Cellular FLICE-Inhibitory Protein Protects Against Cardiac Remodeling Induced by Angiotensin II in Mice

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Abstract—The development of cardiac hypertrophy in response to increased hemodynamic load and neurohormonal stress is initially a compensatory response that may eventually lead to ventricular dilatation and heart failure. Cellular FLICE-inhibitory protein (cFLIP) is a homologue of caspase 8 without caspase activity that inhibits apoptosis initiated by death receptor signaling. Previous studies showed that cFLIP expression was markedly decreased in the ventricular myocardium of patients with end-stage heart failure. However, the critical role of cFLIP on cardiac remodeling remains unclear. To specifically determine the role of cFLIP in pathological cardiac remodeling, we used heterozygote cFLIP+/- mice and transgenic mice with cardiac-specific overexpression of the human cFLIPL gene. Our results demonstrated that the cFLIP+/- mice were susceptible to cardiac hypertrophy and fibrosis through inhibition of mitogen-activated protein kinase kinase-extracellular signal–regulated kinase 1/2 signaling, whereas the transgenic mice displayed the opposite phenotype in response to angiotensin II stimulation. These studies indicate that cFLIP protein is a crucial component of the signaling pathway involved in cardiac remodeling and heart failure. (Hypertension. 2010;56:00-00.)

Key Words: cFLIP ■ cardiac remodeling ■ ERK1/2 ■ apoptosis

Cardiac hypertrophy is a response of the myocardium to increased workload, characterized by increase of myocardial mass and accumulation of extracellular matrix.1–3 The initial cardiac hypertrophy acts likely an adaptive mechanism; however, prolonged and severe hypertrophy is a risk factor for arrhythmias, sudden death, and heart failure. Although much is known about the signal transduction pathways that promote hypertrophic responses, mechanisms that antagonize these pathways have not been as clearly defined. A better understanding of these antagonistic mechanisms in cardiac myocytes may lead to novel strategies for suppressing cardiac hypertrophy.

Cellular FLICE inhibitory protein (cFLIP) is a mammalian homolog of the viral FLICE-inhibitory protein and a natural modulator of tumor necrosis factor signaling.4 Multiple cFLIP splice variants have been found, and 2 main forms have been well characterized, cFLIP short form (cFLIPs; 26 kDa) and long form (cFLIPL; 55 to 60 kDa).4 Both splice variants have death effector domains, with which they bind to Fas-associated death domain at the death-inducing signaling complex and inhibit caspase 8 activation. It has been well documented that elevated cFLIP expression protects cells from death receptor-mediated apoptosis, whereas downregulation of cFLIP by chemicals or small interfering RNA sensitizes cells to death receptor-mediated apoptosis.4 A clear reduction in the expression of cFLIP has been demonstrated in the ventricular myocardium of patients with end-stage heart failure and rodents after myocardial infarction.5,6 cFLIP-/- mice also exhibit severe defects in cardiac trabecula formation, as well as a thinner myocardium.7 In addition, Huber et al8 found that T-cell–specific overexpression of cFLIPL diminished the severity of coxsackievirus B3-induced myocarditis. These data suggest that cFLIP plays a vital role in cardiac development and cardiomyocyte survival after stress. Nevertheless, the role of cFLIP in cardiac hypertrophy remains largely unclear. Recently, Giampietri et al9 reported that mouse cFLIPL mild overexpression reduced transverse aortic constriction–induced hypertrophy. However, it should be noted that the cFLIP transgenic (TG) mice in their study are generated under the control of the testis-specific Stra8 promoter. More importantly, mice with cardiac-specific overexpression or deletion of cFLIPL gene are optimal animals to investigate the role of cFLIP in cardiac hypertrophy and the related mechanisms. In the present study, using cardiac-specific TG mice and cFLIP heterozygous knockout mice, we show for the first time that the cFLIP+/- (HET) mice...
exacerbate cardiac hypertrophy and fibrosis by blocking mitogen-activated protein kinase (MAPK) kinase (MEK)-
extracellular signal-regulated kinase (ERK)1/2 signaling,
whereas the TG mice with cardiac constitutive expression of
human cFLIPpL displayed the opposite phenotype in response
to angiotensin II (Ang II) stimulation. Our study indicates that
cFLIP is a pivotal inhibitor of cardiac remodeling and heart
failure.

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-cFLIPpL (react-
tive with mouse or human) antibody was purchased from Abcam Inc.
[3H]-Proline was purchased from Amersham. The details for other
reagents and adenoviral vectors are given in the online Data

Animals, Ang II Infusion Model, Blood Pressure,
and Echocardiography

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). The details
for mice information, Ang II infusion model, echocardiography, and
cardiac catheterization are given in the online Data Supplement.
Hearts and lungs of the euthanized mice were dissected and weighed
to compare heart weight (HW)/body weight (BW, milligram per gram)
and lung weight (LW)/BW (milligram per gram) ratios in
different groups.

Histological Analysis

Several sections of heart (4 to 5 μm thick) were prepared and stained
with hematoxylin-eosin for histopathology or Picrosirius red (PSR)
for collagen deposition, then visualized by light microscopy. For
myocyte cross-sectional area, a single myocyte was measured with
an image quantitative digital analysis system (National Institutes of
Health Image 1.6). The outline of 100 to 200 myocytes was traced in
each group.

Quantitative Real-Time RT-PCR, Western
Blotting, and Electrophoretic Mobility Shift Assays

For quantitative real-time PCR, Western blot, and electrophoretic
mobility shift assay analyses, please see the online Data Supplement.
Nuclear proteins were isolated as described previously.10

Recombinant Adenoviral Vectors, Cultured
Neonatal Rat Cardiac Myocytes, and Fibroblasts

Recombinant adenoviral vectors include Ad-cFLIP, Ad-LacZ, Ad-
shcFLIP, and Ad-shRNA. Primary cultures of cardiac myocytes and
fibroblasts were prepared as described.10,11 For details, please see the
online Data Supplement.

Cardiac Myocyte Area and Collagen
Synthesis Assay

The surface areas of cardiac myocytes and [3H]-proline incorpora-
tion were measured as described previously.10 For details, please see
the online Data Supplement.

Determination of Apoptosis and Myocardial
Caspase 3, Caspase 8, and Caspase 9 Activity

Apoptosis was evaluated by TUNEL assay and caspase activities
assay, as described previously.12,13

Statistical Analysis

Data are expressed as mean±SEM. Differences among groups were
tested 2-way ANOVA followed by post hoc Tukey test. Compari-
sions between 2 groups were performed by unpaired Student t test. A
value of P<0.05 was considered to be significantly different.

Results

Decreased cFLIP Level Exacerbated Cardiac
Hypertrophy Induced by Ang II In Vivo

Because cFLIP−/− mice exhibit severe defects in cardiac
formation and do not survive beyond day 12 of gestation, we
used HET mice whose cFLIP mRNA and protein levels were
significantly decreased in the heart (Figure S1A and S1B,
available in the online Data Supplement) to assess the impact
of deletion of 1 allele of cFLIP on cardiac hypertrophy. To
investigate whether cFLIP expression is regulated by Ang II
infusion or pressure overload, wild-type (WT) mice were
subjected to Ang II infusion or aortic banding for different
durations. As shown in Figure S1C, cFLIP protein levels
were significantly decreased at week 4 after Ang II infusion.
In addition, cFLIP expression increased by 2.4-fold over
basal levels (n=6) at week 2 of aortic banding. However,
cFLIP expression in the left ventricle (LV) was markedly
decreased compared with basal levels after 8 weeks of aortic
banding (Figure S1D). Thus, cFLIP expression is regulated
during LV remodeling induced by chronic Ang II infusion or
pressure overload.

To examine the potential effect of cFLIP on hypertrophy
mediated by Ang II infusion, osmotic minipumps were
implanted subcutaneously for a 4-week administration period,
followed by cardiac functional assessment. The ratios of
HW/BW and LW/BW cross-sectional area of cardiac myo-
cytes were significantly increased in both WT and HET mice,
and such increases were more pronounced in HET mice than
in WT mice after 4 weeks of Ang II infusion (Figure 1A). We
next assessed cardiac function in WT and HET mice by
echocardiography. The cardiac functional parameters showed
that decreased fractional shortening with dilated LV end-sys-
tolic diameter and LV end-diastolic diameter, as well as
increased LV diastolic septum, and LV posterior wall thick-
ness, were seen in WT mice. In HET mice, significantly
higher LV end-systolic diameter and LV end-diastolic diam-
eter, bigger increased LV diastolic septum, and LV posterior
wall thickness, as well as lower fractional shortening, were
observed compared with WT mice (Figure 1B), indicating
that HET mice have a greater level of cardiac hypertrophy
and dysfunction compared with WT mice in response to
hypertrophic stimuli. Gross heart and wheat germ agglutinin
and hematoxylin-eosin staining analysis further confirmed
the exaggerated effect of inhibition of cFLIP on cardiac
remodeling after Ang II stimulation (Figure 1C). Atrial
natriuretic peptide (ANP), B-type natriuretic peptide (BNP),
myosin heavy polypeptide 7 cardiac muscle (Myh7), and
actin α skeletal muscle (Acta1) are markers for cardiac
hypertrophy.14 To determine whether cFLIP affected the
mRNA expression levels of these markers, we performed
real-time PCR. Our results showed that the expression levels
of ANP, BNP, Myh7, and Acta1 mRNA were higher in HET
mice than those in WT mice (Figure S1E). These findings
suggest that decreased cFLIP level promotes the development of cardiac hypertrophy induced by Ang II stimulation in vivo.

Effect of cFLIP on MEK-ERK1/2 Signaling Pathway

MAPK signal transduction pathways have been shown to be activated by a variety of hypertrophic stresses, including neurohormonal stimuli and hemodynamic overload. To explore the molecular mechanisms through which decreased cFLIP level enhances the hypertrophic response, we examined the state of activation of MAPK in HET and WT hearts in the Ang II–infused hypertrophic model. We found that the increased phosphorylated levels of MEK1/2 and ERK1/2 induced by Ang II were more pronounced in HET hearts (Figure 2A), whereas p38 and Jun kinase (JNK)1/2 were similarly activated in the 2 groups (data not shown). Although Akt signaling plays a crucial role in the regulation of cardiac remodeling and apoptosis, we did not observe any differences in Akt activation between WT and HET mice (data not shown). Collectively, these data suggest that decreased expression of cFLIP increases the activation of MEK-ERK1/2 signaling, although it has no effect on p38, JNK, or Akt activation in hearts subjected to Ang II stimulation.

Figure 1. Decreased cFLIP level exacerbated cardiac hypertrophy induced by Ang II in vivo. A, Statistical results of HW/BW ratio, LW/BW ratio, and myocyte cross-sectional areas (n=200 cells per group) at 4 weeks post-Ang II infusion in WT and HET mice. B, Echocardiography results from WT and HET mice at 4 weeks post-Ang II infusion. C, Gross heart and hematoxylin-eosin staining of 4 weeks of Ang II–infused mice. Scale bar, Gross heart is 20 mm. Hematoxylin-eosin staining is 50 μm. Data represent typical results of 3 to 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.
To further examine the role of cFLIP on MEK-ERK1/2 signaling in the heart, we exposed cultured neonatal rat cardiomyocytes to 1 μmol/L of Ang II infected with Ad-cFLIP or Ad-shcFLIP (recombinant adenoviral vector cFLIP short hairpin RNA). At the beginning, we screened 3 shcFLIP and found that No. 2 shcFLIP markedly inhibited cFLIP expression in cardiac myocytes (Figure S2A). Therefore, we chose No. 2 shcFLIP for the following experiments. Further studies showed that Ang II induced a significant phosphorylation of MEK1/2 and ERK1/2 that was almost completely blocked and sustained for all of the tested time points by overexpression of cFLIP (Figure S2B and S2C). More importantly, decreased cFLIP levels by infection of Ad-shcFLIP resulted in pronounced activation of MEK1/2 and ERK1/2 in cardiac myocytes (Figure S2B and S2C). Our findings suggest that cFLIP inhibits MEK-ERK1/2 signaling in vitro and in vivo in response to hypertrophic stimuli. To examine whether MEK-ERK signaling has a causative role in cFLIP-mediated inhibition of cardiac hypertrophy, further in vitro experiments were performed. As expected, decreased cFLIP levels led to pronounced hypertrophy induced by Ang II as assessed by surface area measurements (Figure S2D). This response was strongly blunted by U0126, an MEK inhibitor that prevented ERK1/2 phosphorylation. These results suggest that cFLIP inhibits cardiac hypertrophy through direct inhibition of MEK-ERK1/2 signaling in cardiac myocytes. The inhibition of MEK1/2 and ERK1/2 phosphorylation by cFLIP prompted us to investigate whether cFLIP directly interacts with MEK-ERK signaling. Inconsistent with recent findings that demonstrated the direct interaction of cFLIP with mitogen-activated protein kinase kinase 7 (MKK7) to suppress JNK activation,17 our immunoprecipitation analysis showed that cFLIP directly interacted with MEK1 (Figure S2E).

Gain- and loss-of-function analyses have indicated that the MEK1-ERK1/2 pathway activates critical transcription factors, including GATA-4, required for the activation of cardiac genes during hypertrophy.18-20 As expected, decreased cFLIP level enhanced GATA-4 phosphorylation and its DNA-binding activity induced by Ang II infusion (Figure 2B). In addition, we found that Ang II-induced GATA-4 phosphorylation and DNA-binding activity were almost completely inhibited by infection with dominant-negative ERK1/2 (Ad-dnERK) or treatment with U0126 but were augmented by infection with constitutive-active ERK1/2 (Ad-caERK1/2; Figure S2F). These data suggest that GATA-4 phosphorylation and activation require MEK-ERK1/2 signaling in cardiac myocytes.

**Decreased cFLIP Level Exacerbated Cardiac Fibrosis Induced by Ang II In Vivo**

Cardiac hypertrophy is associated with increased fibrosis in the myocardium, characterized by the overproduction of extracellular matrix proteins.1 To determine the extent of fibrosis in the heart, paraffin-embedded slides were stained with PSR. Marked perivascular fibrosis and interstitial fibrosis were present in the WT mice subjected to Ang II infusion by PSR staining but much more prominent in the HET mice (Figure 3A). Subsequent analysis of mRNA expression levels

![Figure 2. The effect of cFLIP on MEK-ERK1/2 signaling pathway. A, The effect of cFLIP on MEK1/2 and ERK1/2 phosphorylation and their total protein expression at 4 weeks post-Ang II infusion in WT and HET mice. B, cFLIP knockdown increased the phosphorylation and DNA-binding activity of GATA-4 induced by Ang II infusion (n=4). Top, Quantitative results. Bottom, Representative blots. Values are mean±SEM. The results were reproducible in 3 separate experiments.](image-url)
of known mediators of fibrosis, including transforming growth factor-$\beta 1$ ($Tgf\beta 1$), procollagen type I $\alpha 1$ ($Col1\alpha1$), procollagen type III $\alpha 1$ ($Col3\alpha1$), plasminogen activator inhibitor 1 ($Pai-1$), and connective tissue growth factor ($Ctgf$), demonstrated an increased response in HET mice (Figure S3).

Effect of cFLIP on Transforming Growth Factor-$\beta 1$/Smad Signaling

Transforming growth factor (TGF)-$\beta 1$ induces collagen synthesis via activation of a number of transcription factors, including Smads.21 To further elucidate the cellular mechanisms underlying the antifibrotic effects of cFLIP, we assessed the regulatory role of cFLIP on Smad cascade activation. Our results showed that HET mice induced higher levels of phosphorylation of Smad 2 and more nuclear translocation of Smad 2/3 compared with that of WT mice (Figure 3B). We then infected cardiac fibroblasts with Ad-cFLIP or Ad-shcFLIP and treated with TGF-$\beta 1$ for specific time periods. Western blot analyses revealed significant phosphorylation of Smad 2 and translocation of Smad 2/3 without any significant alterations in Smad 2 after TGF-$\beta 1$ treatment. Ad-cFLIP infection, however, almost completely suppressed Smad 2 phosphorylation, as well as Smad 2/3 nuclear translocation (Figure S4A and S4B). Importantly, Ad-shcFLIP infection enhanced the effects of TGF-$\beta 1$ (Figure S4A and S4B). To further examine the mechanisms involved, we used confluent cardiac fibroblasts infected with Ad-GFP, Ad-caERK1/2, or Ad-dnERK1/2 or treated with U0126. Activation of ERK1/2 by infection with Ad-caERK1/2 revealed a significant increase, while blocking ERK1/2 activity by U0126 or Ad-dnERK1/2 almost completely abrogated collagen synthesis, as well as Smad 2 phosphorylation and Smad 2/3 nuclear translocation in response to TGF-$\beta 1$ (Figure S4C and S4D). These findings suggest that cFLIP blocks collagen synthesis by disrupting MEK-ERK1/2–dependent TGF-$\beta 1$/Smad signaling.

Characterization of Human cFLIP$_L$ TG Mice

The above findings suggested that increased cFLIP levels in the heart will attenuate cardiac remodeling. To confirm this notion, we generated TG mice with full-length human cFLIP$_L$ cDNA under the control of the $\alpha$-myosin heavy chain promoter (Figure S5A). Four lines of TG mice were confirmed by PCR. All of the experiments reported were per-
Figure 4. Human cFLIP<sub>TG</sub> mice develop decreased cardiac hypertrophy and fibrosis. A, Statistical results of HW/BW ratio, LW/BW ratio, and myocyte cross-sectional areas at 4 weeks post-Ang II infusion in WT and TG mice. B, Echocardiography results from WT and TG mice. C, Histological images showing cardiac and lung sections from WT and TG mice treated with saline or Ang II. D, Immunohistochemical analysis of LV collagen volume (%). E, Western blot analysis of P-Smad2/GAPDH and Smad/Lamin B1.
formed with male mice that were 7 to 8 weeks old. We analyzed cFLIP<sub>L</sub> protein levels in various tissues by Western blot analysis using a human-specific anti-cFLIP<sub>L</sub> antibody. We found a robust expression of human cFLIP<sub>L</sub> protein in the heart but did not detect it in other organs (Figure S5B). Among 4 established lines of TG mice, the line that expressed the highest levels of the human cFLIP<sub>L</sub> protein in the heart was used for further experiments (Figure S5C). Western blot analysis further demonstrated that the expression level of the mouse cFLIP<sub>L</sub> protein was not modified by expression of the human cFLIP<sub>L</sub> gene (Figure S5D).

**Human cFLIP<sub>L</sub> TG Mice Develop Decreased Cardiac Hypertrophy and Fibrosis**

The Ang II–induced increase in the ratios of HW/BW, LW/BW, and cardiomyocyte cross-sectional area were attenuated in TG mice compared with WT mice (Figure 4A). Cardiac-specific overexpression of cFLIP abrogated Ang II–induced cardiac chamber dilatation and wall thickness and dysfunction, as evidenced by improvements in LV end-systolic diameter, LV end-diastolic diameter, LV posterior wall thickness, increased LV diastolic septum, and fractional shortening (Figure 4B). Histology analysis further confirmed the inhibitory effect of cFLIP on cardiac hypertrophy mediated by Ang II infusion (Figure 4C). Consistent with the functional data, the mRNA expression levels of hypertrophy markers ANP, BNP, Myh7, and Acta1 were severely blunted in TG mice (Figure S5E). Further studies showed that the cardiomyocyte overexpression of cFLIP<sub>L</sub> abolished MEK and ERK1/2 phosphorylation, as well as GATA-4 phosphorylation and its DNA-binding activity induced by Ang II infusion (Figure S5F and S5G). Moreover, the Ang II–induced interstitial fibrosis and perivascular fibrosis were present in WT mice but were remarkably reduced in the TG mice (Figure 4D). Ang II treatment also led to a lower increase of the mRNA expression of fibrotic markers Tgf<sub>β</sub>1, Colα1, Colα3α1, Pai-1, and Ctgf in TG mice than in WT mice (Figure S5H). Further studies showed that the increased level of Smad 2 phosphorylation and Smad 2/3 nuclear translocation was attenuated in TG mice in response to Ang II infusion (Figure 4E). These results suggest that constitutive expression of cFLIP<sub>L</sub> in the heart leads to decreased cardiac hypertrophy and fibrosis in response to Ang II stimulation.

**Effect of cFLIP on Apoptosis**

It is well known that apoptosis is involved in pathological cardiac remodeling. Because cFLIP has been shown to protect cells from death receptor-mediated apoptosis, we further examined whether the cardioprotective role of cFLIP is related to its antiapoptotic effect in the Ang II–infused hypertrophic model. Myocardial tissue sections were stained with TUNEL staining to detect apoptosis. TUNEL-positive nuclei were present in control mice subjected to Ang II infusion, whereas their number was markedly increased in HET mice and was significantly decreased in TG mice (Figure S6A and S6B). To further elucidate the mechanisms underlying the antia apoptotic effect of cFLIP, we assessed the regulatory role of cFLIP on caspases activation. Ang II stimulation increased caspase 3, caspase 8, and caspase 9 activity modestly in control hearts and massively in HET hearts; however, the activation of these caspas es was rarely seen in TG hearts (Figure S6A and S6B). These results suggest that cFLIP inhibits cardiomyocyte apoptosis, at least in part, by blocking the activation of caspase 3, caspase 8, and caspase 9.

**Discussion**

Previous studies have demonstrated that the expression of cFLIP was significantly decreased in the ventricular myocardium of patients with heart failure. However, its function during cardiac hypertrophy was unclear. In the present study, we examined the role of cFLIP in cardiac hypertrophy by using cardiac-specific cFLIP<sub>L</sub> HET mice and TG mice. The results demonstrated that decreased levels of cFLIP protein expression in HET mice profoundly exaggerated hypertrophy, chamber dilatation, and fibrosis via enhancement of MEK-ERK1/2 signaling after Ang II stimulation. In contrast, elevated cFLIP levels cause a blunted response of pathological cardiac remodeling. These findings suggest that cFLIP protein is a crucial component of the signaling pathway involved in cardiac remodeling and heart failure.

The mechanisms underlying the antihypertrophic effects of cFLIP remain largely unclear. It is well known that the MAPK signaling pathway plays a critical role in the pathogenesis of cardiac hypertrophy. The MAPK pathway consists of a sequence of successively acting kinases, including p38, JNKs, and ERKs, and is initiated in cardiac myocytes by stress stimuli. To examine the molecular mechanisms involved in cFLIP’s ability to suppress cardiac hypertrophy, we examined the activation status of the MAPK pathway in our hypertrophic models. Our data showed that MEK activation and ERK1/2 activation were enhanced further by decreased cFLIP levels, whereas MEK and ERK1/2 phosphorylation levels were almost completely blocked by cardiac expression of human cFLIP<sub>L</sub> in response to Ang II stimulation. However, the phosphorylation of p38, JNK1/2, and Akt was not affected by cFLIP. Therefore, MEK-ERK1/2 signaling seems to be a critical pathway through which cFLIP influences cardiac hypertrophic response. In accordance with our finding, Giampietri et al reported that cFLIP mild overexpression reduces cardiac hypertrophy in response to pressure overload. However, they demonstrated that the antihypertrophic effect of cFLIP is probably achieved through blocking Akt/GSK3β signaling. The discrepancy on mechanisms may be related to a different promoter controlling cFLIP overexpression, the cardiac-specific α-myosin heavy chain promoter in our study and the testis-specific Stra

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**Figure 4 (Continued).** TG mice at 4 weeks post-Ang II infusion. C, Gross heart and hematoxylin-eosin staining at 4 weeks of Ang II-infused WT and TG mice. Scale bar, Gross heart is 20 mm. Hematoxylin-eosin staining is 50 μm. D, PSR staining (scale bar: 32 μm) and quantitative results of fibrotic areas. E, Immunoblot analysis of Smad 2 phosphorylation and Smad 2/3 translocation in indicated groups at 4 weeks of Ang II infusion. For A, B, and D, *P<0.01 was obtained for the WT/saline values; **P<0.01 was obtained for WT/Ang II after Ang II infusion. For E, *P<0.01 was obtained for WT/Ang II.
8 promoter in their study. More importantly, we performed gain- and loss-of-function analyses both in vivo and in vitro, confirming the MEK-ERK1/2–related mechanism in the antihypertrophic effect of cFLIP. Our further study demonstrated that cFLIP can bind directly to MEK1, disrupt its interactions, and finally block its activation. These findings are consistent with a recent study that showed that cFLIP binds to MKK7 and MEK1 in a tumor necrosis factor–dependent manner and abrogates their interactions with MEKK1 and apoptosis signal-regulating kinase 1.17 Conversely, the results of the present study conflict with previous observations that FLIP can spontaneously engage the signaling pathway, leading to the activation of ERK1/2 through recruitment of Raf-1 in T cells.27 In addition, a study from Lüschen et al28 demonstrated that overexpression of cFLIP leads to enhanced and prolonged activation of ERK after tumor necrosis factor treatment in Hela and HEK293 cells. The reason for the discrepancy in the role of cFLIP in the activation of MEK-ERK1/2 signaling remains unclear. We postulate that it may relate to differences in experimental models, cell types, or differences in the strains of mice.

The downstream molecular mechanisms involved in MEK-ERK1/mediated hypertrophy include transcription factors such as GATA-4. Recent studies revealed that ERK1/2 phosphorylates GATA-4 and increases its DNA-binding ability.29,30 In this study, we examined the potential role of cFLIP in regulating these mechanisms. We observed GATA-4 hyperphosphorylation and increased DNA-binding activity in response to hypertrophic stimuli, which is consistent with recent reports demonstrating that the hypertrophic stimuli trigger GATA-4 activation.29 Importantly, in our hypertrophic models, forced expression of cFLIP abolished GATA-4 phosphorylation and DNA-binding activity, whereas decreased cFLIP levels resulted in augmentation. Furthermore, the inhibitory effects of cFLIP on cardiac hypertrophy, as well as on GATA-4 phosphorylation and activation, were through inhibition of MEK-ERK1/2 signaling. These findings suggest that cFLIP blocks cardiac hypertrophy by switching off MEK-ERK1/2–dependent GATA-4 activation.

Cardiac fibrosis, which is characterized by interstitial and perivascular fibrosis, is another classic feature of pathological cardiac hypertrophy, and approaches to limit collagen deposition in the heart have been limited to date.31,32 This study for the first time revealed that cFLIP blocks cardiac fibrosis in vivo and inhibits TGF-β1–induced collagen synthesis in vitro. In an attempt to elucidate the mechanisms underlying the antifibrotic effect of cFLIP, we analyzed key components of TGF-β1-Smad signaling, which is a crucial pathway in the regulation of fibrosis.10 Our results demonstrated that cFLIP abrogates Smad 2 phosphorylation and Smad 2/3 translocation in both cultured cardiac fibroblasts and hypertrophied hearts, thus inhibiting collagen synthesis and fibrosis. Recent studies suggest that the TGF-β1/Smad pathway can be regulated by the MEK-ERK1/2 pathway.33 We further investigated the effects of MEK-ERK1/2 activation on fibrosis and found that blocking MEK-ERK1/2 activation results in significant inhibition, whereas activation of MEK-ERK1/2 resulted in upregulation of collagen synthesis, as well as Smad 2 phosphorylation and Smad 2/3 translocation. The results indicate that cFLIP blocks cardiac fibrosis by inhibiting the MEK-ERK1/2 pathway.

In conclusion, this is the first study that defines the role of cFLIP in reducing cardiac remodeling in response to hypertrophic stimuli. The molecular mechanisms for the cardioprotective effects of cFLIP, at least in part, ascribe to inhibition of the MEK-ERK1/2 signaling pathway. We propose that targeting of the cFLIP signaling pathway may develop novel promising strategies for the treatment of cardiac hypertrophy and heart failure.

Perspectives
In the present study, we provide evidence for the protective role of cFLIP in cardiac remodeling through the use of Ang II–induced hypertrophic model in cardiac-specific cFLIP TG mice and cFLIP heterozygous knockout mice. Therapies designed to overexpress cFLIP in the heart might be beneficial in the prevention and treatment of cardiac hypertrophy and heart failure worthy of further validation and investigation.

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Disclosures
None.

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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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ONLINE SUPPLEMENT

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. $[^3]$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-β1 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 α-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 α-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 [γ-³²P] ATP using T₄ polynucleotide kinase and separated from unincorporated [γ-³²P] 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, Stratagene). 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 1×10⁶/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, 1×10⁵/well cardiac myocytes or cardiac fibroblasts were cultured in 6-well plates and exposed to 2×10⁸ pfu each of virus in 1 ml of serum-free medium for 24
hours. 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 $[^3]$H-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 $[^3]$H-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


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 normalized to GAPDH.

C

Saline Ang II

D

Sham 1W 2W 4W 8W

E

WT HET Saline WT HET Ang II

WT HET Saline WT HET Ang II

WT HET Saline WT HET Ang II

WT HET Saline WT HET Ang II
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 S2

A

B

C

Ad-LacZ+Ang II  Ad-cFLIP+Ang II  Ad-lacZ  Ad-cFLIP  Ad-shRNA  Ad-shcFLIP

0  15  30  60  0  15  30  60

P-MEK1/2
T-MEK1/2
P-ERK1/2
T-ERK1/2

GAPDH

P-MEK1/2
P-ERK1/2

Normalized proteins/GAPDH

Minutes
0  15  30  60  0  15  30  60  0  15  30  60  0  15  30  60

* * *
D) PBS, Ang II, Ang II+U0126

Ad-chRNA

Ad-sheFLIP

E)

Flag-ERK + 
HA-cFLIP-L - 
IB: 
Flag 
HA 
IP: Flag 
HA 
WCE 
Flag 
P: HA 
Flag 
HA 
Flag 
HA 
Flag 
HA 
Flag 
HA 
Flag 
HA

F)

P-GATA4 
GAPDH 
GATA4 
Oct-1 

Control 
Ang II 
Ad-caERK12 
Ad-caERK12+Ang II 
U0126+Ang II 

Normalized P-GATA4/GAPDH 

GATA DNA Binding activity (%) 

Control 
Ang II 
Ad-caERK12 
Ad-caERK12+Ang II 
U0126+Ang II 

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 \( \text{Tgfb}1 \), \( \text{Col1a1} \), \( \text{Col3a1} \), \( \text{Pai-1} \), and \( \text{Ctgf} \) 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. 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

WT1 WT2 WT3 WT4 Tg1 Tg2 Tg3 Tg4

Human cFLIP-L

GAPDH

WT TG

Human cFLIP-L

mouse cFLIP-L

GAPDH

ANP

BNP

Myh7

Acta1

Fold Change

WT TG

Saline Ang II

WT TG

Saline Ang II

WT TG

Saline Ang II

WT TG

Saline Ang II

WT TG

Saline Ang II

WT TG

Saline Ang II

* $
Figure S5. Human cFLIP_L transgenic mice develop decreased cardiac hypertrophy and fibrosis. (A) Diagram of the transgenic construct used for the generation of cFLIP_L TG mice. (B) Representative Western blot of human cFLIP_L protein from different tissue of TG mice as indicated. (C) Representative Western blots of human cFLIP_L protein in the heart tissue from 4 lines of both TG and WT mice. (D) Representative Western blots of transgenic cFLIP_L and endogenous cFLIP_L 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, Col1α1, Col3α1, 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.