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Original article

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 IκB kinase/nuclear factor (NF)-κB and JNK/activator protein (AP)-1 pathways in autoimmune and inflammatory diseases. Since Ang-II is a potent activator of NF-κB 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-κB and JNK/AP-1 activation, p65 and c-Jun phosphorylation and nuclear translocation, NF-κB- 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-κB 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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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]. Ang-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

Abbreviations: Act1, activator of NF-κB; 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'-dichlorofluorescin-diacetate; DCF, dichlorofluorescein; Dn, dominant negative; DPI, diphenylene iodonium; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GLP, guanine nucleotide-exchange factor-like protein; GST, glutathione-S-transferase; IκB, inhibitory κB; IKK, IκB 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-κB essential modulator; NF-κB, nuclear factor kappa B; Nox, NADPH oxidase; NADPH, nicotinamide adenine dinucleotide phosphate; N17rac1, a dominant negative form of rac1; OCT, optimun 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, TFAF3 interacting protein 2; TNF, tumor necrosis factor; UTR, untranslated region; WT, wild-type.

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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-κB and AP-1 in cardiomyocytes *in vitro* and myocardium *in vivo* [12,13]. Both NF-κB 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-κB 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-κB and AP-1 contribute to Ang-II-induced pathological hypertrophy [12,13].

The adapter molecule CIKS (connection to IκB kinase and c-Jun-N-terminal kinase), also known as Act1 or TRAF3IP2, has been shown to mediate the activation of both NF-κB and AP-1 in an IKK and JNK-dependent manner respectively [15,16]. CIKS, a ubiquitin ligase, signals via both TRAF6-dependent and -independent mechanisms to induce NF-κB 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-κB and AP-1 activation [12,13], we hypothesized that CIKS could mediate this process. In addition, since the proinflammatory cytokine interleukin (IL)-18 is an NF-κB 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-κB 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 Ang-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 1 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 N_2 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 or tibial length, (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% phosphomolybdic 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 μM)-enhanced chemiluminescence (NADPH 300 μM; 100 μg protein; 37 °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

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