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CIKS (Act1 or TRAF3IP2) mediates Angiotensin-II-induced Interleukin-18 Expression, and Nox2-dependent Cardiomyocyte Hypertrophy

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Abstract

Chronic elevation of angiotensin (Ang)-II can lead to myocardial inflammation, hypertrophy and cardiac failure. The adaptor molecule CIKS (connection to IKK and SAPK/JNK) activates the I κ B kinase/nuclear factor (NF)- κ B and JNK/activator protein (AP)-1 pathways in autoimmune and inflammatory diseases. Since Ang-II is a potent activator of NF- κ B and AP-1, we investigated whether CIKS is critical in Ang-II-mediated cardiac hypertrophy. Here we report that Ang-II induced CIKS mRNA and protein expression, CIKS binding to IKK and JNK perhaps functioning as a scaffold protein, CIKS-dependent IKK/NF- κ B and JNK/AP-1 activation, p65 and c-Jun phosphorylation and nuclear translocation, NF- κ B- and AP-1-dependent IL-18 and MMP-9 induction, and hypertrophy of adult cardiomyocytes isolated from WT, but not CIKS-null mice. These results were recapitulated in WT-cardiomyocytes following CIKS knockdown. Infusion of Ang-II for 7 days induced cardiac hypertrophy, increased collagen content, and upregulated CIKS mRNA and protein expression in WT mice, whereas cardiac hypertrophy and collagen deposition were markedly attenuated in the CIKS-null mice, despite a similar increase in systolic blood pressure and DPI-inhibitable superoxide generation in both types of animals. Further, Ang-II-induced IKK/p65 and JNK/c-Jun phosphorylation, NF- κ B and AP-1 activation, and IL-18 and MMP-9 expression were also markedly attenuated in CIKS-null mice. These results demonstrate that CIKS is critical in Ang-II-induced cardiomyocyte hypertrophy and fibrosis, and that CIKS is an important intermediate in Ang-II induced redox signaling. CIKS is a potential therapeutic target in cardiac hypertrophy, fibrosis, and congestive heart failure.

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Disclosures

None declared

Keywords

RAAS; NADPH oxidase; Act1; TRAF3IP2; fibrosis; cardiac hypertrophy

1. Introduction

Angiotensin-II (Ang-II) is a major component of the renin-angiotensin-aldosterone system (RAAS) and an important mediator of vascular tone [1–4]. Chronic elevation of Ang-II contributes to a variety of pathological conditions including myocardial hypertrophy [1–4]. Angiotensin-II exerts its biological effects through the G-protein coupled angiotensin II type 1 (AT1) and type II (AT2) receptors. There is growing evidence however, that some of these pathways are G-protein-independent [42]. In the heart, the majority of the pathological effects of chronically elevated Ang-II are thought to be mediated through AT1.

We and others have reported that Ang-II exerts its pro-hypertrophic effects in both neonatal and adult cardiomyocytes via induction of diphenylene iodonium (DPI)-inhibitable oxidative stress [5]. DPI inhibits flavoprotein oxido-reductases, including the NOX family of NADPH oxidases, which contribute significantly to induced reactive oxygen species (ROS) generation in all major cardiac resident cells [6, 7]. Although cardiovascular tissues express a number of NOX isoforms, Nox2 and Nox4 predominate in the cardiomyocytes, and were shown to be critical in Ang-II signaling [8]. Several reports have indicated a significant role of Nox2 in AT1 signaling in cardiomyocytes, and in Ang-II-induced cardiac hypertrophy *in vivo* [8–11]. We recently confirmed those reports, and further demonstrated that Nox2 can associate physically with the cytoplasmic, carboxy terminal domain of AT1 [5]. Moreover, this interaction was enhanced in response to Ang-II *in vivo*, and knockdown of Nox2 blunted oxidative stress and the hypertrophic response in cardiomyocytes [5].

Ang-II is a powerful activator of oxidative stress-responsive transcription factors NF- κ B and AP-1 in cardiomyocytes *in vitro* and myocardium *in vivo* [12, 13]. Both NF- κ B and AP-1 are ubiquitously expressed and regulate various genes involved in apoptosis, cell survival and growth, and matrix degradation, all of which are involved in myocardial hypertrophy and failure [14]. Thus, either individually or in combination, AP-1 and NF- κ B regulate the transcription of a wide range of genes implicated in inflammation, and cellular injury, survival, and growth. Reports from several laboratories have shown clearly that both NF- κ B and AP-1 contribute to Ang-II-induced pathological hypertrophy [12, 13].

The adapter molecule CIKS (connection to I κ B kinase and c-Jun-N-terminal kinase), also known as Act1 or TRAF3IP2, has been shown to mediate the activation of both NF- κ B and AP-1 in an IKK and JNK-dependent manner respectively [15, 16]. CIKS is an adapter molecule that possesses ubiquitin ligase activity, and signals via both TRAF6-dependent and -independent mechanisms to induce NF- κ B and AP-1 responsive genes [17]. Its pathological role has been demonstrated in diverse autoimmune and inflammatory diseases. Since Ang-II-induced myocardial hypertrophy is associated with enhanced NF- κ B and AP-1 activation [12, 13], we hypothesized that CIKS is a critical mediator in this process. In addition, since the proinflammatory cytokine interleukin (IL)-18 is an NF- κ B and AP-1-responsive gene [18], and a potent growth factor for cardiomyocytes [19–22], we further hypothesized that Ang-II-induced myocardial hypertrophy is mediated by CIKS-dependent, IL-18 induction.

Our results demonstrate for the first time that Ang-II induces CIKS in cardiomyocytes via AT1 and Nox2 dependent ROS generation, and that CIKS knockdown or gene deletion blunts Ang-II-induced NF- κ B and AP-1 activation, IL-18 and MMP-9 induction, and cardiomyocyte hypertrophy both *in vitro* and *in vivo*. Further, CIKS gene deletion blunts

Ang-II induced cardiac fibrosis *in vivo*. These results indicate that CIKS is a critical mediator of Ang-II-induced cardiomyocyte hypertrophy and adverse remodeling, and an important intermediate in Ang-II induced redox signaling. Thus CIKS is a potential therapeutic target in cardiac hypertrophy, fibrosis, and congestive heart failure.

2. Materials and methods

2.1. Animals

This 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). All protocols were approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center, San Antonio and Tulane University, New Orleans, LA. The CIKS-null mice (C57Bl/6 background) used in this study were generated by Dr. Ulrich Siebenlist at the National Institute of Allergy and Infectious Diseases, and have been previously described [23]. All mice used in the study were male, ~3 months old, and weighed ~25 g. Absence of CIKS expression was confirmed by immunoblotting using left ventricular tissue (Supplementary Fig. S1-A) and isolated cardiomyocytes (Supplementary Fig. S1-B) from CIKS null mice selected at random. The CIKS-null mice exhibited no basal phenotypic abnormalities. Breeding, litter size and the sex ratio, growth, body and heart weights were comparable between CIKS-null and C57Bl/6 control mice.

2.2. Infusion of Angiotensin-II

Animals were trained for systolic blood pressure (SBP) measurement using a tail-cuff method without anesthesia (CODA Noninvasive Blood Pressure System, Kent Scientific, Torrington, CT) [5]. A subset of animals was infused with 1.5 µg/kg/min of Ang-II for one week *via* subcutaneously implanted (midscapular region) Alzet miniosmotic pumps (n=8/group) [5]. 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 N₂ for not more than 3 days prior to analysis. The third piece was embedded in OCT for histo-morphometric analysis. Tibial lengths were also recorded.

2.3. Echocardiography

Left ventricular function was analyzed by 2D echocardiography (Acuson 128XP/10) as described before [24]. We measure left ventricular wall thickness, end-diastolic dimension (LVDd) and fractional shortening (FS).

2.4. Hypertrophy of heart

Hypertrophy was characterized [5] by: (i) the ratio of heart weight to body weight, (ii) the diameter of cardiomyocytes in the region of the cell nucleus in H & E stained cryosections (100 cells/heart, 4 hearts/group, and 4 groups), (iii) phosphorylation of ribosomal S6 protein and p70 S6 kinase (p70S6K), and (iv) atrial natriuretic factor (ANF) mRNA expression as detailed in the Supplement.

2.5. Assessment of cardiac remodeling

Since collagen synthesis is a significant feature of pathological cardiac remodeling, we quantified fibrosis by picrosirius red staining. Sirius red F3BA dissolved in saturated picric acid stains collagens type I and III. In brief, 8 µM cryosections were submerged in 0.2 % phosphomolybden acid to clear cytoplasm, and then incubated with 0.1 % Sirius red F3BA

dissolved in saturated picric acid for 90 min. The slides were washed for 2 min in 0.01 N HCl, dehydrated and mounted. Digital photographs were acquired using Zeiss AXIO Imager.A2 and analyzed with NIH ImageJ software.

2.6. NADPH oxidase activity in vivo

DPI-inhibitable, NADPH-dependent, superoxide production was measured using left ventricular homogenates and lucigenin (5 μ M)-enhanced chemiluminescence (NADPH 300 μ M; 100 μ g protein; 37 $^{\circ}$ C), as described previously [5, 11]. The assays were performed at least 3 times.

2.7. Isolation of cardiomyocytes

Calcium-tolerant adult mouse cardiomyocytes (ACM) were isolated from WT and CIKS-null mice by a modified Langendorff perfusion and collagenase digestion technique, as previously described [5, 25]. The yield, shape, and viability of cardiomyocytes from wild-type and CIKS-null mice were similar (data not shown).

2.8. Adeno and lenti viral transduction

Cardiomyocytes were infected at ambient temperature with adenovirus in PBS at the indicated multiplicities of infection (MOI) as detailed in the Supplement. After 2 h, adenovirus was replaced with culture medium supplemented with 0.5% BSA. Assays were carried out 24 h later. Lentiviral infection was carried out for 48 h. The transfection efficiency with the adenovirus (*e.g.*, Ad.GFP) and lentiviral particles (copepod GFP [copGFP] or control) was near 100%, and infection with the viral vectors at the indicated MOI and for the duration of the study had no significant effect on cardiomyocyte shape, adherence, or viability (data not shown).

2.9. Hypertrophy in isolated cardiomyocytes

Cardiomyocyte hypertrophy *in vitro* was assessed [5, 19] by: (i) increased protein, but not DNA, synthesis, (ii) cell surface area, (iii) phosphorylation of ribosomal S6 protein and p70S6 kinase, and (iv) ANF mRNA expression, as detailed in the Supplement.

2.10. AT1/Nox2 binding interaction in vitro - GST pull-down assay

A plasmid containing the cDNA for mouse Nox2 was obtained from Origene Technologies, Rockville, MD (#MC204867). The Nox2 coding sequence was amplified by PCR with sequence-specific primers that also inserted a strong Kozak sequence at the 5' end, and cloned into the expression vector pcDNA3.1/Zeo(-) (Life Technologies). Orientation and identity were confirmed by nucleotide sequencing. The carboxy-terminal domain of mouse angiotensin II type 1 receptor (AT1 – amino acids 303 to 359) was prepared by PCR from the complete mouse AT1 cDNA (#MC201798, OriGene Technologies) and cloned in-frame with the GST coding sequence in pGEX-3X (GE Healthcare Life Sciences). Details are in the Supplement. The GST pull-down assays between GST-AT1 and Nox2 were carried out exactly as before for rat GST-AT1 and Nox2 [5].

2.11. AT1/Nox binding interaction in vivo - immunoprecipitation and immunoblotting

For immunoprecipitation, equal amounts of membrane extracts were incubated overnight at 4 $^{\circ}$ C with specific antibodies immobilized on agarose beads under slow rotation. After washing 3 times in a buffer containing 50 mM Tris-Cl, 150 mM NaCl, and 0.1% Nonidet P-40, the bound proteins were eluted by boiling in SDS sample buffer for subsequent SDS-PAGE and immunoblotting. Antibodies against AT1 and Nox2, and immunoblotting and immunoprecipitation are performed as previously described [5.]

2.12. Detection of intracellular ROS

Intracellular ROS levels were determined by oxidation of the cell-permeable, redox-sensitive fluorophore, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR) into fluorescent 2',7'-dichlorofluorescein (DCF). DCF fluorescence is thought to be a measure of a number of ROS, particularly hydrogen peroxide, peroxynitrite and hydroxyl radicals [5, 25, 26]. Prior to Ang-II addition, cardiomyocytes were incubated in medium containing 30 μ M non-fluorescent DCFH-DA for 1 h to obtain stable intracellular levels of the probe. Similar concentrations were maintained during Ang-II treatment. The intensity of DCF fluorescence in the cells is proportional to the intracellular levels of ROS. Image acquisition and analysis were performed using a Plan-Apo $\times 60$ oil immersion objective mounted on an inverted Olympus microscope with an Olympus LSM GB200 confocal imaging system attached. Excitation of dyes was carried out using the 488 nm line of a 15-milliwatt argon ion laser. All images were collected at room temperature. Mean fluorescence intensity was calculated by averaging area intensities from a number of outlined cells. For each condition described, six images of different cells were collected, and experiments were repeated at least three times.

2.13. Transcription factor activation

NF- κ B DNA binding activity in nuclear protein extracts was analyzed by electrophoretic mobility shift assay (EMSA) [5, 19, 25, 27] using the double-stranded sequence from inducible *III8* promoter (exon 1; sense, 5'-CCCTGATAAAATGTAGATTCCTATTATAC-3'), the κ B site from the *MMP9* promoter (sense, 5'-CTGCGGAAGACAGGGGGTTGCCCA GTGGAATTCCC-3') or a consensus double stranded κ B sequence (sense, 5'-AGTTGAGGGGACTTTCCCA GGC-3'; Santa Cruz Biotechnology, Inc.). Cytoplasmic and nuclear phospho-p65 (Ser536) levels were analyzed by immunoblotting. Similarly, AP-1 activation was analyzed by EMSA using *III8* (exon 1; ATCTCCTCTTCTGAATCAGCTCTT CCACCAGCA) and *MMP9* (sense, 5'-CTGACCCCTGAGTCAGCACTT-3') promoter-specific, and consensus (5'-CGCTTGATGACTCAGCCGAA-3'; Santa Cruz Biotechnology, Inc.) double-stranded oligonucleotides. Cytoplasmic and nuclear phospho-c-Jun (Ser63) levels were analyzed by immunoblotting.

2.14. mRNA expression

IL-18 and MMP-9 expression levels were analyzed by Northern blotting or real time quantitative PCR (RT-qPCR) [27]. All data were normalized to corresponding 28S rRNA or actin mRNA, and expressed as the fold difference in gene expression in Ang-II-treated cardiomyocytes relative to untreated controls. ANF mRNA expression was also analyzed by Northern blotting. Details are provided in the Supplement.

2.15. Immunoblotting

Extraction of whole cell lysates, membrane, cytoplasmic and nuclear protein extracts, immunoprecipitation, immunoblotting, chemiluminescence, and densitometry were performed as detailed in the Supplement.

2.16. Kinase activity

IKK activity in cardiomyocytes treated with Ang-II (10^{-7} M for 15 min) was determined by an *in vitro* kinase assay using glutathione *S*-transferase-I κ B fusion protein as the substrate [25]. JNK activity was measured using a commercially available kit (Stress-activated protein kinase/JNK assay kit, # 9810, Cell Signaling Technology). A c-Jun fusion protein (glutathione *S*-transferase fused to the N terminus of c-Jun, amino acids 1–89) was used to pull down JNK enzyme from cell extracts, which in the presence of kinase buffer and ATP

phosphorylates c-Jun (1–89). Phosphorylated c-Jun was detected by immunoblotting using anti-phospho-c-Jun (Ser63) antibody.

2.17. Cell death analysis

To determine whether transduction of viral vectors, pharmacological inhibitors or over-expression of mutant proteins affects cell viability, cell death was analyzed using the Cell Death Detection ELISA^{PLUS}, trypan blue dye exclusion, and microscopic visualization of cell shape and for cells floating in the media.

2.18. 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. Ang-II induces cardiomyocyte hypertrophy in vitro via CIKS

In a previous study, we showed that Ang-II potently induces hypertrophy in cardiomyocytes (ACM) isolated from adult WT mice, through a mechanism mediated by the AT1 but not AT2 Ang-II receptor [5]. Since both NF- κ B and AP-1 are known to be induced by Ang-II and contribute to Ang-II induced cardiomyocyte hypertrophy, and both can be activated through CIKS, we investigated the effect of CIKS knockdown on Ang-II-induced hypertrophy. Confirming our earlier results [5], Ang-II induced ACM hypertrophy as evidenced by increased protein, but not DNA, synthesis and cell surface area (Supplementary Figure S2-A). In addition, Ang-II induced hypertrophy-associated markers and pre-treatment with the AT1 antagonist, but not AT2 antagonist, markedly attenuated ACM hypertrophy (Supplementary Fig. S2-B and C). Notably, transduction of ACM with lentivirus for CIKS shRNA significantly reduced Ang-II induced cardiomyocyte growth (Fig. 1A, CIKS knockdown confirmed by immunoblotting and is shown on the right). Consistent with these results, Ang-II failed to induce hypertrophic growth in ACM isolated from CIKS-null mice (Fig. 1B, ³H-leucine incorporation; Fig. 1C, cell surface area), despite similar levels of AT1 expression (Fig. 1B, inset). These results indicate that Ang-II induction of cardiomyocyte hypertrophy is mediated by AT1 and in part by CIKS (Fig. 1).

3.2. Ang-II induces CIKS in cardiomyocytes via AT1 and ROS

Since CIKS plays a critical role in Ang-II induced hypertrophy (Fig. 1), we next investigated whether Ang-II stimulates CIKS expression. Indeed, Ang-II (10^{-7} M) induced a rapid increase in CIKS mRNA expression that declined with time, but was still significantly elevated at 24 h (Fig. 2A). Further, Ang-II induced an increase in CIKS protein level that peaked between 1 – 2 h. This also decayed, but at 24 h was still elevated compared with untreated controls (Fig. 2B). As we have shown previously [5], Ang-II also induced the generation of ROS in ACM (Fig. 2C) that was significantly inhibited by losartan and by the flavoprotein inhibitor DPI. Similarly, the induction of CIKS protein was also inhibited by losartan and DPI (Fig. 2D). Thus Ang-II induces CIKS expression in cultured ACM via AT1-dependent, DPI-inhibitable ROS generation (Fig. 2).

3.3. Ang-II induces CIKS expression via Nox2 and Rac1

Previously we showed that Ang-II induces the pro-hypertrophic factor WISP1 in ACM through DPI-inhibitable ROS generation that was dependent on the NADPH oxidase Nox2 [5]. Further, Nox2 interacted with the C-terminal cytoplasmic domain of AT1 *in vitro*, and AT1-Nox2 association *in vivo* was enhanced by Ang-II treatment [5]. To determine if this is

also the case for the induction of CIKS, Nox2 expression was targeted by lentivirus shRNA. Results show that Nox2 knockdown markedly inhibited Ang-II-induced CIKS protein expression (Fig. 3A; knockdown of Nox2 is shown on the right) and ROS generation (Supplementary Fig. S3). Since Rac1, a member of the Rho family of GTPases, is critical for the activation of the Nox2 NADPH oxidase complex and cardiomyocyte hypertrophy [28–30], we next investigated its role in Ang-II-induced CIKS expression. Forced expression of a dominant-negative mutant of Rac1 (N17rac1) by adenoviral transduction, or pre-treatment with the Rac1-specific small molecule inhibitor NSC23766 (NSC), significantly inhibited Ang-II-mediated ROS generation (Fig 3B) and CIKS protein expression (Fig 3C). Importantly, N17rac1 over-expression and Nox2 knockdown blunted Ang-II induced cardiomyocyte hypertrophy (Fig 3D). We also carried out a preliminary investigation on the subcellular localization of CIKS. Although it is known to be a cytoplasmic protein that interacts with the cell surface receptors such as IL-17RA, analysis of the amino acid sequence of mouse CIKS (NM_134000) using online prediction algorithms WoLF PSORT II (www.wolfpsort.org) and PSORT II (www.psort.hgc.jp/form2.html) suggest that it may also be localized to the nucleus. Of note, we identified a putative nuclear localization (NLS) signal in CIKS that is conserved across the species, and this sequence is very similar to the classical NLS of SV40 Large T-antigen (Table 1). In fact, immunoblot analysis demonstrated the presence of CIKS in both cytoplasmic and nuclear fractions of ACM and levels in both fractions were increased by Ang-II (Fig. 3E). Although the role of CIKS in the nucleus is unclear at the moment and requires further investigation, our results indicate that Ang-II induces CIKS expression via Nox2/Rac1-dependent ROS generation, and the increased CIKS protein is not confined to the cytoplasmic compartment (Fig. 3).

3.4. Ang-II activates NF- κ B via CIKS in ACM

Since CIKS is critical in Ang-II-mediated hypertrophic growth in adult cardiomyocytes (Fig. 1), and overexpression of phosphorylation-deficient I κ B in a cardiomyocyte-specific manner blunts Ang-II-induced cardiomyocyte hypertrophy both *in vitro* and *in vivo* [12], we next investigated whether Ang-II-induced NF- κ B activation is CIKS-dependent. Ang-II induced IKK β phosphorylation and kinase activity in ACM and these effects were markedly attenuated by losartan and DPI (Supplementary Fig. S4-A and B). Further, the Rac1 inhibitor NSC, over-expression of N17rac1, and Nox2 knockdown also inhibited IKK β phosphorylation (Fig. 4A). Importantly, knockdown of CIKS by shRNA blunted Ang-II-mediated IKK β activation (Fig. 4B). Since CIKS is reported to oligomerize with the IKK complex [31], we next investigated whether CIKS in fact complexes with IKK β in ACM. Using reciprocal IP/IB, we found that that CIKS does physically associate with IKK β in ACM, and their interaction appears to be enhanced by Ang-II exposure (Fig. 4C). No specific binding was detected when lysates were pre-adsorbed with specific antibodies or when control antibodies were used in immunoprecipitation (data not shown). Further, Ang-II enhanced time-dependent phosphorylation of p65, and this was markedly attenuated by CIKS knockdown (Fig. 4D). CIKS knockdown also blunted Ang-II mediated nuclear translocation of phosphorylated p65 (Fig. 4E). Using *III8* gene-specific NF- κ B oligonucleotides, we also found that over-expression of kinase-deficient (kd) kdIKK β or CIKS knockdown blunted NF- κ B DNA binding activity (Fig. 4F). Similar results were obtained with *MMP9* gene-specific and consensus NF- κ B probes (Supplementary Fig. S4-C). These results indicate that CIKS physically associates with IKK β , and mediates Ang-II-induced NF- κ B activation in ACM (Fig. 4).

3.5 Ang-II activates AP-1 via CIKS in ACM

Ang-II has also been reported to activate AP-1 in the heart [32]. Since CIKS activates JNK [15], we next investigated whether Ang-II activates the JNK/c-Jun pathway in ACM via CIKS. Ang-II-induced JNK phosphorylation and kinase activity, and these responses were

markedly inhibited by DPI (Supplementary Fig.S5-A and B). Further, JNK phosphorylation was inhibited by NSC, N17rac1 over-expression and Nox2 knockdown (Fig. 5A). Importantly, CIKS knockdown blunted JNK activation (Fig. 5B). In addition, using reciprocal IP/IB, we found that CIKS physically associates with JNK in ACM, and that Ang-II enhances this interaction (Fig. 5C). Since IKK γ has been shown to physically associate with JNK [33], and as CIKS binds IKK γ [15], we next investigated whether CIKS physically associates with IKK γ in the ACM. In IP/IB studies, we found that CIKS binds IKK γ (Fig. 5D, upper left panel), JNK binds IKK γ (Fig. 5D, lower left and upper right), and knockdown of IKK γ inhibits Ang-II-mediated c-Jun phosphorylation (Fig. 5D, lower right). Importantly, CIKS gene deletion blunts IKK γ physical association with JNK (Fig. 5E). Further, CIKS knockdown blunts Ang-II-mediated c-Jun phosphorylation (Fig. 5F, upper panel) and nuclear translocation (Fig. 5F, lower panel). Using an *III8* gene-specific AP-1 probe, we further show that CIKS knockdown, similar to overexpression of dnJNK1, blunts AP-1 DNA binding activity (Fig. 5G). Comparable results were obtained when *MMP9* gene-specific and consensus AP-1 oligonucleotides were used in the EMSA (Supplementary Fig. S5-C). These results indicate that CIKS may serve as a scaffold for JNK/IKK γ interactions, and their associations may mediate Ang-II-induced AP-1 activation in ACM (Fig. 5).

3.6 Ang-II induces IL-18 and MMP-9 expression in cultured ACM via CIKS

The proinflammatory cytokine IL-18 is a potent pro-growth factor for cardiomyocytes, inducing hypertrophic growth *in vitro* and myocardial hypertrophy *in vivo* [19–22]. Further, *III8* gene deletion markedly attenuates pressure overload-induced myocardial hypertrophy [34]. Since Ang-II induces CIKS expression and activates both NF- κ B and AP-1, we next investigated whether CIKS mediates Ang-II induction of IL-18. Ang-II induced time-dependent IL-18 mRNA (Fig. 6A, left panel) and protein expression (Fig. 6A, middle panel), and enhanced its secretion (Fig. 6A, right panel) from ACM. Importantly, knockdown of CIKS blunted Ang-II-induced IL-18 protein expression (Fig. 6B), as did forced expression of kdIKK β , dnp65 (Fig. 6C), dnJNK1 and dnc-Jun (Fig. 6D). Further, and supporting our earlier report [35], treatment with IL-18BP or IL-18 neutralizing antibodies blunted Ang-II-induced cardiomyocyte hypertrophy (Fig. 6E). Ang-II also induced the MMP-9 mRNA expression (Fig. 6F) and activation (Fig. 6G), effects blunted by CIKS knockdown. These results indicate that Ang-II induces NF- κ B- and AP-1-responsive IL-18 and MMP-9 expression via CIKS (Fig. 6).

3.7. Deficiency of CIKS abrogates Ang-II induced myocardial hypertrophy

Continuous infusion of Ang-II for 7 days increased systolic blood pressure (SBP) (Fig. 7A) and enhanced superoxide generation (Fig. 7B) to comparable levels in WT and CIKS-null mice. However, whereas Ang-II induced cardiac hypertrophy in the WT mice, it failed to do so in the CIKS-null mice (Fig. 7C). Moreover, echocardiographic analysis revealed that while left ventricular wall thickness (Fig. 7D), end-diastolic dimension (LVDd; Fig. 7E) and fractional shortening (FS; Fig. 7F) were significantly modulated in Ang-II infused WT mice, these effects were blunted in CIKS null mice. Further, the hypertrophy-associated markers (ANF mRNA expression, phospho-ribosomal S6 protein, phospho-p70S6K, and phospho-Akt) were also markedly attenuated in CIKS null mice (Figs. 8A and B). These results indicate that CIKS deficiency blunts Ang-II-induced myocardial hypertrophy, and its effects are blood pressure-independent (Figs. 7 and 8).

3.8. Deficiency of CIKS is associated with reduced Ang-II-induced cardiac NF- κ B and AP-1 activation and IL-18 and MMP-9 expression in vivo

Compared with WT mice, our results also show reduced Ang-II-induced phospho-IKK β and phospho-p65 levels (Fig. 9A), and NF- κ B DNA binding activity (Supplementary Fig. S8-A) in CIKS-null mouse hearts. Absence of CIKS also blunted Ang-II-induced myocardial

phospho-JNK and phospho-c-Jun levels (Fig. 9B), and AP-1 DNA binding activity (Supplementary Fig. S8-B). Importantly, infusion of Ang-II induced a marked elevation of CIKS expression in the cardiac tissue of WT mice, supporting the *in vitro* observations that *CIKS* is an Ang-II-responsive gene. Furthermore, myocardial IL-18 expression was markedly attenuated in Ang-II-infused CIKS-null mice (Supplementary Fig. S8-C). These results demonstrate that CIKS deficiency blunts Ang-II induced myocardial NF- κ B and AP-1 activation and IL-18 expression *in vivo* (Fig. 9).

3.9. Deficiency of CIKS is associated with reduced collagen content/fibrosis

Our results also demonstrate that while Ang-II infusion induces myocardial MMP-9 mRNA expression in WT mice (Supplementary Fig. 8-D), an effect that was markedly attenuated in CIKS null mice. In addition, Ang-II infusion increased myocardial collagen content/fibrosis in WT, but not CIKS null mice (Fig. 10A). These results indicate that CIKS deficiency blunts adverse remodeling by suppressing MMP-9 expression and collagen content *in vivo* (Fig. 10A).

4. Discussion

Here we report the novel observation that Ang-II-induced cardiomyocyte hypertrophy, both *in vivo* and *in vitro*, is mediated by the adapter protein CIKS. Furthermore, Ang-II induces the expression of CIKS in cardiomyocytes, and both CIKS induction and CIKS-dependent cardiomyocyte hypertrophic growth are mediated through AT1 and Nox2-dependent ROS generation. Ang-II increased AT1 physical association with Nox2 both *in vitro* and *in vivo*, increased ROS generation, increased physical association of CIKS with IKK β and with JNK, induced NF- κ B and AP-1 activation, and enhanced the NF- κ B- and AP-1-responsive *Il18* gene expression. Further, CIKS knockdown or gene deletion blunted Ang-II-induced NF- κ B and AP-1 activation, as well as IL-18 and MMP-9 expression. Importantly, while continuous infusion of Ang-II for 7 days led to an increase in systolic blood pressure and oxidative stress in both WT and CIKS-null mice, cardiac hypertrophy and collagen content were markedly attenuated in the CIKS-deficient animals. These results indicate a critical role for CIKS in Ang-II-induced myocardial hypertrophy and adverse remodeling (Fig. 10B), and demonstrate for the first time that CIKS is an important intermediate in Ang-II induced redox signaling. Our results suggest that CIKS is a potential therapeutic target in cardiac hypertrophy, adverse remodeling, and congestive heart failure.

CIKS plays major roles in various immune and inflammatory diseases, particularly those involving T helper type-17 lymphocytes that express interleukin-17 [36, 37]. IL-17 signals through the IL-17 receptor, and activates both NF- κ B- and AP-1-dependent signaling. Knockdown of CIKS or its gene deletion abrogates IL-17 signaling, indicating that IL-17 signals almost exclusively through CIKS [15, 16]. In cardiomyocytes, Ang-II treatment led to phosphorylation and enhanced kinase activity of IKK β and the subsequent release of p65 and p50 for translocation to the nucleus. This was abrogated by knockdown of either CIKS or Nox2, and by losartan. Furthermore, while confirming earlier reports that CIKS can physically associate with IKK β [31], we also found that in cardiomyocytes, Ang-II increased this association. Ang-II also induced the activation of JNK, c-Jun and the translocation of AP-1 DNA binding activity to the nucleus. As with the NF- κ B pathway, these activities were blocked by losartan, and either CIKS or Nox2 knockdown. We also demonstrate that CIKS is complexed with JNK and this interaction is increased by Ang-II. Recently, it has been shown that IKK γ /NEMO binds JNK [33], raising the possibility that it is the CIKS-bound IKK γ that in fact associates with JNK. This is supported by our results showing that CIKS associates with IKK γ , and IKK γ physically associates with JNK. Notably, CIKS gene deletion markedly attenuated IKK γ /JNK interactions, indicating that CIKS may act as a scaffold in their interaction. Thus a CIKS/IKK γ /JNK complex may

mediate c-Jun phosphorylation and activation, a mechanism that remains to be further investigated.

Although our data strongly suggest a major role for CIKS in mediating the NF- κ B and AP-1 proinflammatory signaling pathways induced by the Ang-II/AT1 interaction, the precise mechanism linking CIKS to AT1 is unclear. In IL-17 signaling, IL-7 receptors and CIKS interact through their SEFIR (SEF-similar expression to fibroblast growth factor genes, and IL17R) domains present in their C-terminal regions to recruit a kinase TAK1 and TRAF6 to induce downstream signaling events [38, 39]. The SEFIR domains also share some homology with, and are structurally similar to, the Toll-IL-1 receptor (TIR) domains of Toll-like receptors and IL-1Rs that mediate the TIR-TIR homotypic interactions. Very recently, a molecular structure in the SEFIR domain responsible for the SEFIR-SEFIR interaction was identified, and it appears to differ from the structures mediating the TIR-TIR interaction [39]. In addition to the SEFIR domain, CIKS contains two TRAF-binding sites in its N-terminus, and a U-box-like region N-terminal to the SEFIR/TIR domain that functions as an E3 ubiquitin ligase [17]. Analysis of the amino acid sequence of AT1 indicates that it contains neither TIR nor SEFIR domains. Further, our preliminary experiments aimed at identifying an interaction between the cytoplasmic C-terminal domain of AT1 and CIKS by a GST pull-down assay *in vitro*, or an AT1/CIKS interaction *in vivo* by IP/IB, have so far been unsuccessful (data not shown). Therefore, if CIKS does not interact with AT1 in a manner similar to its interaction with IL17R, then by what mechanism does CIKS mediate the Ang-II/AT1 stimulus-response pathway? There is evidence that CIKS can be phosphorylated *in vivo* [40] suggesting that phosphorylation/dephosphorylation events may regulate its function. Therefore one possible mechanism may be that an intermediate kinase is activated by AT1, and this in turn activates CIKS. Among kinases that are known to be activated following Ang-II binding to AT1 are Src, PKC, Jak2, and ROCK [41]. Currently however, the amino acid residues of CIKS phosphorylated in response to agonist stimulation, and the effects of these modifications on its function, have not been clearly characterized, therefore more intensive studies on CIKS will be required to fully investigate this potential mechanism. Another possibility is that Ang-II binding to AT1 may result in the intracellular transactivation of an unrelated receptor that does interact with CIKS. Currently it is known that interaction of Ang-II with AT1 can lead to the transactivation of PDGF, EGF and possibly IGF receptors [42], and induction of their downstream signaling pathways. Potential candidates would have to contain a SEFIR domain such as IL-17R, or possibly a TIR domain such as the TLRs and members of the IL-1/IL-18 family of receptors. Since IL-18 is a critical mediator of Ang-II-induced cardiomyocyte hypertrophy, the IL-18 receptor may be a prominent candidate.

Although CIKS is known to be localized to cytoplasm, and associate with the C-termini of IL-17 receptors (and possibly others), and transduce downstream signal transduction pathways, here we show that CIKS can also be found in the nucleus in the resting cell, and Ang- II treatment increases both cytoplasmic and nuclear levels. Moreover, we identified a potential nuclear localization signal in CIKS that is very similar to the NLS of the SV-40 large T antigen, and notably, this sequence is highly conserved across species. Currently, we do not know the significance of the nuclear localization of CIKS, whether for instance it is an integral part of the NF- κ B and AP-1 activation pathways, or if it acts to regulate gene transcription. Future studies will determine its role in the transcriptional regulation of inflammatory genes, including *II18* and *MMP9*.

The NF- κ B- and AP-1-responsive proinflammatory cytokine IL-18 plays a critical role in cardiomyocyte hypertrophy *in vitro* and *in vivo* [19–22]. Pre-treatment with IL-18 neutralizing antibodies or with IL-18BP markedly attenuates the Ang-II-induced hypertrophic growth response. Importantly, our results here show that CIKS knockdown or

gene deletion markedly attenuates both IL-18 expression and Ang-II-induced cardiomyocyte hypertrophy. Thus Ang-II-induced hypertrophy may be mediated, at least in part, by CIKS/NF- κ B/AP-1/IL-18 pathways. Interestingly, a recent report indicates that mechanical stretch applied to cardiomyocytes *in vitro* also results in the induction of IL-18, and this is mediated by Endothelin-1 receptor and AT1 through a Rho/Rho kinase pathway [43]. Here we show that Ang-II induced CIKS expression via AT1- and Nox2-dependent ROS generation. Importantly, a preliminary analysis of the mouse *CIKS* promoter region has identified potential binding sites for AP-1, NF- κ B, CREB, IRF and C/EBP β transcription factors (unpublished observations). Since IL-18 is also a potent inducer of NF- κ B, AP-1 and CREB activation [27], this suggests that IL-18 may also induce CIKS expression in cardiomyocytes, and that elevation of CIKS may be a critical reinforcement mechanism in the cardiomyocyte hypertrophic growth pathway. Studies aimed at identifying a role for CIKS in the IL-18/IL-18R signaling pathway, and the role of Ang-II/AT1 interaction on the possible transactivation of the IL-18 receptor are currently in progress in our laboratory.

We recently demonstrated in rat cardiomyocytes, that AT1 not only signals via Nox2, but also exhibits a physical association with Nox2 that increases with Ang-II treatment [5]. Similarly, here we show that mouse AT1 also binds Nox2 in mouse cardiomyocytes both *in vitro* and *in vivo*, and Ang-II enhances this binding activity. Our GST pull-down assays indicate that this interaction appears to be between Nox2 and the C-terminal cytoplasmic domain of AT1. This particular region of the AT1 receptor is known to bind a number of cytoplasmic proteins that may regulate non-G-protein AT1 signaling [41], including ATRAP, ARAP1, GLP, JAK2 and PLC γ 1. Previously, we suggested that the interaction between Nox2 and AT1 might serve to colocalize the two proteins in lipid rafts, perhaps comprising an integral feature of the functional AT1 membrane complex that mediates the downstream responses to Ang-II. That Nox2 is the critical isoform required for the redox-sensitive, Ang-II-induced, CIKS-mediated, hypertrophic response in cardiomyocytes is further supported by our observations that the Rac1 inhibitor NSC, and forced expression of dominant negative Rac1 (N17rac1) abrogated Ang-II-mediated cardiomyocyte hypertrophy.

In support of our *in vitro* results, Ang-II infusion induced CIKS expression and myocardial hypertrophy in wild-type mice, but this was markedly attenuated in CIKS-null mice. Furthermore, Ang-II-induced hypertrophy-associated markers were attenuated in CIKS-null hearts, as were levels of phosphorylated IKK β , p65, JNK and c-Jun. Similarly, Ang-II-induced myocardial NF- κ B and AP-1 DNA-binding activities and IL-18 expression were significantly reduced in CIKS-null mice. Notably, Ang-II induced collagen content, a surrogate marker for fibrosis [44, 45], is also markedly attenuated by CIKS deficiency.

In summary, we report several novel findings. (i) Ang-II-induced cardiomyocyte hypertrophy *in vitro* and *in vivo* is mediated in part via CIKS. (ii) CIKS deficiency blunts Ang-II induced cardiac fibrosis *in vivo*. (iii) Ang-II enhances AT1 binding to Nox2. (iv) Ang-II induces CIKS expression via AT1 and Nox2-mediated ROS generation. (v) CIKS binding to IKK β and IKK γ , and association with JNK is elevated in response to Ang-II. Notably, CIKS gene deletion blunts IKK γ physical association with JNK, indicating that CIKS may serve as a scaffold in their interaction. (vi) Ang-II induces p65 and c-Jun phosphorylation, NF- κ B and AP-1 activation, and IL-18 and MMP-9 induction in CIKS-dependent manner. These results indicate that CIKS is a critical intermediate in Ang-II signaling, and therefore a potential therapeutic target in cardiac hypertrophy, adverse remodeling and congestive heart failure.

Research highlights

► Ang-II induces cardiomyocyte hypertrophy and fibrosis via CIKS. ► Ang-II induces CIKS expression via AT1/Nox2-mediated ROS generation. ► Ang-II enhances CIKS binding to IKK β and JNK. ► CIKS acts as a scaffold in IKK γ /JNK interaction. ► CIKS is a potential therapeutic target in cardiac hypertrophy and adverse remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

Act1	activator of NF- κ B
ACM	adult cardiomyocytes
ANF	atrial natriuretic factor
AP-1	activator protein-1
AT1	angiotensin II type 1 receptor
ATRAP	AT1-receptor-associated protein
ARAP1	type 1 Ang II-receptor-associated protein 1
C/EBP	CCAAT/enhancer-binding protein
CIKS	Connection to IKK and SAPK/JNK
CREB	cAMP response element-binding protein
DCFH-DA	2',7'-dichlorofluorescein
DCF	2',7'-dichlorofluorescein
dn	dominant negative
DPI	diphenylene iodonium
EMSA	electrophoretic mobility shift assay
GFP	green fluorescent protein
GLP	glucagon-like peptide
GST	glutathione-S-transferase
IκB	inhibitory κ B
IKK	I κ B kinase
IL	interleukin
IP/IB	immunoprecipitation/immunoblotting
IRF	IFN regulatory factor

JAK2	janus kinase 2
JNK	c-Jun amino-terminal kinase
kd	kinase deficient
MOI	multiplicity of infection
MMP	matrix metalloproteinase
NEMO	NF- κ B essential modulator
NF-κB	nuclear factor kappa B
Nox	NADPH oxidase
NADPH	nicotinamide adenine dinucleotide phosphate
N17rac1	a dominant negative form of rac1
OCT	optimum cutting temperature
PLC	Phospholipase C
RAAS	Renin-angiotensin-aldosterone system
Rac1	ras-related C3 botulinum toxin substrate 1
ROCK	Rho-associated, coiled-coil containing protein kinase 1
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SBP	systolic blood pressure
SEF	similar expression to fibroblast growth factor genes
SEFIR	SEF-IL-17R
TAK1	Transforming growth factor β -activated kinase 1
TIR	Toll-IL-1 receptor
TRAF	TNF Receptor Associated Factor
TRAF3IP2	TRAF3 interacting protein 2
TNF	tumor necrosis factor
UTR	untranslated region
WT	wild-type.

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Fig. 1. Angiotensin-II induces cardiomyocyte hypertrophy *in vitro* through CIKS

(A) Ang-II induces cardiomyocyte hypertrophy via CIKS. Cardiomyocytes isolated from adult WT mice (ACM) were infected with lentivirus expressing CIKS shRNA or non-targeting control shRNA (MOI 5) for 48 h. After an additional 12 h culture in medium containing 0.5% BSA, cells were exposed to Ang-II (10^{-7} M) and analyzed for cell growth ($[^3\text{H}]$ leucine incorporation normalized to total DNA). Knockdown of CIKS was confirmed by immunoblotting, and is shown on the right. Both α Tubulin and GAPDH served as invariant controls. * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II + control shRNA ($n = 12$). (B) CIKS deficiency blunts Ang-II-induced cardiomyocyte hypertrophy. ACM isolated from CIKS-null mice were exposed to Ang-II and analyzed for cell growth by $[^3\text{H}]$ leucine incorporation (B) and cell surface area (C). AT1 levels in the membrane fraction were analyzed by immunoblotting (inset). * $P < 0.01$ versus untreated ($n = 12$).

Fig. 2. Angiotensin-II induces CIKS expression via AT1/ROS in WT-cardiomyocytes

(A) Ang-II induces time-dependent CIKS mRNA expression in WT-ACM. Quiescent ACM exposed to Ang-II (10^{-7} M) were analyzed for CIKS mRNA expression by RT-qPCR. 28S served as an invariant control. * $P < 0.01$ versus untreated ($n = 12$). (B) Ang-II induces time-dependent CIKS protein expression in WT-ACM. ACM treated as in A were analyzed for CIKS protein levels by immunoblotting using cleared whole cell homogenates. A representative of three independent experiments is shown. The intensity of immunoreactive bands was semiquantified. Densitometric analysis is summarized on the right. * $P < 0.05$ versus untreated. (C) The AT1 antagonist losartan and the NADPH oxidase inhibitor DPI attenuate Ang-II-induced ROS generation. ACM loaded with the ROS-sensitive fluorophore dichlorofluorescein diacetate (DCFH-DA) were incubated with losartan ($10 \mu\text{M}$ for 1 h) or DPI ($10 \mu\text{M}$ in DMSO for 30 min) prior to Ang-II exposure (10^{-7} M for 15 min). Mean fluorescence intensity was calculated as detailed in 'Material and methods'. * $P < 0.001$ versus untreated, † $P < 0.001$ versus Ang-II + DMSO ($n = 12$). (D) Losartan and DPI inhibit Ang-II induced CIKS protein expression. Quiescent ACM incubated as in C, but for 2 h with Ang-II, were analyzed for CIKS protein levels by immunoblotting ($n = 3$; densitometric analyses is summarized on the right). * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II.

Fig. 3. Ang-II induces CIKS expression via Nox2 and Rac1

(A) Nox2 knockdown blunts Ang-II-induced CIKS expression. ACM infected with lentiviral Nox2 shRNA or control (MOI 5 for 48 h), were exposed to Ang-II (10^{-7} M) for 2 h and analyzed for CIKS protein expression by immunoblotting ($n = 3$; Densitometric analysis is summarized in the middle panel. $*P < \text{at least } 0.01 \text{ versus untreated}$). Knockdown of Nox2 was confirmed by immunoblotting, and is shown on the right. (B) Ang-II induces ROS generation via Rac1. ACM were infected with adenoviral Myc-tagged dominant negative Rac1 (Ad.N17rac1, MOI 50 for 24 h) and then loaded with DCFH-DA. Alternatively, ACM were loaded with DCFH-DA and then treated with the Rac1 inhibitor NSC23766 (NSC; 100 nM in water for 21 h) prior to Ang-II addition (10^{-7} M for 15 min). ROS generation was quantified as in Fig. 2C. Expression of Myc was confirmed by immunoblotting, and is shown as an inset. $*P < 0.001 \text{ versus untreated}$, $\dagger P < 0.01 \text{ versus Ang-II}$ ($n = 12$). (C) Ang-II induces CIKS expression via Rac1. ACM infected or treated as in B, but for 2 h with Ang-II, were analyzed for CIKS protein expression by immunoblotting ($n = 3$; Densitometric analyses is shown on the right. $*P < 0.01 \text{ versus untreated}$, $\dagger P < \text{at least } 0.05 \text{ versus Ang-II}$. (D) Ang-II induces cardiomyocyte hypertrophy via Rac1. ACM treated as in C, but for 48 h with Ang-II, were analyzed for cell growth as detailed in Fig. 1A. $*P < 0.01 \text{ versus untreated}$, $\dagger P < 0.05 \text{ versus Ang-II}$ ($n = 12$). (E), Ang-II promotes CIKS localization to both cytoplasm and nucleus. ACM treated with Ang-II (10^{-7} M for 1 h) were analyzed for CIKS protein expression in cytoplasmic and nuclear protein extracts. GAPDH (cytoplasmic), Lamin A/C (nuclear), and V-β (mitochondrial) served as loading and purity controls ($n = 3$). Densitometric analysis is shown on the right. $*P < 0.05 \text{ versus untreated}$.

Fig. 4. Ang-II activates NF-κB via CIKS

(A) Ang-II activates IKKβ via Nox2 and Rac1. ACM infected with Nox2 shRNA or control lentivirus (MOI 5 for 48 h), Ad.N17rac1 (MOI 50 for 24 h) or treated with the Rac1 inhibitor NSC (100 nM in water for 21 h) were exposed to Ang-II (10^{-7} M for 1 h). IKKβ phosphorylation (Ser180/181) was analyzed by immunoblotting ($n = 3$). Densitometric analysis is shown in the lower panel. * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II. (B) CIKS knockdown blunts Ang-II-induced IKKβ phosphorylation. ACM infected with lentiviral CIKS shRNA or non-targeting control shRNA (MOI 5 for 48 h) were treated with Ang-II (10^{-7} M for 1 h). Phospho-IKKβ levels were analyzed by immunoblotting as in A ($n = 3$). Densitometric analysis is shown in the lower panel. * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II. (C) Ang-II enhances CIKS physical association with IKKβ. Quiescent ACM were treated or not with Ang-II (10^{-7} M for 15 min), and then analyzed by reciprocal immunoprecipitation (IP) and immunoblotting (IB) using antibodies to CIKS and IKKβ ($n = 3$). Densitometric analysis is shown in the lower panel. * $P < 0.05$ versus untreated. (D and E) CIKS knockdown blunts p65 activation and nuclear translocation. ACM treated as in B were analyzed for phospho-p65 levels in cytoplasmic (D) and nuclear (E) extracts by immunoblotting. GAPDH and Lamin A/C served as respective loading and purity controls ($n = 3$). Densitometric analysis is shown in the lower panels. * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II. (F) Ang-II activates NF-κB via CIKS/IKKβ. ACM were infected as in B or with Ad.kdIKKβ prior to Ang-II (10^{-7} M for 1 h) addition. NF-κB DNA-binding activity was analyzed by EMSA using nuclear protein extracts ($n = 3$). Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls (lower panels).

Fig. 5. Ang-II activates AP-1 via CIKS

(A) Ang-II activates JNK via Nox2 and Rac1. ACM were infected with Nox2 shRNA or control lentivirus (MOI 5 for 48 h), Ad.N17rac1 (MOI 50 for 24 h) or treated with the Rac1 inhibitor NSC (100 nM in water for 21 h) prior to Ang-II addition (10^{-7} M for 1 h). Total JNK and phospho-JNK (Thr183/Tyr185) levels were analyzed by immunoblotting ($n = 3$). (B) CIKS knockdown blunts Ang-II-induced JNK phosphorylation. ACM infected with CIKS shRNA or control lentivirus (MOI 5 for 48 h) were exposed to Ang-II (10^{-7} M for 1 h). Phospho-JNK levels were analyzed by immunoblotting as in A ($n = 3$). (C) Ang-II enhances CIKS physical association with JNK. Quiescent ACM treated with Ang-II (10^{-7} M for 15 min) were analyzed by reciprocal IP/IB using antibodies to CIKS and JNK as detailed in Materials and methods ($n = 3$). (D) Ang-II enhances CIKS/IKK γ and IKK γ /JNK physical association. Quiescent ACM treated with Ang-II (10^{-7} M for 15 min) were analyzed by IP/IB using antibodies to CIKS and IKK γ as detailed in Materials and methods ($n = 3$). (E) CIKS gene deletion blunts IKK γ physical association with JNK. Quiescent ACM from WT and CIKS null mice were treated with Ang-II (10^{-7} M for 15 min), and then analyzed by IP/IB using antibodies to IKK γ and JNK ($n = 3$). (F) CIKS knockdown blunts c-Jun activation. ACM treated as in B were analyzed for phospho-c-Jun levels in cytoplasmic (upper panel) and nuclear (lower panel) extracts by immunoblotting. GAPDH and Lamin A/C served as respective loading and purity controls ($n = 3$). (G) Ang-II activates AP-1 via CIKS/JNK. ACM were infected as in B or with Ad.dnJNK1 prior to Ang-II (10^{-7} M for 1 h). AP-1

DNA-binding activity was analyzed by EMSA using nuclear protein extracts ($n = 3$). Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls (inset). Densitometric analyses of three independent experiments from panels A – F are summarized in Supplementary Fig. S6.

Fig. 6. Ang-II induces IL-18 and MMP-9 expression via CIKS

(A) Ang-II induces time-dependent IL-18 expression. Quiescent ACM exposed to Ang-II (10^{-7} M) were analyzed for IL-18 mRNA (RT-qPCR; left hand panel; $n=6$), mature IL-18 protein levels (immunoblotting; middle panel; $n=3$), and secreted IL-18 levels (24 h; ELISA; right hand panel). * $P < 0.001$ versus untreated. (B) CIKS knockdown blunts Ang-II-induced IL-18 protein expression. ACM infected with CIKS shRNA or control lentivirus (MOI 5 for 48 h) were exposed to Ang-II (10^{-7} M). Mature IL-18 protein levels were analyzed at 2 h by immunoblotting ($n=3$). (C) Ang-II induces IL-18 via IKK β and p65. ACM infected (MOI 50 for 24 h) with Ad.kdIKK β or Ad.dnp65 were exposed to Ang-II (10^{-7} M for 2 h). Mature IL-18 protein levels were analyzed by immunoblotting as in A ($n=3$). (D) Ang-II induces IL-18 via JNK and c-Jun. ACM infected (MOI 50 for 24 h) with

Ad.dnJNK1 or Ad.dnc-Jun were exposed to Ang-II (10^{-7} M for 2 h). Mature IL-18 protein levels were analyzed by immunoblotting as in A ($n = 3$). (E) Ang-II induces cardiomyocyte hypertrophy in part via IL-18. Quiescent ACM were incubated with IL-18-neutralizing antibodies or IL-18BP-Fc (10 μ g/ml for 1 h) prior to Ang-II (10^{-7} M for 48 h) addition. Cell growth was analyzed as in Fig. 1A. * $P < 0.01$ versus untreated, † $P < 0.05$ versus Ang-II ($n = 12$). (F) CIKS knockdown blunts Ang-II-induced MMP-9 mRNA expression. ACM infected with CIKS shRNA or control lentivirus (MOI 5 for 48 h) were exposed to Ang-II (10^{-7} M for 2 h). MMP-9 mRNA expression was analyzed by RT-qPCR. * $P < 0.001$ versus untreated, † $P < 0.01$ versus Ang-II ($n = 6$). (G) Ang-II activates MMP-9 in a time dependent manner and is attenuated by CIKS knockdown. ACM infected or not with lentiviral CIKS shRNA (MOI 5 for 48 h) were exposed to Ang-II (10^{-7} M). Latent and mature MMP-9 protein levels were analyzed by immunoblotting (time-dependent activation, left hand panel) ($n = 3$). Densitometric analyses of three independent experiments from panels B–D and G are summarized in Supplementary Fig. S7.

Fig. 7. CIKS deficiency fails to modulate blood pressure and ROS generation, but abrogates Ang-II induced myocardial hypertrophy

(A) CIKS deficiency fails to modulate Ang-II induced systolic blood pressure. Male WT and CIKS-null mice were infused with Ang-II (1.5 $\mu\text{g/kg}$ body wt/min) for 7 days via miniosmotic pumps. Saline served as a control. SBP was measured using tail cuff plethysmography in conscious animals before sacrifice. * $P < 0.01$ versus naïve or saline-infusion ($n = 6/\text{group}$). (B) CIKS deficiency fails to modulate Ang-II-induced superoxide generation. WT and CIKS-null mice infused with Ang-II as in A were analyzed for superoxide production by lucigenin chemiluminescence using heart homogenates. * $P < 0.01$ versus saline-infused ($n = 6/\text{group}$). (C) CIKS deficiency blunts Ang-II induced myocardial hypertrophy. WT and CIKS-null mice infused with Ang-II as in A. Heart and body weights were measured to quantify myocardial hypertrophy. * $P < 0.05$ versus saline-infused WT mice ($n = 6/\text{group}$). (D–F). Echocardiographic analysis of myocardial function. CIKS deficiency blunts Ang-II induced changes in left ventricular wall thickness (D), end-diastolic dimension (E) and fractional shortening (F). * $P < 0.01$ versus saline ($n = 6/\text{group}$).

Fig. 8. Deficiency of CIKS is associated with reduced hypertrophy-associated markers
 (A and B), CIKS deficiency blunts hypertrophy-associated markers. WT and CIKS-null mice infused with Ang-II as in Fig. 7 were analyzed for myocardial ANF mRNA expression by Northern blotting, and levels of phosphorylated ribosomal S6P, p70S6K and Akt by immunoblotting. Densitometric analysis is shown in the lower panels. ** $P < 0.01$, * $P < 0.05$ versus saline-infused WT mice ($n = 3-6$ /group).

Fig. 9. Deficiency of CIKS is associated with reduced Ang-II-induced cardiac NF- κ B and AP-1 activation and IL-18 and MMP-9 expression *in vivo*

(A) CIKS deficiency is associated with reduced Ang-II induced myocardial phospho-IKK β and phospho-p65 levels. WT and CIKS-null mice infused with Ang-II were analyzed for p-IKK β and p-p65 levels by immunoblotting using cleared heart homogenates. Densitometric analyses are shown in the lower panels. * $P < 0.01$ versus CIKS null ($n = 3$ /group selected at random). (B) CIKS deficiency is associated with reduced Ang-II induced myocardial p-JNK and p-c-Jun levels. WT and CIKS-null mice infused with Ang-II were analyzed for p-JNK and p-c-Jun by immunoblotting using cleared heart homogenates. Densitometric analysis is shown in the lower panels. * $P < 0.01$ versus CIKS null ($n = 3$ /group selected at random). (C) Ang-II induces myocardial CIKS expression in WT mice. WT-mice infused with Ang-II were analyzed for CIKS protein levels in three randomly selected mice. Densitometric analyses are shown in the lower panels. * $P < 0.01$ versus saline ($n = 3$ /group).

Fig. 10. CIKS deficiency blunts Ang-II-induced cardiac fibrosis

(A) Ang-II-induced collagen deposition is attenuated in CIKS null mice. WT and CIKS null mice were infused with Ang-II as in Fig. 7. Cryosections (8 μ M) were analyzed for collagen content by Sirius red F3BA dissolved in saturated picric acid and photographed at 100X magnification. (B) Schema showing the critical role of CIKS in Ang-II induced cardiomyocyte hypertrophy and myocardial fibrosis. Ang-II promotes AT1 physical association with Nox2, stimulates AT1/Nox-2 dependent superoxide generation, CIKS induction, CIKS/TRAF6 dissociation, binding of CIKS with IKK β and JNK, IKK β and JNK phosphorylation, NF- κ B and AP-1 activation, and IL-18 and MMP-9 induction. Ang-II-mediated cardiomyocyte hypertrophy is markedly attenuated by IL-18 neutralization. IL-18, via IL-18R and CIKS, may perpetuate the inflammatory signaling in the heart. CIKS is a potential therapeutic target in pathological cardiac hypertrophy. Solid blue arrows indicate protein-protein interactions. Broken line indicates our hypothesis that Ang-II induced cardiomyocyte hypertrophy and fibrosis are mediated via IL-18R/CIKS interaction.

Table 1

(A) Multispecies alignment of putative nuclear localization signal (NLS) in CIKS (TRAF3 interacting protein 2).				
Species	GenBank Acc#	N-term	"NLS"	C-term
Human	BC002823	493	MQIEFIKQGS MNFRFIPVLF PNAKKEHVPTWLQNT H VYSWPKNKKNI LLR	542
Chimpanzee	XM_001157429	85	MQIEFIKQGS MNFRFIPVLF PNAKKEHVPTWLQNT H VYSWPKNKKNI LLR	134
Rhesus	XM_001086102	500	MQIEFIKQGS MNFRFIPVLF PNAKKEHVPTWLQNT H VYSWPKNKKNI LLR	549
Cow	NP_001030560	491	MQIEFIKQGS MNFRFIPVLF PNAKKEHVPTWLQNT H IYSWPKNKKNI LLR	540
Mouse	NM_134000	483	MQIEFISQGS MNFRFIPVLF PNAKKEHVPTWLQNT H VYSWPKNKKNI LLR	532
Rat	NP_001037713	486	MQIEFINQGS MNFRFIPVLF PNAKKEHVPTWLQNT H VYSWPKNKKNI LLR	535
Chicken	XM_419782	460	MQIEFIQQGS MNFRFIPVLF PNAKKEHVPTWLQNT H IYNWPKNKKNI LLR	509

(B) The putative NLS of CIKS is similar to the classical NLS of SV40 large T antigen.	
CIKS (TRAF3 interacting protein 2)	P K N K K N I
SV40 large T antigen	P K K K R K V

Analysis of mouse CIKS amino acid sequence by PSORT II Prediction (<http://psort.hgc.jp/form2.html>) identified PKNKKNI in the C-terminal region as a potential nuclear localization sequence. Comparison of sequences shows it to be completely conserved in the seven species examined (highlighted).