ang-II addition.

2. 4. Promoter reporter assays

A *WISP1* promoter reporter construct spanning the region from −4764 to −39 bp relative to the transcription start site (pWISP4764) and containing one CREB and three TCF/LEF binding sites, a deletion construct lacking all three TCF/LEF sites, and the deletion construct lacking the CREB site in pGL2-Basic vector were previously described [15] and generously provided by Arnold J. Levine (Rockefeller University, New York, New York). pGL2-Basic served as a vector control. Cells were transfected with 3 μg of the reporter vectors, and cotransfected with 100 ng of the internal reference plasmid *Renilla* luciferase (pRL-TK; Promega) using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After incubation for 12 h, the cells were harvested for the dual-luciferase assay. Data were normalized by dividing firefly luciferase activity with that of corresponding *Renilla* luciferase activity. The transfection efficiency of NRCM is ~33% with only 9% cell death as determined using the pEGFP-N1 vector (Clontech, Mountain View, CA). All plasmids were purified using EndoFree Plasmid Maxi kit (QIAGEN Inc., Valencia, CA).

2. 5. Transcription factor activation

TCF-LEF DNA binding and transactivation activity were analyzed by EMSA and a reporter assay as detailed in the Supplementary file. Analysis of CREB activation by EMSA is also described in the Supplementary file.

2. 6. mRNA expression

WISP1 mRNA expression was analyzed by real time quantitative PCR as described in the Supplementary file. All data were normalized to corresponding Actin mRNA, and expressed as the fold difference in gene expression in Ang-II treated cardiomyocytes relative to untreated controls. WISP1 mRNA expression was confirmed by Northern blotting with 28S rRNA serving as a control.

2. 7. Western blotting

Extraction of nuclear, cytoplasmic, membrane and whole cell lysates, immunoblotting, chemiluminesence, and densitometry were performed as detailed in the Supplementary file.

2. 8. AT1/NOX2 binding interaction *in vitro*-GST pull-down assay

NOX2 cDNA was amplified from rat spleen cDNA by PCR using primers based on the rat NOX2 sequence (NM_023965); Sense: 5′-gct agc gga ccA TGG GGA ACT GGG CTG TGA ATG AGG-3′; and Antisense: 5′-TTA GAA GTT TTC CTT GTT GAA GAT GA-3′. *Nhe*I and Kozak consensus sequences were included in the sense primers (lower case). The PCR product was cloned into pCR2.1-TOPO (Invitrogen), excised with *Nhe*I and *Xba*I and cloned into the vector pcDNA3.1/Zeo(-) (Invitrogen). Orientation and identity were confirmed by nucleotide sequencing. The cDNA for rat angiotensin II type 1 receptor (AT1; NM_030985) was prepared with a similar approach using the primers Sense: 5′-gct agc gga ccA TGG CCC TTA ACT CTT CTG CTG A-3′; and Antisense: AAC CTG TCA CTC CAC CTC AAA AC-3′. Rat ventricle cDNA was used as template. For binding studies, a fusion protein of glutathione-S-transferase (GST) and the C terminal 57 amino acids of ATI (303–359) were prepared. cDNA encoding $ATI_{(303–359)}$ was amplified from rat ATI cDNA using the Sense primer 5′-gaa ttc CGG CTT TCT GGG GAA GAA ATT TA-3′ and the above antisense primer. The PCR product was cloned into pCR2.1-TOPO, excised with *EcoR*I and cloned into the *EcoR*I site of pGEX-3X (GE Healthcare). GST and GST-$ATI_{(303–359)}$ were induced in the Rosetta™ strain of E. coli (EMD Chemical Inc) with 1mM IPTG and bacterial cell lysates prepared using standard procedures.

Binding interactions were carried out between GST and GST-$ATI_{(303–359)}$ fusion protein immobilized on GSH-Sepharose (GE Health Care), and *in vitro* synthesized [^{35}S]-labeled rat NOX2. Volumes of fusion protein lysates, previously determined to give equal loading of the GSH-Sepharose, were mixed for 30 min at room temperature with 50 μl of a 50% suspension of washed GSH-Sepharose in PBS (145 mM NaCl, 10 mM Na_2HPO_4, pH 7.3). The suspensions were washed x3 with PBS and x3 with PBS containing 0.05% Triton X-100, and resuspended to 476 μl in PBS/0.05%. Rat NOX2, was synthesized and labeled *in vitro* with L-[^{35}S]-methionine (PerkinElmer NEG709A, 1175Ci/mmol) using the TNT® Coupled Reticulocyte Lysate System (Promega) and T7 RNA polymerase. A 24 μl aliquot of the labeling reaction was mixed with the resuspended immobilized GST fusion protein for 30 min at room temperature. The gel pellet was washed x3 with PBS/0.05%TX-100 and treated with 100 μl 2X SDS treatment buffer on ice for 20 min. After centrifugation for 5 min at 14,000 rpm, an aliquot of the supernatant (10 μl) was separated on 9% SDS-PAGE and analyzed by fluorography. For comparison, an aliquot of the labeled NOX2 equivalent to 2% of the input was included on the gel. To confirm the equivalent loading of the GST

proteins, 5 μl of the supernatant was separated on 12% SDS PAGE and the gel stained for protein (GelCode® Blue Stain Reagent, Thermo Fisher Scientific Inc).

2. 9. AT1/NOX2 binding interaction *in vivo*- immunoprecipitation and immunoblotting

For immunoprecipitation, equal amounts of membrane extracts were incubated overnight with specific antibodies attached to agarose beads at 4 °C under slow rotation. After washing 3 times in a buffer containing 50 mM Tris-Cl, 150 mM NaCl, 0.1% Nonidet P-40, the bound proteins were eluted from the beads by boiling in SDS sample buffer for subsequent SDS-PAGE and immunoblotting. The antibodies against AT1 (AT1 (306): sc-579) were used for immunoprecipitation as well as immunoblotting. Similarly, anti-gp91 phox (NOX2) antibodies (Millipore, catalog# 07-024) were used in immunoblotting. The same antibodies were used in immunoprecipitation, and were previously described (16).

2. 10. Akt and GSK3β activities

Akt and GSK3β activities were assayed as detailed in the Supplementary file.

2. 11. Cardiomyocyte hypertrophy

Cardiomyocyte hypertrophy was assessed by two independent methods: increased protein, but not DNA, synthesis and cell surface area (13). Details are provided in the Supplementary file.

2. 12. Cell death analysis

To determine whether transduction of viral vectors and pharmacological inhibitors negatively affect cell viability, cell death was analyzed using the Cell Death Detection ELISA^{PLUS}. Cell viability was also tested by trypan blue dye exclusion and microscopic visualization of shape and for cells floating in the media.

2. 13. Statistical analysis

Comparisons between controls and various treatments were performed by analysis of variance with post hoc Dunnett's *t* tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E.

3. Results

3. 1. Angiotensin-II induces cardiomyocyte hypertrophy via AT1 and WISP1

Ang-II and WISP1 share similar downstream signal transduction pathways and since both induce cardiomyocyte growth [8,11], we investigated whether Ang-II induced cardiomyocyte hypertrophy is WISP1 dependent. Treatment of neonatal rat cardiomyocytes (NRCM) with Ang-II significantly increased protein synthesis in a dose-dependent manner with peak levels of incorporation detected at 10^{-7}M (Fig. 1A). However, Ang-II failed to significantly modulate DNA synthesis (Fig. 1B), implying cardiomyocyte hypertrophy. Increased surface area confirmed this pro-hypertrophic effect (data not shown). Since Ang-II signals via AT1 and AT2, we next investigated the receptor specificity. While pretreatment with AT1 antagonist Losartan significantly attenuated Ang-II-mediated cardiomyocyte hypertrophy, the AT2 antagonist PD 12339 had minimal effect (Fig. 1C). Further, knockdown of WISP1 by lentiviral transduction of WISP1 shRNA significantly blunted Ang-II-induced cardiomyocyte growth (Fig. 1D). Knockdown of WISP1 also blunted Ang-II induced activation of p70S6 kinase (Fig. 1E) and ribosomal S6 protein (Fig. 1F), two indices of protein synthesis. These results indicate that Ang-II induces cardiomyocyte growth, and its pro-hypertrophic effects are mediated in part via AT1 and WISP1 (Fig. 1).

3. 2. Angiotensin-II induces WISP1 expression via AT1 and NOX2

NOX2 is the predominant NOX isoform in NRCM [8]. Our semiquantitative PCR (RT-PCR) data demonstrated that NOX2 is readily detectable in NRCM at basal conditions (Supplementary Figure; Fig. S1A). Further, real-time quantitative RT-PCR (RT-qPCR) using TaqMan probes demonstrated increased NOX2 mRNA expression in NRVM (Supplementary Figure, Fig. S1B). Therefore, we next investigated whether Ang-II induced WISP1 expression requires NOX2. NRCM express low levels of WISP1 mRNA (Fig. 2A) and protein (Fig. 2B) under basal conditions, but treatment with Ang-II significantly upregulated both in a time dependent manner. These effects were blunted when NRCM were pre-treated with the AT1 antagonist losartan (Fig. 2C), but not by the AT2 antagonist PD-123319 (PD; Fig. 2C). Further, knockdown of NOX2 significantly inhibited Ang-II mediated WISP1 expression (Fig. 2D), as did treatment with DPI, a NADPH oxidase inhibitor (Fig. 2E). These results indicate that Ang-II is a potent inducer of WISP1 expression in cardiomyocytes, and Ang-II-mediated WISP1 expression is AT1 and NOX2 dependent (Fig. 2).

3. 3. AT1 physically associates with NOX2

Ang-II has been shown to activate NOX2 in cardiomyocytes and other cell types in AT1-dependent manner [8,14]. Also, NOX2 is the predominant NOX isoform in Ang-II-treated NRCM (Supplementary Fig. 1 and [8]). Although Ang-II has been shown to signal via NOX2, it is however not known whether AT1 can physically associate with NOX2. Using GST pull-down assays, we identified a direct interaction between the C-terminal cytoplasmic domain of AT1 with NOX2 (Fig. 3A). Further, using reciprocal immunoprecipitation and immunoblotting, we identified an AT1-NOX2 protein-protein interaction *in vivo* which appeared to increase following Ang-II treatment (Fig. 3B). This suggests a regulatable interaction between AT1 and NOX2 that may be important for signaling activity (Fig. 3).

3. 4. Angiotensin-II induces superoxide generation

Superoxide is a critical intracellular activator of various second messenger molecules, including the pro-survival factor Akt [16]. Therefore, we first investigated Ang-II-mediated superoxide generation in NRCM using a lucigenin assay. While low levels of superoxide were detectable under unstimulated basal conditions, treatment with Ang-II markedly increased its generation (Fig. 4), and this increase was significantly attenuated by the cell permeable non-enzymatic superoxide scavenger Tiron, the NOX inhibitor DPI, or by NOX2 knockdown (Fig. 4). Similar inhibitory effects were observed with TEMPOL, another superoxide scavenger, and with adenoviral transduction of CuZnSOD (Fig. 4). These results indicate that Ang-II-induced superoxide generation in NRCM is produced chiefly by NOX2 (Fig. 4).

3. 5. Angiotensin-II induces cardiomyocyte hypertrophy via Akt1

Since Ang-II-induced cardiomyocyte hypertrophy is WISP1-dependent (Fig. 1), and Ang-II upregulated WISP1 expression (Fig. 2), we next investigated some of the molecular mechanisms involved in Ang-II-induced WISP1 expression. Ang-II induced Akt phosphorylation (Fig. 5A) and stimulated its activity (Fig. 5B), and these effects were significantly inhibited by DPI, Ad.CuZnSOD, Ad.dnAkt1 and Akti-X (Fig. 5C). Further, pretreatment with Akti-X or adenoviral transduction of dnAkt1 prior to Ang-II addition, significantly inhibited Ang-II-induced cardiomyocyte hypertrophy (Fig. 5D). These results indicate that Ang-II-induced cardiomyocyte hypertrophy is Akt dependent (Fig. 5).

3.6. Angiotensin-II induces β-catenin activation via Akt and GSK3β

Further, Ang-II induced time-dependent GSK3β phosphorylation (Fig. 6A), and this effect was inhibited the adenoviral transduction of dnAkt and pretreatment with the pharmacological inhibitor of Akt, Akti-X (Fig. 6B). Significantly, Ang-II induced β-catenin accumulation in the cytoplasm (Fig. 6C), and promoted its nuclear translocation and stabilization (Fig. 6D). These effects were attenuated by Ad.dnAkt1 (Fig. 6E) and phosphorylation-deficient GSK3β mutant (Ad.GSK3β (S9A); Fig. 6F). Importantly, forced expression of ICAT (Adeno-ICAT (Myc)-IRES-GFP), the inhibitor of β-catenin-TCF interaction, significantly inhibited Ang-II-induced cardiomyocyte hypertrophy (Fig. 6G; expression Myc tag is shown as an insert). Together, these results indicate that Ang-II is a potent inducer of β-catenin activation and its nuclear translocation in NRCM (Fig. 6).

3. 7. Ang-II induces TCF-LEF activation

The 5′ *cis* regulatory region of *WISP1* contains multiple TCF-LEF binding elements (TBE; [15]), suggesting that it may be a TCF-LEF-responsive gene. Since Ang-II promoted β-catenin nuclear translocation (Fig. 6), and since β-catenin associates with TCF-LEF in the nucleus and induces gene expression [17], we next investigated Ang-II effects on TCF-LEF activation. Results show that indeed Ang-II induces TCF/LEF DNA binding activity (Fig. 7A) and reporter gene activation (Fig. 7B), effects that were markedly attenuated by the forced expression of phosphorylation-deficient GSK3β mutant (Ad.GSK3β (S9A)) (Fig. 7A) or the inhibitor of the β-catenin/TCF binding interaction (Ad.ICAT) (Fig. 7A and 7B). These results were further confirmed in reporter assays using NRCM transfected with wild type and TCF/LEF mutant *WISP1* promoter-reporter vectors (Fig. 7C). Further, forced expression of dnAkt1, GSK3β (S9A), or ICAT by adenoviral transduction inhibited Ang-II-induced WISP1 expression (Fig. 7D). These results indicate that Ang-II induces WISP1 expression via superoxide/Akt/GSK3β/β-catenin/TCF/LEF signaling (Fig. 7).

3. 8. Angiotensin-II induces CREB activation

We and others have previously reported that WISP1 is a CREB responsive gene [12,15]. Since superoxide is known to mediate CREB activation[18], we next investigated whether Ang-II-induced WISP1 expression is CREB dependent. At first we determined whether Ang-II activates CREB in NRCM. Indeed, our results show that Ang-II is a potent inducer of CREB activation, as evidenced by an increase in CREB DNA binding activity (Fig. 8A). This effect was significantly attenuated by the forced expression of mutant CREB (Ad.KCREB or Ad.MCREB). Further, forced expression of dnAkt1 by adenoviral transduction significantly attenuated Ang-II-mediated CREB activation (Fig. 8B), as were the superoxide scavenger Tiron, the NADPH oxidase inhibitor DPI and NOX2 knockdown (Fig. 8C). Importantly, mutation in the lone CRE site in the *WISP1* proximal promoter region significantly inhibited *WISP1* transcription (Fig. 8D). Further, infection with Ad.KCREB attenuated Ang-II-mediated WISP1 mRNA expression (Fig. 8E), indicating that Ang-II activates CREB via Akt, NOX2, and superoxide, and Ang-II-induced WISP1 expression is CREB dependent (Fig. 8).

3. 9. Angiotensin-II induces CREB activation via mitogen activated protein kinases p38 and ERK1/2

Since superoxide has been reported previously to activate CREB via p38MAPK and ERK1/2 [6], we next investigated whether these MAP kinases play a role in Ang-II-mediated CREB activation in NRCM. Results show that Ang-II did activate p38 MAPK in NRCM in a time-dependent manner (Fig. 9A), an effect that is significantly attenuated by SB203580 (SB; Fig. 9B). Further, DPI pre-treatment and NOX2 knockdown also inhibited p38MAPK activation (Fig. 9B). Similar to its stimulatory effects on p38MAPK, Ang-II also

induced time-dependent ERK1/2 activation (Fig. 9C), an effect that is significantly inhibited by PD98509 (PD), DPI and NOX2 knockdown (Fig. 9D). Importantly, both SB and PD inhibited Ang-II-mediated CREB activation (Fig. 9E) and WISP1 expression (Fig. 9F). These results indicate that Ang-II induces WISP1 expression via superoxide-mediated p38MAPK- and ERK1/2-dependent CREB activation (Fig. 9).

3. 10. Angiotensin-II induces myocardial hypertrophy and WISP1 expression *in vivo*

We have demonstrated that Ang-II induces cardiomyocyte hypertrophy *in vitro* in part via WISP1 (Fig. 1). We next investigated whether Ang-II can induce WISP1 expression *in vivo*. As shown in Fig. 10, continuous infusion of rats with Ang-II for one week increases systolic blood pressure (SBP; Fig. 10A, left hand panel), and induces significant hypertrophy, as evidenced by increased left ventricular weight (LVW) to body weight ratio (Fig. 10A, right hand panel). Further, Ang-II infusion increased p-p38 MAPK and p-ERK1/2 levels (Fig. 10B), total and phospho-Akt levels (Fig. 10C), and WISP1 protein (Fig. 10C) and mRNA (Fig. 10D) expression in the LV tissue, recapitulating Ang-II's effects *in vitro* in NRCM (Fig. 5A and 5B, Fig. 9A and 9C). These results demonstrate that Ang-II-induced myocardial hypertrophy is associated with Akt, p38 MAPK and ERK1/2 activation and WISP1 upregulation (Fig. 10).

4. Discussion

The Renin-Angiotensin-Aldosterone System (RAAS) is a critical mediator of myocardial hypertrophy, adverse remodeling, and failure[2–4]. Here we show that Ang-II induced cardiomyocyte hypertrophy is WISP1 dependent. Ang-II induces WISP1 mRNA and protein expression via AT1/superoxide/Akt/GSK3β/β-catenin/TCF/LEF and AT1/superoxide/p38MAPK- and ERK-dependent CREB activation pathways. Knockdown experiments showed that NOX2 NADPH oxidase is the principal source of the superoxide involved in Ang-II/AT1 signaling. Further, we demonstrate that NOX2 and AT1 can physically interact *in vivo* in cardiomyocytes and this interaction is increased by Ang-II treatment. Such interaction may constitute a potential mechanism of enhancement of the Ang-II/AT1 stimulus-response axis. Continuous infusion of Ang-II in rats for 7 days induces myocardial hypertrophy, and is associated with increased levels of Akt and phospho-Akt, activation of p38 MAPK and ERK, and upregulation of WISP1. Together, these data show that hypertrophic growth in cardiomyocytes results from the interaction of the RAAS, NOX and WISP1, and suggest a therapeutic potential for WISP1 in cardiomyocyte survival and growth following myocardial injury and remodeling.

Many of the pathophysiologic effects of Ang-II are mediated through the AT1 receptor. Inhibition of AT1 has been shown in a number of studies to blunt Ang-II-induced myocardial hypertrophy[19–25]. AT1 is also a therapeutic target in renal diseases, hypertension, and myocardial failure, and blockade of AT1 or its signaling reduces complications associated with these diseases, and improves function and survival[19–25]. Similarly, in our studies, Ang-II-stimulated cardiomyocyte growth was significantly inhibited by the AT1 antagonist losartan. We also demonstrate that Ang-II induced the generation of superoxide in cardiomyocytes and this was a necessary intermediate in the induction of WISP1 via both Akt/GSK3β/β-catenin/TCF/LEF and p38MAPK- and ERK-dependent CREB activation pathways, as well as for the hypertrophic response. DPI, an NADPH oxidase inhibitor, and forced expression of CuZnSOD by adenoviral transduction both attenuated Ang-II-induced superoxide generation and cardiomyocyte growth. The source of the superoxide appeared to be NOX2 NADPH oxidase since lentiviral transduction of the NRCM with NOX2 shRNA blunted Ang-II-induced superoxide generation, downstream signaling, WISP1 induction and cardiomyocyte hypertrophy. Although the role of NADPH oxidase-dependent superoxide generation in cardiac hypertrophy *in vivo* and

cardiomyocyte growth *in vitro* is known[9,10], here we show for the first time that AT1 can in fact directly interact with NOX2. Immunoprecipitation and immunoblotting showed that AT1 physically associates with NOX2 in the unstimulated cell, and that this association is increased following Ang-II treatment. Our results are consistent with those reported by Hingtgen, et al. who showed that NOX2 is the predominant isoform in NRCM[8]. Using quantitative PCR and isoform-specific siRNA, these authors reported that NOX2, but not NOX4, is highly induced following Ang-II treatment and that NOX1 was barely detectable. AT1 is a 7 transmembranous domain-containing G-protein coupled receptor that contains a relatively short (~55 amino acid) cytoplasmic C-terminal domain. To determine whether this domain may be a potential site of interaction between NOX2 and AT1, GST pull down studies were carried out between *in vitro* transcribed/translated NOX2 and a GST-$AT1_{(303–359)}$ fusion protein. The increased binding between NOX2 and GST-$AT1_{(303–359)}$ compared to that seen with just GST alone strongly suggest that the C-terminal amino acids of AT1 contribute to the binding of NOX2. The binding of Ang-II to AT1 has also been shown to enhance the interaction of the receptor with caveolin-1 and promote its trafficking into caviolae/lipid rafts which in turn promotes Rac1 translocation and increased local ROS production [26,27]. Further, binding of Ang-II induces the interaction of AT1 with clathrin adapter protein 2 and β-arrestin to induce c-Src-regulated, clathrin-mediated, receptor internalization[28]. Further, NOX is also shown localized to lipid rafts[29,30], suggesting colocalization of AT1 and NOX2 in lipid rafts, and thus their interaction may represent an integral part of a functional AT1 membrane complex that mediates the downstream responses to Ang-II.

We also show that both DPI and forced expression of CuZnSOD inhibited Ang-II-mediated Akt activation and cardiomyocyte growth, suggesting a role for NADPH oxidase-generated superoxide in Akt activation. Similar results were reported previously in Ang-II treated smooth muscle cell (SMC) growth[16]. In that study, Ang-II-induced Akt activation and SMC hypertrophy were significantly inhibited by DPI, indicating that Akt is redox-sensitive [16]. Upon activation, Akt regulates several down-stream signal transduction pathways, including IKK, Bad, caspase-9, and GSK3 [31].

GSK3β, a ubiquitously expressed serine-threonine kinase, plays a role in both survival and death in a cell- and stimulus-specific manner. At steady state, it is present in an active form in the cytoplasm complexed with β-catenin, axin and adenomatous polyposis coli[32]. Akt-mediated GSK3β phosphorylation at Ser9 results in its degradation by the ubiquitin-proteasome pathway[32], liberating β-catenin. In fact, GSK3β has been shown to negative regulate cardiomyocyte growth [33]. Released β-catenin translocates to the nucleus, where it regulates target gene expression after co-activation of members of the T-cell-specific factor (TCF)/lymphoid-enhancer-binding factor (LEF) family. Here we show that Ang-II treatment of cardiomyocytes results in GSK3β phosphorylation and that Ang-II promotes β-catenin nuclear translocation and stabilization in an Akt and GSK3β-dependent manner. Moreover, forced expression of ICAT[34], the inhibitor of the β-catenin/TCF binding interaction, attenuated Ang-II-induced TCF activation and WISP1 expression. Importantly, mutation in TCF/LEF binding elements in *WISP1* 5′ *cis* regulatory region blunted Ang-II-stimulated *WISP1* promoter-reporter activity, indicating that Ang-II stimulates WISP1 expression via superoxide/Akt/GSK3β/β-catenin-dependent TCF/LCF activation. Of note, the pro-survival factor Akt is also a TCF/LEF-responsive gene[35]. This suggests that, in addition to activating Akt, Ang-II may regulate its transcription in TCF/LEF-dependent manner, and thus amplify Akt pro-survival signaling. Since WISP1 phosphorylates GSK3β, and activates β-catenin and TCF/LEF [36], it is plausible that Ang-II-induced WISP1 may regulate its own expression, and its perpetual expression may mediate Ang-II's pro-growth effects. Further, Ang-II also activates IKK-dependent NF-κB[37], a pro-survival factor, and as IKK is a substrate for Akt[31], our results together with other published reports indicate that Ang-

II stimulates a series of non-redundant, inter-related pro-survival signal transduction pathways in cardiomyocytes, resulting in their survival and growth. Since we also observed Ang-II infusion to induce WISP1 expression and myocardial hypertrophy, we hypothesize that Ang-II-induced myocardial hypertrophy *in vivo* involves activation of signal transduction pathways similar to those identified in the cell studies. In fact, recapitulating our *in vitro* results, our *in vivo* results show increased levels of Akt, phospho-Akt, p-p38 MAPK, p-ERK1/2, and WISP1 expression in Ang-II infused mouse hearts.

We and others have previously demonstrated that *WISP1* is also a CREB-responsive gene[12,15]. Overexpression of mutant CREB and mutation in the single CRE in *WISP1* proximal promoter region blunted Ang-II-induced WISP1 expression, indicating that Ang-II-induced WISP1 expression is both TCF/LEF and CREB responsive. We also demonstrate that Ang-II-induced superoxide stimulates CREB activation in a p38MAPK and ERK1/2-dependent manner. The AT1 antagonist losartan, the NOX inhibitor DPI, NOX2 knockdown, and the cell permeable superoxide scavengers Tiron and TEMPOL, all inhibited p38MAPK and ERK1/2 activation. Further, the p38MAPK and ERK1/2 inhibitors SB 203580 and PD 98059, respectively, inhibited Ang-II-mediated CREB activation and WISP1 upregulation. Similar results were reported previously in Ang-II-treated cardiac fibroblasts [6]. In that study, Ang II was shown to increase intracellular ROS in cardiac fibroblasts, and this increase was completely inhibited by the AT1 blocker candesartan and the NOX inhibitor DPI. They further confirmed that the antioxidant N-acetylcysteine, superoxide scavenger Tiron, and DPI suppressed Ang II-induced CREB phosphorylation and IL-6 transcription[6], suggesting that Ang-II-mediated DPI-inhibitable superoxide activates multiple and similar signal transduction pathways in at least two major myocardial constituent cell types. Of note, CREB has been shown to be a regulatory target of Akt [38]. Since Ang-II is a potent activator of Akt, it is plausible that Ang-II may induce CREB activation via multiple signaling pathways. Although WISP1 also activates Akt[36], it needs to be determined whether WISP1 induces its own expression via CREB activation. We will investigate this possibility in our future studies. Importantly, and in agreement with our *in vitro* studies, continuous infusion of Ang-II for 7 days induces myocardial hypertrophy, and is associated with increased levels of total Akt and phospho-Akt, and WISP1 upregulation.

Together, we show that Ang-II, a major components of the RAAS, induces cardiomyocyte hypertrophy in part via WISP1, and may involve the direct interaction of AT1 with NOX2. Since WISP1 plays a role in cardiomyocyte survival and growth, our studies suggest a therapeutic potential for WISP1 in myocardial injury and remodeling, disease states characterized by cardiomyocyte death and hypertrophy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

Ang-II	angiotensin-II
AT1	angiotensin-II type 1 receptor

CCN	cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed
CRE	cAMP response elements
CREB	CRE-binding protein
dn	dominant negative
DPI	diphenyleneiodonium chloride
ECM	extracellular matrix
ERK	extracellular signal-regulated protein kinase
GFP	green fluorescent protein
GSK-3	glycogen synthase kinase–3
GSK-3β (S9A)	phosphorylation-deficient mutant of GSK-3β with a serine 9-to-alanine substitution
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
NF-κB	nuclear factor κB
NRCM	neonatal rat cardiomyocytes
Nov	nephroblastoma-overexpressed gene
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NOX2	NADPH oxidase 2
shRNA	short hairpin RNA
TCF/LEF	T-cell-specific transcription factor/lymphoid enhancer binding factor 1
WISP1	WNT1-inducible signaling pathway protein-1

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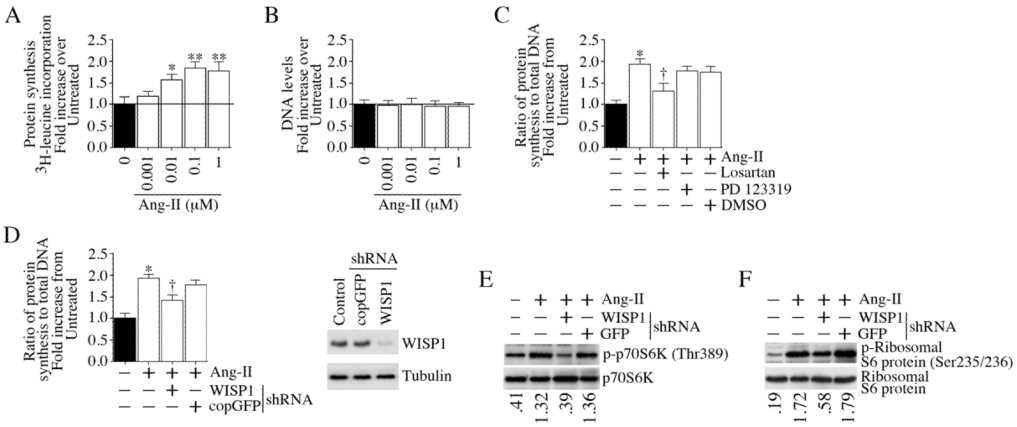

Fig. 1. Ang-II induces cardiomyocyte hypertrophy in part via WISP1

A, Ang-II enhances protein synthesis. Quiescent NRCM were treated with indicated concentrations of Ang-II for 48 h. [^{3}H]leucine (0.5 μCi) was added for an additional 6 h, and leucine incorporation was determined. **P* < 0.05, ** *P* < 0.01 *versus* untreated (n=6). *B*, Ang-II had no effect on DNA synthesis. Quiescent NRCM were treated with Ang-II (10^{-7} M for 48 h) and total DNA content was quantified. *C*, Ang-II induces cardiomyocyte hypertrophy via AT1. Quiescent NRCM were treated with the AT1 antagonist Losartan (10 μM for 1 h), AT2 antagonist PD (10 μM for 1 h), or their solvent control DMSO for 1 h prior to Ang-II addition (10^{-7} M for 48 h). Protein synthesis and DNA levels were quantified as *A* and *B*. **P* < 0.001 *versus* untreated, †*P* < 0.05 *versus* Ang-II+DMSO (n=6). *D*, WISP1 knockdown blunts Ang-II induced cardiomyocyte hypertrophy. NRCM infected with lentivirus expressing WISP1 or control GFP shRNA for 48 h were incubated with Ang-II (10^{-7} M for 48 h). Cardiomyocyte hypertrophy was determined as in *C*. **P* < 0.01 *versus* untreated, †*P* < 0.05 *versus* Ang-II (n=6). Knockdown of WISP1 was confirmed by immunoblotting (right hand panel). *E*, Ang-II induced p70 S6 kinase activation is blunted by WISP1 knockdown. Quiescent NRCM were treated with Ang-II (10^{-7} M) for 30 min. Cell lysates were analyzed by immunoblotting with phospho-p70 S6K(Thr389) antibody. The *lower panel* shows total p70 S6 kinase levels. Ratio of p-p70S6K to corresponding total p70S6K is shown at the bottom. *F*, Ang-II induced ribosomal S6 protein activation. Quiescent NRCM were treated with Ang-II (10^{-7} M) for 30 min. Cell extracts were prepared and analyzed by immunoblotting for total and phospho-ribosomal S6 protein. Ratio of p-Ribosomal S6 protein to total Ribosomal S6 protein is shown at the bottom. Blots in *E* and *F* are representatives from 3 independent experiments.

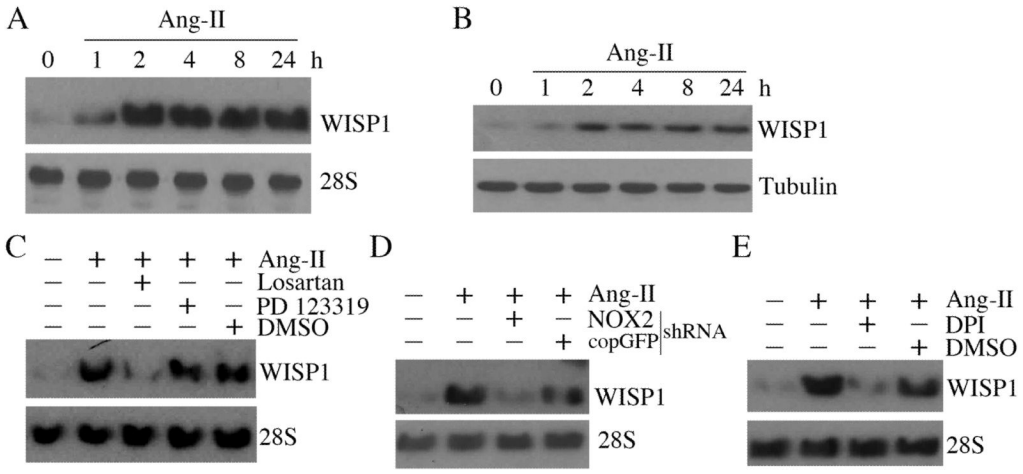

Fig. 2. Angiotensin-II induces WISP1 expression via AT1 and NOX2

A, Time-dependent upregulation of Ang-II mediated WISP1 mRNA expression in NRCM. Quiescent NRCM were treated with Ang-II (10^{-7} M), and 20 μg total RNA, isolated at the indicated time points, analyzed for WISP1 and 28S rRNA (control) expression by Northern blotting *B*, Ang-II induces WISP1 protein expression. NRCM treated as in *A* were analyzed for WISP1 protein expression by Western blotting. Tubulin served as a loading control (n=3). *C*, Ang-II induces WISP1 mRNA expression in AT1-dependent manner. Quiescent NRCM were treated with the AT1 antagonist Losartan (10 μM for 1 h), AT2 antagonist PD (10 μM for 1 h), or their solvent control DMSO for 1 h prior to Ang-II addition (10^{-7} M for 2 h). WISP1 mRNA expression was analyzed as in *A. D*, NOX2 knockdown blunts Ang-II-mediated WISP1 mRNA expression. NRCM were transduced with NOX2 or GFP shRNA by lentiviral transduction for 48 h and then incubated with to Ang-II (10^{-7} M) for 2 h. WISP1 mRNA expression was analyzed as in *A. E*, The NADPH oxidase inhibitor DPI blocks Ang-II –induced WISP1 mRNA expression. Quiescent NRCM were treated with DPI (10 μM in DMSO for 30 min) prior to Ang-II addition (10^{-7} M for 2 h). WISP1 mRNA expression was analyzed as in *A*. All blots are representatives from three independent experiments.

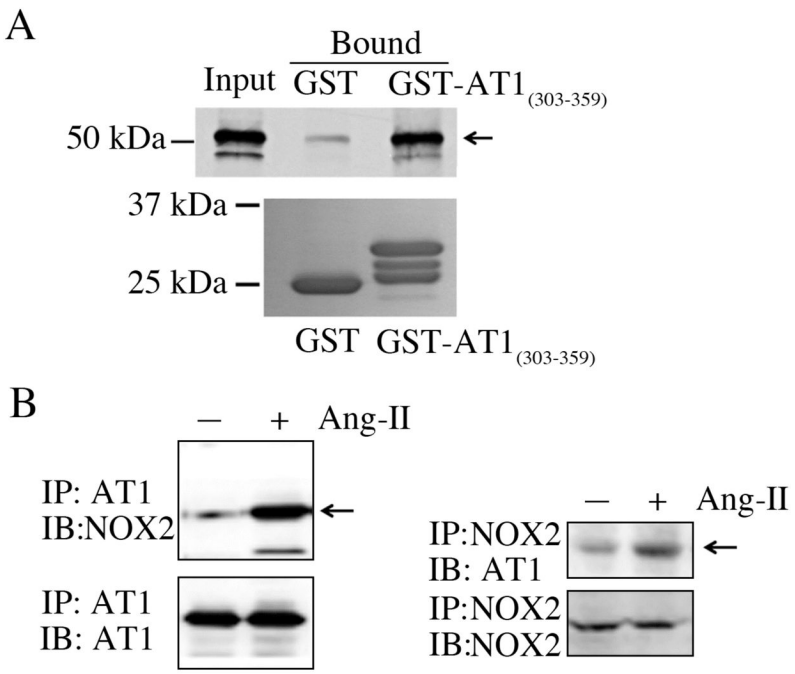

Fig. 3. Ang-II increases the physical association between AT1 and NOX2 *in vivo*

A, NOX2 binds the C-terminal domain of AT1 *in vitro*. An *in vitro* pulldown assay was carried out between GSH-sepharose loaded with either GST, or a fusion of GST and the cytoplasmic domain of AT1 (residues 303–359), and *in vitro* transcribed/translated, ^{35}S-methionine labeled human NOX2. Upper panel input (2% of total) and bound NOX2 (10% of reaction) analyzed by SDS-PAGE and fluorography. Lower panel, equal aliquots of the loaded GSH-sepharose were analyzed by SDS-PAGE and GelCode Blue staining to show equivalent protein loading. *B*, AT1 and NOX2 association *in vivo* is increased by Ang-II. Quiescent NRCM were treated with or without Ang-II (10^{-7}M for 15 min), and reciprocal immunoprecipitation (IP) and immunoblotting (IB) were performed with antibodies to AT1 and NOX2 as detailed in ‘Materials and Methods’ All blots are representatives from three independent experiments.

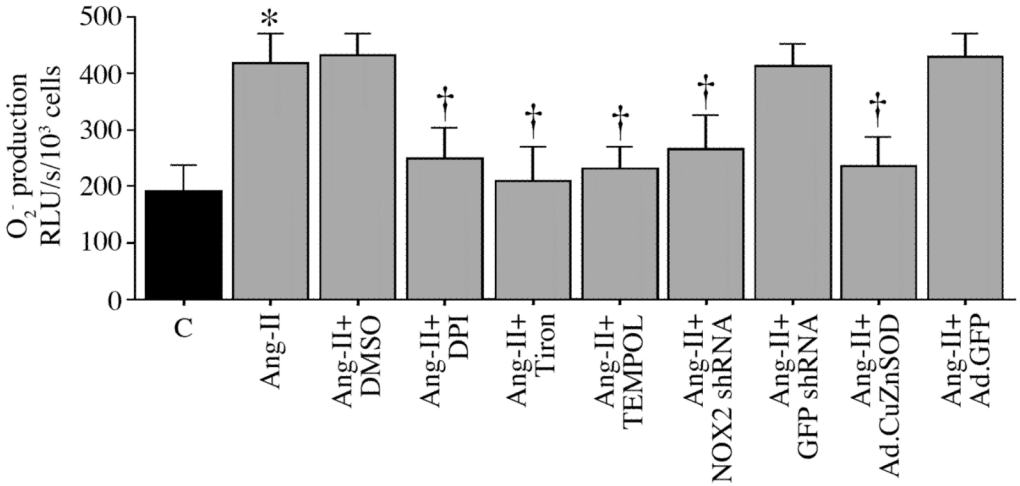

Fig. 4. Angiotensin-II stimulates superoxide generation

NRCM were incubated with Lucigenin (5×10^{-6} M) and ANG II (10^{-7} M) or ANG II + Tiron, Ang-II + DPI, or Ang-II+TEMPOL. Studies were also performed using NRCM infected with Ad.CuZnSOD (100 moi for 24 h) or lentivirus expressing NOX2-specific shRNA (48 h) prior to Ang-II addition. Superoxide (O_2^-) production was quantified using a lucigenin-enhanced chemiluminescence assay as detailed in Materials and Methods. Luminescence was measured for 10 s with a delay of 5 s. After subtracting background luminescence, results are expressed as RLU/second/1000 cells.

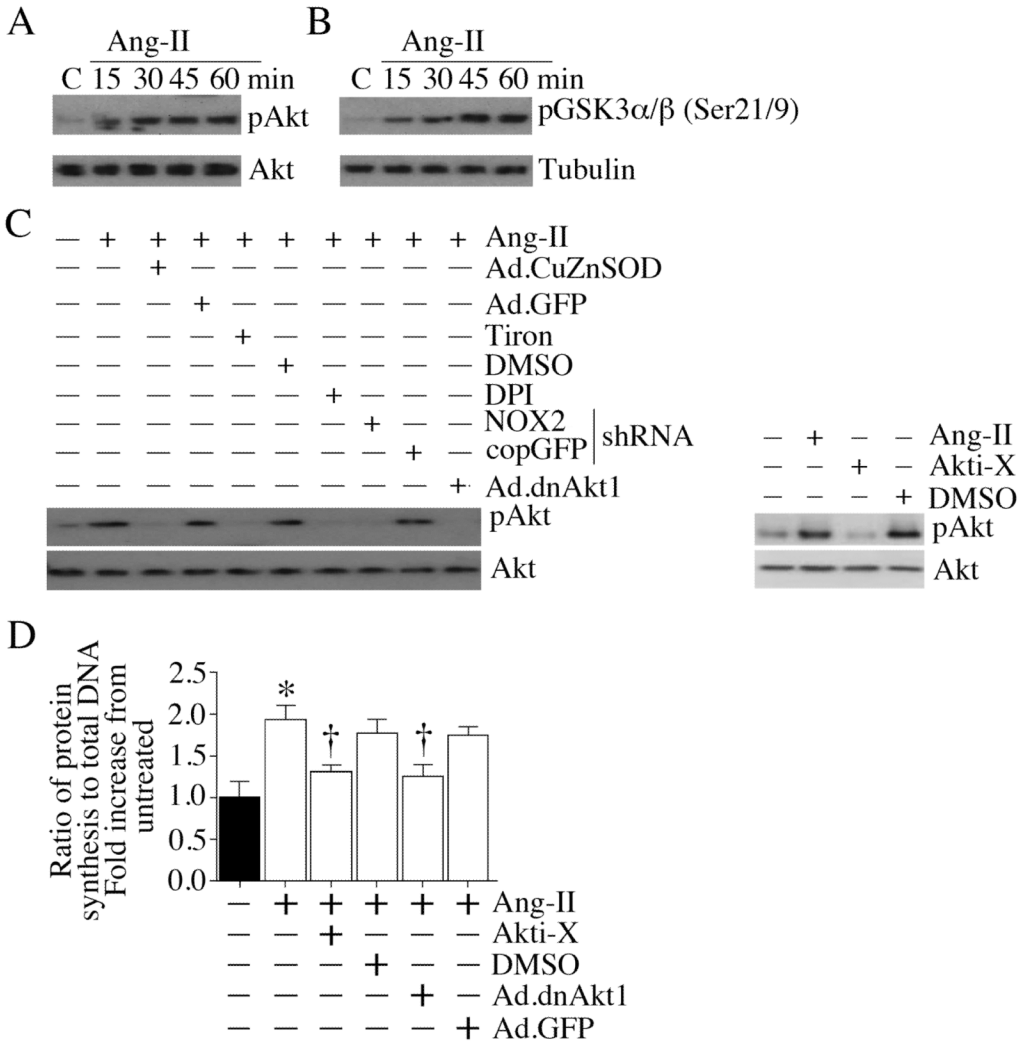

Fig. 5. Angiotensin-II induces cardiomyocyte hypertrophy via Akt1

A, Ang-II induces Akt phosphorylation. Quiescent NRCM were incubated with Ang-II (10^{-7}M). At the indicated time periods, cleared cell lysates were analyzed for total and phospho-Akt (Ser473) levels by immunoblotting (n=3). *B*, Ang-II stimulates Akt kinase activity. NRCM treated as in A were analyzed for Akt kinase activity using GSK-3a/β as a substrate (n=3). *C*, Ang-II induces Akt activation via NOX2 and superoxide. NRCM were treated with Tiron (5 mM for 1 h), DPI (10 μM in DMSO for 30 min), or transduced with Ad.CuZnSOD, Ad.dnAkt1, Akti-X (right hand panel) or infected with lentiviral NOX2 shRNA prior to Ang-II addition (10^{-7} M for 60 min). Phospho-Akt (Ser473) levels were analyzed as in *A* (n=3). D, Angiotensin-II induces cardiomyocyte hypertrophy via Akt1. Quiescent NRCM were treated with Akti-X (2.5 μM in water for 1 h) prior to Ang-II addition (10^{-7} M for 48 h). Protein synthesis and DNA levels were quantified as 1*A* and 1*B*. **P* < 0.001 *versus* untreated, †*P* < 0.05 *versus* Ang-II (n=6).

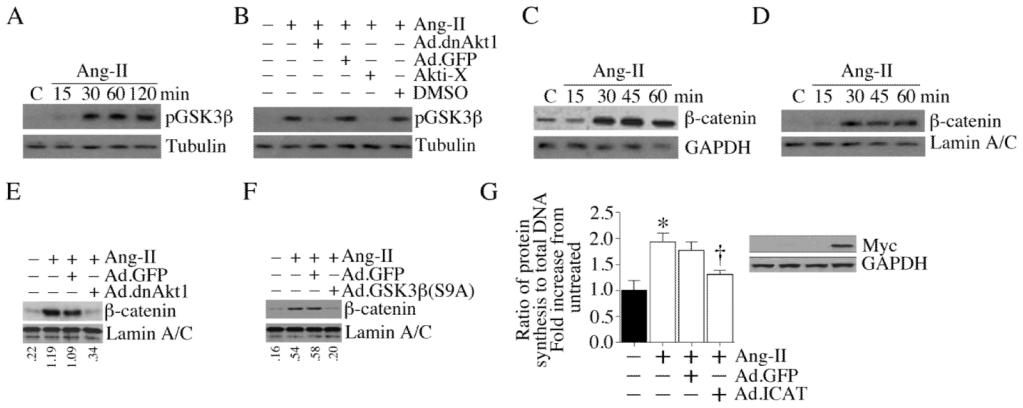

Fig. 6. Angiotensin-II induces Akt/GSK3β-dependent β-catenin nuclear translocation

A, Ang-II induces GSK3β phosphorylation. NRCM treated as in 5*A* were analyzed for phospho-GSK3β (Ser9) level by immunoblotting (n=3). *B*, Ang-II induces GSK3β phosphorylation via Akt. NRCM infected with A.dnAkt1 (50 moi for 24 h) or treated with Akti-X (2.5 μM in water for 1 h) prior to Ang-II addition (10^{-7}M for 60 min). Phospho-GSK3β (Ser9) levels were analyzed by immunoblotting (n=3). *C*, Ang-II induces time-dependent β-catenin accumulation in cytoplasm. Quiescent NRCM treated with Ang-II (10^{-7}M) were analyzed by immunoblotting for β-catenin levels in cytoplasm. GAPDH served as a loading control (n=3). *D*, Ang-II induces β-catenin accumulation in the nucleus. NRCM were treated as in *C*, and were analyzed for β-catenin levels in nuclear extracts by immunoblotting. Lamin A/C served as a loading control (n=3). *E*, Ang-II induces β-catenin nuclear translocation is Akt dependent. NRCM infected with Ad.dnAkt1 (50 moi for 24 h) were treated with Ang-II (10^{-7}M for 60 min). β-catenin levels in nuclear extracts were analyzed as in *D* (n=3). *F*, Forced expression of phosphorylation-deficient GSK3β inhibits Ang-II-induced β-catenin nuclear translocation. NRCM were infected with Ad.GSK3β(S9A) (50 moi for 24 h) prior to Ang-II treatment (10^{-7}M for 60 min). β-catenin levels in nuclear extracts were analyzed as in *D* (n=3). *G*, Forced expression of ICAT inhibits Ang-II-mediated cardiomyocyte hypertrophy. NRCM were infected with Ad.ICAT (50 moi for 24 h) prior to Ang-II addition (10^{-7} M for 48 h). Protein synthesis and DNA levels were quantified as 1*A* and 1*B*. Myc expression from ICAT was confirmed by immunoblotting using cytoplasmic extracts, and is shown on the top. GAPDH served as a loading control. *$P < 0.001$ *versus* untreated, †$P < 0.05$ *versus* Ang-II (n=6).

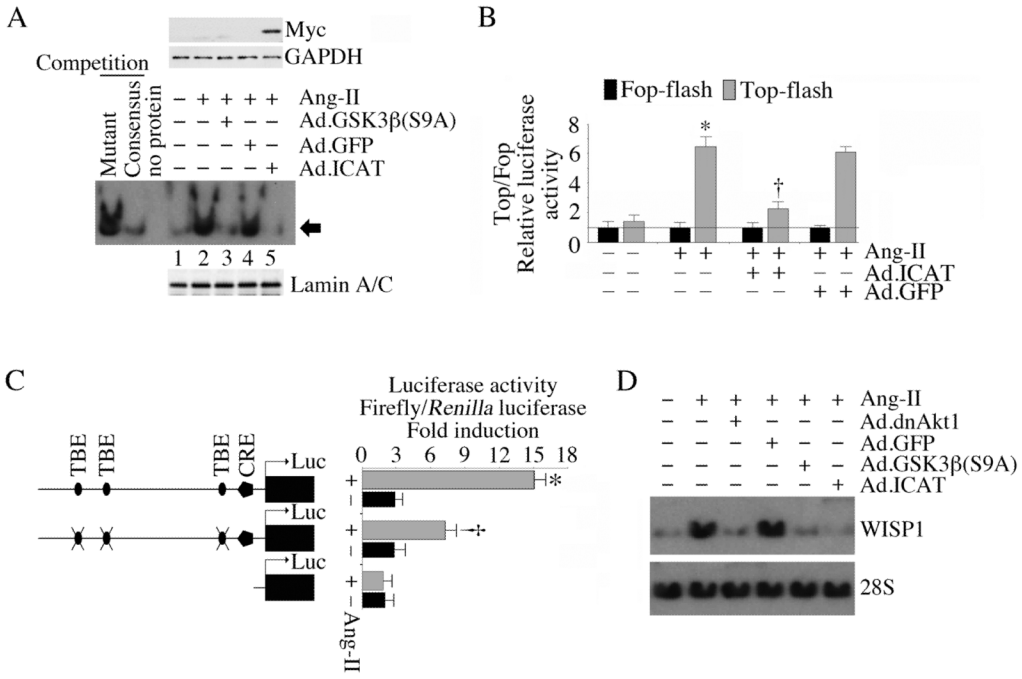

Fig. 7. Ang-II induces WISP1 expression via TCF-LEF

A, Ang-II induces TCF/LEF DNA binding activity via GSK3β and β-catenin. NRCM were infected with adenovirus (50 moi for 24 h) expressing GSK3β(S9A) or ICAT, and then incubated with Ang-II (10^{-7}M for 2 h). Ad.GFP served as a control. TCF-LEF activation was analyzed by EMSA using nuclear protein extracts (n=3). Specificity of DNA binding, indicated by an arrow, was verified in competition experiments as described in 'Materials and Methods'. Unincorporated labeled probe that runs to the bottom of gel is not shown. Lamin A/C served as a loading control, and is shown in the bottom panel. Myc expression from ICAT was confirmed by immunoblotting using cytoplasmic extracts, and is shown on the top. GAPDH served as a loading control. *B*, Ang-II stimulates TCF-LEF-dependent reporter gene activation. NRCM transfected with the Top flash vector (2 μg) for 48 h were treated or not with Ang-II. The FOP-flash vector with mutated LEF sites was used as a negative control. NRCM were co-transfected with *Renilla* luciferase vector to control for transfection efficiency (100 ng, pRL-TK). Firefly and *Renilla* luciferase activities were analyzed after 12 h. **P* < 0.001 *versus* untreated; †*P* < 0.001 *versus* Ang-II or Ang-II +Ad.GFP (n=6). *C*, Ang-II induced WISP1 promoter-reporter activation is TCF-LEF dependent. NRCM transfected with wild type or TBE (TCF-LEF binding element) mutated WISP1 promoter reporter vector (3 μg, 48 h) were incubated with Ang-II (10^{-7}M for 12 h). The cells were co-transfected with pRL-TK (100 ng), and processed as in *B*. **P* < 0.001 *versus* untreated; †*P* < 0.01 *versus* Ang-II or Ang-II+Ad.GFP (n=6). *D*, Ang-II induces WISP1 mRNA expression via TCF-LEF. NRCM were infected (50 moi for 24 h) with Ad.dnAkt1, Ad.GSK3β(S9A), or Ad.ICAT and then treated with Ang-II (10^{-7}M for 2 h). WISP1 mRNA expression was analyzed as in 2*A* (n=3).

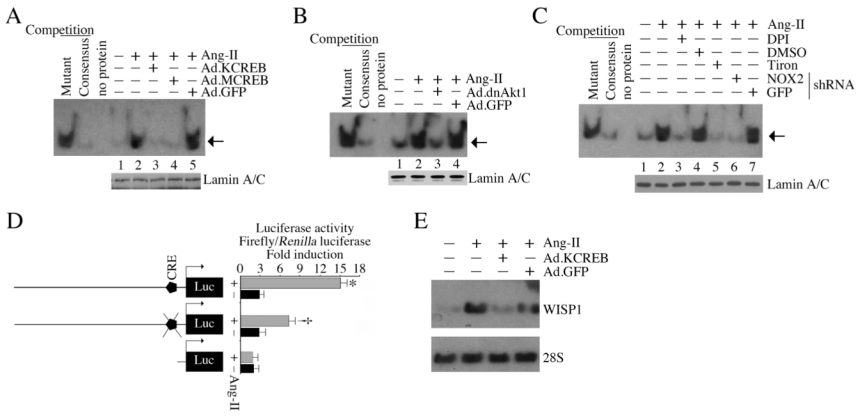

Fig. 8. Angiotensin-II induces WISP1 expression in part via CREB activation

A, Ang-II induces CREB DNA binding activity. NRCM were infected with adenovirus (50 moi for 24 h) expressing mutant CREB (KCREB or MCREB; 50 moi for 24 h) prior to Ang-II addition (10^{-7}M for 2 h). Ad.GFP served as a control. CREB DNA binding activity was analyzed by EMSA as described in Materials and Methods (n=3). Lamin A/C served as a loading control, and is shown in the bottom panel. *B*, Ang-II induced CREB activation is Akt dependent. NRCM infected with Ad.dnAkt1 (50 moi for 24 h) were incubated with Ang-II (10^{-7}M for 2 h), and then processed for CREB DNA binding activity as in *A* (n=3). *C*, Ang-II induced CREB activation is NOX2 and superoxide dependent. NRCM treated as in 5*C*, but for 2h, were processed for CREB DNA binding activity as in *A* (n=3). *D*, Ang-II induced WISP1 promoter-reporter activation is CREB dependent. NRCM transfected with the wild type or CREB mutated WISP1 promoter reporter vector (3 μg, 48 h) were incubated with Ang-II (10^{-7}M for 12 h). The cells were co-transfected with pRL-TK (100 ng), and processed as in *6C*. **P* < 0.001 *versus* untreated; †*P* < 0.01 *versus* Ang-II or Ang-II +Ad.GFP (n=6). *E*, Ang-II induces WISP1 mRNA expression in part via CREB. NRCM were infected with Ad.KCREB (50 moi for 24 h) prior to Ang-II addition (10^{-7}M for 2 h). WISP1 mRNA expression was analyzed as in 2*A* (n=3).

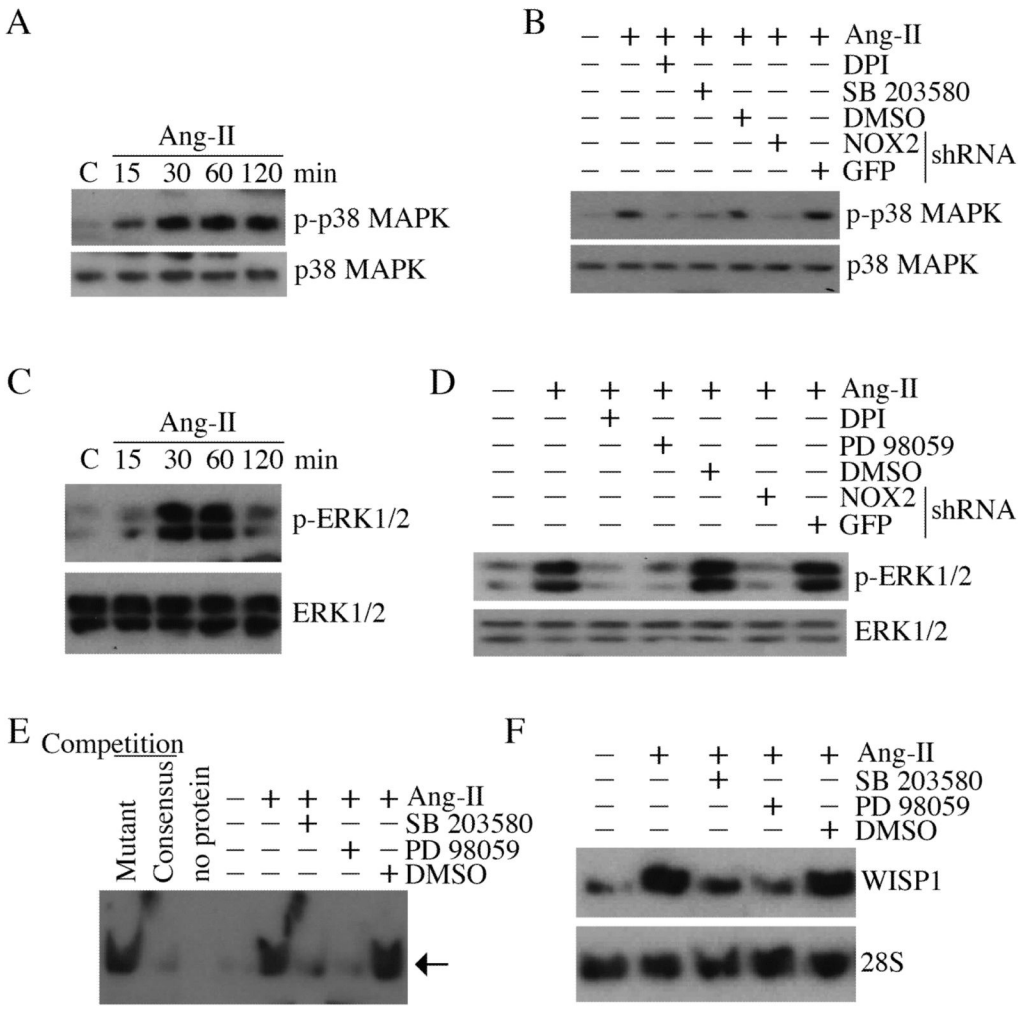

Fig. 9. Angiotensin-II induces CREB activation via p38 MAPK and ERK1/2

A, Ang-II activates p38MAPK. Quiescent NRCM were treated with Ang-II (10^{-7}M) for the indicated times. Cleared cell lysates were analyzed for total and phospho-p38 MAPK levels by immunoblotting (n=3). *B*, Ang-II induced p38MAPK activation is dependent on NOX2. Quiescent NRCM treated with DPI (10 μM in DMSO for 30 min) or SB 203580 (SB; 1 μM in DMSO for 30 min) or NRCM infected with lentivirus expressing NOX2 shRNA prior to Ang-II addition (10^{-7} M for 1 h) were analyzed for total and phospho-p38 MAPK levels by immunoblotting (n=3). *C*, Ang-II activates ERK1/2. Quiescent NRCM treated as in *A* were analyzed for total and phospho-ERK1/2 levels by immunoblotting (n=3). *D*, Ang-II induced ERK1/2 activation is dependent on NOX2. Quiescent NRCM treated with PD 98059 (PD; 10 μm in DMSO for 1 h) and as in *B* were analyzed for total and phospho-ERK1/2 levels by immunoblotting (n=3). *E*, Inhibition of p38MAPK and ERK1/2 blunts Ang-II-mediated CREB activation. Quiescent NRCM treated with SB or PD prior to Ang-II (10^{-7}M for 2 h) were analyzed for CREB DNA binding activity by EMSA (n=3). *F*, Inhibition of p38MAPK and ERK1/2 blunts Ang-II-induced WISP1 mRNA expression. Quiescent NRCM treated as in *E* were analyzed for WISP1 mRNA expression by Northern blotting (n=3).

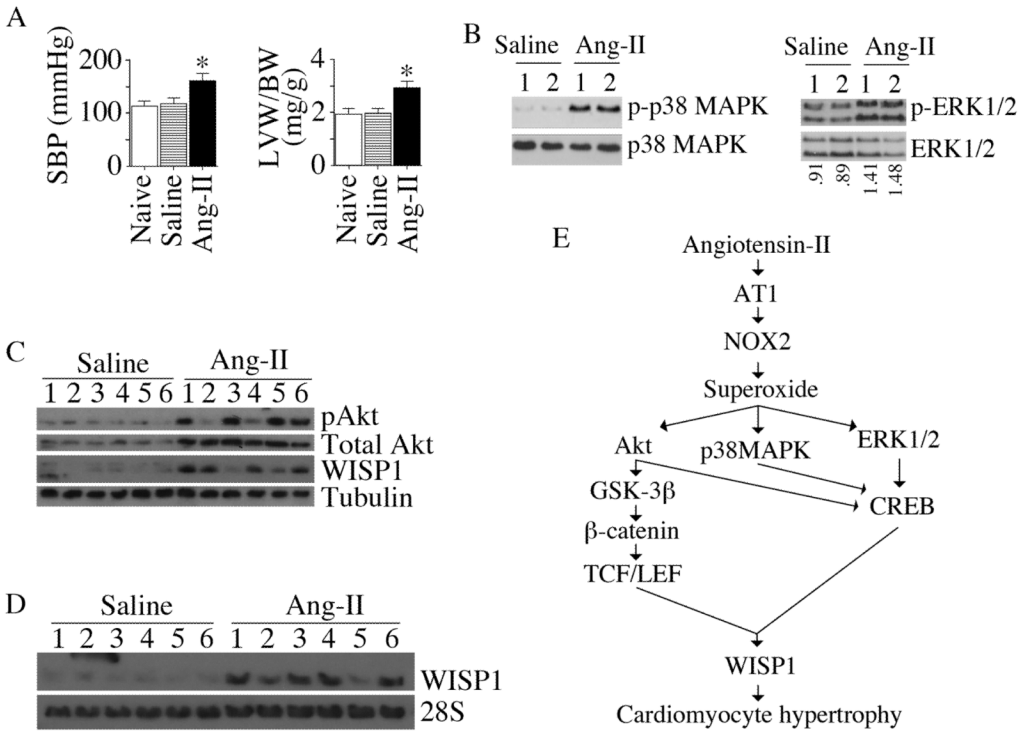

Fig. 10. Ang-II induced myocardial hypertrophy *in vivo* is associated with WISP1 expression

A, Ang-II infusion increases systolic blood pressure (SBP), and myocardial hypertrophy. Male SD rats were infused with Ang-II via miniosmotic pumps for 7 days. Saline served as a control. SBP was measured using tail-cuff method (left hand panel) in conscious animals before sacrifice. Body weight (BW) and left ventricular weight (LVW) were noted (right hand panel). * $P < 0.05$ vs. naïve or saline-infusion. *B*, Ang-II infusion activates p38 MAPK and ERK1/2. LV tissues from two animals/per group were analyzed as in 8*A* and 8*B* for total and activated p38 MAPK and ERK1/2 by immunoblotting. *B*, right hand panel, ratio of p-ERK1/2 to total ERK1/2 is shown at the bottom. *C*, Ang-II infusion induces WISP1 mRNA expression. LV tissues from animals infused with Ang-II or saline were analyzed for WISP1 mRNA expression by Northern blotting. 28S rRNA served as a loading control. Numbers at the top denote individual animal numbers (n=6/group). *D*, Ang-II infusion enhances WISP1 and Akt protein levels. LV tissue from animals infused with Ang-II or saline were analyzed for WISP1, total Akt and phospho-Akt protein levels by immunoblotting. Tubulin served as a loading control. Numbers at the top denote individual animal numbers (n=6/group). *E*, Schema showing the signal transduction pathway involved in Ang-II-mediated WISP1 induction and cardiomyocyte hypertrophy.