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Cellular FLICE-inhibitory protein protects against cardiac remodeling induced by Angiotensin II in mice

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Running Title: cFLIP inhibits cardiac hypertrophy

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Methods and Materials

Materials
Antibodies for the MAPK and Smad pathways were purchased from Cell Signaling Technology. The antibody used to recognize GATA-4 was purchased from Upstate Biotechnology. The anti-cFLIP\textsubscript{L} (reactive with mouse or human) antibody was purchased from Abcam Inc. \textsuperscript{3}H-proline was purchased from Amersham. The BCA protein assay kit was purchased from Pierce. All other antibodies were purchased from Santa Cruz Biotechnology. TGF-\textbeta\textsubscript{1} was purchased from R&D Systems. FCS was obtained from Hyclone. Cell culture reagents and all other reagents were obtained from Sigma.

Animals and animal models
All protocols were approved by the Animal Care and Use Committee of University Health Network (Toronto, Ontario, Canada) and Renmin Hospital of Wuhan University (Wuhan, China). All surgeries and subsequent analyses were performed in a blinded fashion. Human cFLIP\textsubscript{L} cDNA construct containing full-length human cFLIP\textsubscript{L} cDNA was cloned downstream of the cardiac myosin heavy chain (MHC) promoter. Transgenic mice were produced by microinjection of the \textalpha-MHC-cFLIP\textsubscript{L} construct into fertilized mouse embryos (CD1 background). Four independent transgenic lines were established and studied. Transgenic mice were identified by PCR analysis of tail genomic DNA. Functional data and gene expression levels were analyzed in pairs of \textalpha-MHC-cFLIP\textsubscript{L} (TG) and littermate nontransgenic (WT) male mice ranging in age from 7 to 8 weeks. Male cFLIP heterozygous knockout mice (cFLIP\textsuperscript{+-}, CD1 background; provided by Dr. Wen-Chen Yeh) and their wild-type littermates aged 7 to 8 weeks were used in the studies. Genotyping was performed by PCR as described previously.\textsuperscript{1}

To examine the role of cFLIP in cardiac hypertrophy, the experiments were performed in an Ang II Infusion model. Ang II (1.4 mg/kg/day and dissolved in 0.9% NaCl) was subcutaneously infused for 4 weeks using an osmotic minipump (Alzet model 2004; Alza Corp) implanted in each mouse. Saline-infused animals served as infusion controls and were subjected to the same procedures as the experimental animals with the exception of Ang II infusion. The control group for these experiments was given the same volume of PBS. The internal diameter and wall thickness of the LV were assessed by echocardiography at the indicated time after infusion. Hearts and lungs of the sacrificed mice were dissected and weighed to compare HW/BW (mg/g) and LW/BW (mg/g) ratios in different groups.

Blood pressure and echocardiography
A microtip catheter transducer (SPR-839, Millar Instruments, Houston, Tex) was inserted into the right carotid artery and advanced into the left ventricle. After stabilization for 15 minutes, the pressure signals and heart rate were recorded continuously with an ARIA pressure-volume conductance system coupled with a Powerlab/4SP A/D converter, stored, and displayed on a personal computer as described previously.\textsuperscript{2} Echocardiography was performed by SONOS 5500 ultrasound (Philips Electronics, Amsterdam) with a 15-MHz linear array ultrasound transducer. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. LVEDD and LVESD were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.
Quantitative real-time RT-PCR and Western blotting
Real-time PCR was used to detect the mRNA expression levels of hypertrophic, fibrotic, and inflammatory markers. Total RNA was extracted from frozen, pulverized mouse cardiac tissue using TRIzol (Invitrogen) and synthesized cDNA using oligo (dt) primers with the Advantage RT-for-PCR kit (BD Biosciences). Sequences of primers used for RT-PCR are shown in supplemental Table 1. We quantified PCR amplifications using SYBR Green PCR Master Mix (Applied Biosystems) and normalized results against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression. Cardiac tissue and cultured cardiac myocytes or fibroblasts were lysed in RIPA lysis buffer. Nuclear protein extracts were isolated as described previously.\(^2,3\) Fifty micrograms of cell lysate was used for SDS-PAGE, and proteins were then transferred to an immobilon-P membrane (Millipore). Specific protein expression levels were normalized to either the GAPDH protein for total cell lysate and cytosolic proteins, or the Lamin-B1 protein for nuclear proteins on the same nitrocellulose membrane.

Electrophoretic mobility shift assays (EMSA) and Histological analysis
Nuclear proteins were isolated as described previously.\(^2,3\) EMSA was performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega). Synthetic, double-strand oligonucleotides containing GATA-4 binding domains were labeled with \([\gamma-^{32}\text{P}]\) ATP using T\(_4\) polynucleotide kinase and separated from unincorporated \([\gamma-^{32}\text{P}]\) ATP by gel filtration using a Nick column (Pharmacia). Hearts were excised, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (4-5 µm thick) were prepared and stained with H&E for histopathology or PSR for collagen deposition and then visualized by light microscopy. To determine the cross-sectional area of myocytes, HE-stained sections were used. A single myocyte was measured using an image quantitative digital analysis system (Image-Pro Plus 6.0). Between 100 and 200 myocytes in the left ventricles were outlined in each group.

Recombinant Adenoviral Vectors, and cultured neonatal rat cardiac myocytes and fibroblasts
We used replication-defective adenoviral vectors encoding for the entire coding region of cFLIP gene (kindly provided by Dr. Yeh) under the control of the cytomegalovirus promoter, and as a control, a similar adenoviral vector encoding for the LacZ gene (AdEasy XL adenoviral Vector system, Strategene). We ordered three rat shcFLIP constructs from SuperArray (Cat. No. KR06420G) and then generated three Ad-shcFLIP adenovirus, and selected one that led to a significant decrease in cFLIP levels for further experiments. Ad-shRNA was used as control. We infected cardiomyocytes with Ad-cFLIP and Ad-LacZ as well as Ad-shcFLIP and Ad-shRNA at a multiplicity of infection (MOI) of 100, resulting in 95-100% of cells expressing the transgenes without toxicity. Primary cultures of cardiac myocytes were prepared as described previously.\(^2,3\) Cells from the hearts of 1- to 2-day old Sprague-Dawley rats (Charles River Laboratories) were seeded at a density of \(1 \times 10^6\)well onto six well culture plates coated with fibronectin (Becton Dickinson) in plating medium consisting of F10 medium supplemented with 10% FCS and penicillin/streptomycin. After 48 hours, the culture medium was replaced with F10 medium containing 0.1% FCS and BrdU (0.1 mM). Viability was determined by cell number, frequency of contractions, cellular morphology, and trypan blue exclusion. Cultures of neonatal rat ventricular nonmyocytes, which have been shown to be predominantly fibroblasts, were prepared as described previously by Sadoshima and Izumo.\(^4\) All experiments were performed on cells from the first or second passages which were placed in DMEM medium containing 0.1% FCS for 24 hours before the experiment. The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. For the cell infection, \(1\times10^7\)well cardiac myocytes or cardiac fibroblasts were cultured in 6-well plates and exposed to \(2\times10^8\) pfu each of virus in 1 ml of serum-free medium for 24
The cells were then washed and incubated in serum-containing media for 24 hours. Additional treatments are described in the figure legends.

**Cardiac myocytes area and collagen synthesis assay**

For the surface areas, the cardiac myocytes were fixed with 3.7% formaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS, and stained with α-actinin (Sigma) at a dilution of 1:100 by standard immunocytochemical techniques. Collagen synthesis was evaluated by measuring [³H]-proline incorporation as described previously. Briefly, cardiac fibroblasts were infected with different adenoviruses and then made quiescent by being cultured in 0.1% FCS DMEM for 24 h, and subsequently incubated with TGF-β1 and 5 μCi/ml [³H]-proline for the indicated time. Cells were washed with PBS twice, treated with ice-cold 5% trichloroacetic acid (TCA) for one hour, and washed with distilled water twice. Cells were then lysed with 1 N NaOH solution and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number.

**Determination of apoptosis and myocardial caspase-3, caspase-8, and caspase-9 activity**

Cell death by apoptosis was evaluated after measuring oligonucleosomal DNA fragments by a TUNEL assay that was performed in sections with use of the CardiaoTACS in situ Apoptosis Detection Kit (R&D Systems) as described previously. Myocardial caspase-3, caspase-8, and caspase-9 activation was evaluated by fluorometric assay kits (Biomol Research Laboratories) as described previously by Haudek et al. Briefly, frozen mouse cardiac tissues were lysed in lysis buffers for caspase-3, caspase-8, and caspase-9, respectively. 0.5 mg protein was incubated either with fluorogenic substrates specific for caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), and caspase-9 (LEHD-AFC) cleavage or was pretreated with a specific inhibitor for caspase-3 (Ac-DEVD-CHO), caspase-8 (Ac-IETD-CHO) or caspase-9 (Ac-LEHD-CHO) prior to adding the substrate. Kinetic measurements were obtained at room temperature for 4 hours using a FLx800 Microplate Fluorescence Reader (excitation, 360/40 nm; emission, 530/20 nm; Bio-Tek Instruments). Measurements of caspase activity were determined from the steepest slope of the resulting curve, which was determined using KCjunior software (Bio-Tek Instruments). Each sample (with and without inhibitors) was measured in duplicate. The values obtained for noninhibitable protease cleavage were subtracted from the value of the same sample obtained without inhibitor pretreatment in order to obtain specific activity. Final values for caspase-3, caspase-8, and caspase-9 activity were normalized by the total protein concentration for each sample.

**Statistical analysis**

Data are expressed as means±SEM. Differences among groups were tested two-way ANOVA followed by post hoc Tukey test. Comparisons between two groups were performed by unpaired Student's t test. A value of $P<0.05$ was considered to be significantly different.
References


Online Supplement Figures

Figure S1

(A) The mRNA expression of cFLIP in the heart of HET mice (n=4). (B) The protein expression of cFLIP. (C) Saline vs Ang II for mouse cFLIP-L. (D) AB vs Sham for mouse cFLIP-L. (E) ANP, BNP, Myh7, and Acta1 expression levels.

Figure S1. Decreased cFLIP level exacerbated cardiac hypertrophy induced by Ang II in vivo. (A) The mRNA expression of cFLIP in the heart of HET mice (n=4). (B) The protein expression of cFLIP.
cFLIP in the heart of HET mice (n=4). *P<0.01 for difference from WT values. (C) Representative Western blots of mouse cFLIPL in heart tissue from WT mice subjected to 4 weeks of saline or Ang II infusion (n=4). (D) Representative Western blots of mouse cFLIPL in heart tissue from WT mice after aortic banding at time points indicated (n=4). (E) Analysis of hypertrophic markers. Total RNA was isolated from hearts of mice of the indicated groups, and expression of transcripts for ANP, BNP, Myh-7 and Acta1 induced by Ang II infusion were determined by real-time PCR analysis. Data represent typical results of 3-4 different experiments as mean±SEM (n=4 to 6 mice/per group). *P<0.01 was obtained for the WT/saline values; § P<0.01 was obtained for WT/Ang II after Ang II infusion.
Figure 2. The effect of cFLIP on MEK-ERK1/2 signaling pathway
(A) The protein expression level of cFLIP after infection with Ad-cFLIP or Ad-shcFLIP. Left, representative blots. Right, quantitative results. Values are mean±SEM. *P<0.01 for difference from Ad-LacZ group values. (B and C) The effect of cFLIP on MEK1/2 and ERK1/2 activation after treated with Ang II for indicated time in different adenovirus infected primary cardiac myocytes. B, representative blots. C, quantitative results. The results were reproducible in three separate experiments. (D) The effect of U0126 on the enlargement of myocytes area induced by Ang II after infection with adenovirus. Cardiac myocytes were pretreated with U0126 1 hour, and treated with Ang II for 48 hours after infection with Ad-shRNA or Ad-shcFLIP for 24 hours. The results were reproducible in three separate experiments as mean±SEM. *P<0.01 was obtained for the PBS-treated groups; § P<0.01 was obtained for Ang II treated alone after Ang II treatment. (E) immunoprecipitation analysis showed that cFLIPL directly interacted with MEK1. (F) Effect of ERK1/2 activation on the phosphorylation and DNA-binding activity of GATA-4 induced by Ang II. Cells were infected with different adenovirus for 24 hours or pretreated with U0126 1 hour, and then treated with 1 μM Ang II for 24 hours, the extracts were assayed for GATA-4 phosphorylation and DNA-binding activity. Left, representative blots. Right, quantitative results. Values are mean±SEM. The results were reproducible in three separate experiments.
Figure S3

Figure S3. The effect of cFLIP on the expression of fibrosis markers in vivo.
Real-time PCR analyses of Tgfβ1, Col1α1, Col3α1, Pai-1, and Ctgf were performed to determine mRNA expression levels in indicated groups. GAPDH was used as the sample loading control. Data represent typical results of 3 different experiments as mean±SEM (n=4 to 6 mice/per group). *P<0.01 was obtained for the WT/saline values; § P<0.01 was obtained for WT/Ang II after Ang II infusion.
Figure S4

A

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CE

Phos-smad-

Smad 2

GAPDH

NE

Smad-2

Smad-3

Lamin-B1

B

Normalized P-Smad2/GAPDH

Normalized Smad/Lamin B1

Smad2

Smad3

minutes
Figure S4. The effect of cFLIP on TGF-β/Smad signaling in vitro.

(A and B) The effect of cFLIP on Smad-2 phosphorylation and Smad-2/3 translocation induced by TGF-β1 in cardiac fibroblasts after infection with different adenovirus. A, representative blots. B, quantitative results. CE: Cytoplasmic extracts; NE: Nuclear extracts. Values are mean±SEM. The results were reproducible in three separate experiments. *P<0.01 for difference from Ad-LacZ group values.

(C) The effect of ERK1/2 activation on collagen synthesis. Cells were infected with or without indicated adenovirus for 24 hours or pretreated with U0126 1 hour, and then incubated with 15 ng/ml TGF-β1 for up to 48 hours. [3H]-proline incorporation was performed as described in Materials and Methods. The results were reproducible in three separate experiments. *P<0.01 was obtained for the control group values.

(D) The effect of ERK1/2 activation on Smad-2 phosphorylation and Smad-2/3 translocation.
Figure S5

A

B

C

D

E

**ANP**

**BNP**

**Myh7**

**Acta1**

WT  |  TG  |  WT  |  TG  |  WT  |  TG  |  WT  |  TG  |  WT  |  TG

Saline |  |  |  |  |  |  |  |  |  |

Ang II |  |  |  |  |  |  |  |  |  |
Figure S5. Human cFLIP<sub>L</sub> transgenic mice develop decreased cardiac hypertrophy and fibrosis.  
(A) Diagram of the transgenic construct used for the generation of cFLIP<sub>L</sub> TG mice. (B) Representative Western blot of human cFLIP<sub>L</sub> protein from different tissue of TG mice as indicated. (C) Representative Western blots of human cFLIP<sub>L</sub> protein in the heart tissue from 4 lines of both TG and WT mice. (D) Representative Western blots of transgenic cFLIP<sub>L</sub> and endogenous cFLIP<sub>L</sub> protein levels in the heart from WT and TG mice. (E) Analysis of hypertrophic markers. Total RNA was isolated from hearts of WT and TG mice, and expression of transcripts for ANP, BNP, Myh7 and Acta1 induced by Ang II infusion were determined by real-time PCR analysis. (F) Representative blots of MEK1/2 and ERK1/2 activation at 4 weeks post-Ang II infusion in WT and TG mice. (G) The phosphorylation and DNA-binding activity of GATA-4 induced by Ang II infusion were determined. (H) mRNA expression levels of Tgfβ1, Col1a1, Col3a1, Pai-1, Ctgf in indicated groups at 4 weeks of Ang II infusion. GAPDH was used as the sample loading control.
Figure S6. The effect of cFLIP on apoptosis

(A) Statistical results of number of TUNEL-positive cells/10,000 cells, and relative caspase-3, caspase-8, and caspase-9 activity at 4 weeks post-Ang II infusion in WT and HET mice. (B) Statistical results of number of TUNEL-positive cells/10,000 cells, and relative caspase-3, caspase-8, and caspase-9 activity at 4 weeks post-Ang II infusion in WT and TG mice. The results were reproducible in three separate experiments. *P<0.01 was obtained for the WT/saline values; § P<0.01 was obtained for WT/Ang II after Ang II infusion.