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Tumor Suppressor A20 Protects Against Cardiac Hypertrophy and Fibrosis by Blocking Transforming Growth Factor-β–Activated Kinase 1–Dependent Signaling

He Huang, Qi-Zhu Tang, Ai-Bing Wang, Manyin Chen, Ling Yan, Chen Liu, Hong Jiang, Qinglin Yang, Zhou-Yan Bian, Xue Bai, Li-Hua Zhu, Lang Wang, Hongliang Li

Abstract—A20 or tumor necrosis factor–induced protein 3 is a negative regulator of nuclear factor κB signaling. A20 has been shown previously to attenuate cardiac hypertrophy in vitro and postmyocardial infarction remodeling in vivo. In the present study, we tested the hypothesis that overexpression of A20 in the murine heart would protect against cardiac hypertrophy in vivo. The effects of constitutive human A20 expression on cardiac hypertrophy were investigated using in vitro and in vivo models. Cardiac hypertrophy was produced by aortic banding in A20 transgenic mice and control animals. The extent of cardiac hypertrophy was quantitated by echocardiography, as well as by pathological and molecular analyses of heart samples. Constitutive overexpression of human A20 in the murine heart attenuated the hypertrophic response and markedly reduced inflammation, apoptosis, and fibrosis. Cardiac function was also preserved in hearts with increased A20 levels in response to hypertrophic stimuli. Western blot experiments further showed A20 expression markedly blocked transforming growth factor-β–activated kinase 1–dependent c-Jun N-terminal kinase/p38 signaling cascade but with no difference in either extracellular signal-regulated kinase 1/2 or AKT activation in vivo and in vitro. In cultured neonatal rat cardiac myocytes, [3H]proline incorporation and Western blot assays revealed that A20 expression suppressed transforming growth factor-β–induced collagen synthesis and transforming growth factor-β–activated kinase 1–dependent Smad 2/3/4 activation. In conclusion, A20 improves cardiac functions and inhibits cardiac hypertrophy, inflammation, apoptosis, and fibrosis by blocking transforming growth factor-β–activated kinase 1–dependent signaling. (Hypertension. 2010;56:232-239.)

Key Words: A20 ■ cardiac remodeling ■ inflammation ■ apoptosis

Heart failure is increasing in prevalence and is a debilitating disease with high rates of mortality and morbidity.1,2 Cardiac hypertrophy is a common precursor to many forms of heart failure, of which molecular and cellular determinants remain largely unknown. After a period of compensatory adaptation, hypertrophy is associated with functional and histological deterioration of the myocardium, fibrosis, inflammation, and altered cardiac gene expression.3,4 Accumulating evidence suggests that the nuclear factor κB (NF-κB) signaling system is a critical regulator of this process.5–7 Modulation of NF-κB signaling in the heart may provide a novel approach to attenuate the development of heart failure after cardiac hypertrophy.

A20 is a zinc finger protein originally identified as a tumor necrosis factor (TNF)–responsive gene in endothelial cells.8 It is an inducible and broadly expressed cytoplasmic protein that inhibits TNF-induced NF-κB activity. Recent studies showed that A20 expression protects various cell types from TNF-mediated apoptosis.9,10 We also found that A20 expression protects against oxidized low density lipoprotein–induced macrophage apoptosis and inhibits the proliferation of vascular smooth muscle cells.11,12 A20-deficient mice demonstrate spontaneous inflammation, cachexia, and premature death, and A20-deficient fibroblasts cannot properly terminate TNF-induced NF-κB activity.13 A20 is also an inducible ubiquitin-editing enzyme that restricts both toll-like receptor and TNF-induced responses by regulating the ubiquitination of key signaling proteins.14 Our data demonstrated that forced expression of A20 in the heart resulted in markedly improved functional recovery, decreased inflammation, reduced apo-
ptosis, and diminished interstitial fibrosis after acute myocardial infarction.\textsuperscript{15} Cook et al\textsuperscript{16} reported that A20 is dynamically regulated during acute biomechanical stress in the heart and functions to attenuate cardiac hypertrophy in vitro. Despite the potentially significant roles of A20 in attenuating NF-κB–dependent apoptotic, inflammatory, and hypertrophic signaling, it has remained unclear whether A20 could regulate cardiac hypertrophy in vivo and whether targeted myocardial overexpression of A20 is cardioprotective. Thus, in the present study, our aim was to investigate the role of A20 in cardiac hypertrophy mediated by pressure overload and to clarify the related molecular mechanisms.

**Methods and Materials**

**Materials**

The antibodies against extracellular signal-regulated kinase (ERK) 1/2, p38, c-Jun N-terminal kinase (JNK), caspase 3/8/9, phospho-Smad2, transforming growth factor (TGF)-β–activated kinase 1 (TAK1), phospho-p65, inhibitor of NF-κB (IκB) kinase (IKK)-α, IKKβ, phospho-IκBα, and IκBα were purchased from Cell Signaling Technology. [\textsuperscript{3}H]-leucine and [\textsuperscript{3}H]-proline were purchased from Amersham. The details for other reagents and adenoviral are given in the online Data Supplement.

**Animals, Aortic Banding Surgery, Blood Pressure, and Echocardiography**

The study protocol was approved by the animal care and use committee of our hospital. The details for mice information, aortic banding (AB) model, echocardiography, and cardiac catheterization are given in the online Data Supplement. Hearts and lungs of euthanized mice were dissected and weighed to compare heart weight/body weight (in milligrams per gram) and lung weight/body weight (in milligrams per gram) in transgenic (TG) and control mice.

**Histological Analysis and Determination of Apoptosis**

Several sections of heart (4 to 5 μm thick) were prepared and stained with hematoxylin-eosin for histopathology or Picrosirius red 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. Apoptosis was evaluated by TUNEL assay and caspase activities assay.

**Western Blot Analysis, Northern Blot, and Reporter Assays**

For Northern blot and Western blot analyses, as well as atrial natriuretic peptide promoter activity, please see the online Data Supplement.

**Electrophoretic Mobility Shift Assay, IKK Assay, and TAK1 Kinase Assay**

Electrophoretic mobility shift assays were performed according to the manufacturer’s instructions (Gel Shift Assay System E3300, Promega). Nuclear proteins were isolated using our method described previously.\textsuperscript{6,7} To determine the effect of A20 on IKK activation, the IKK assay was performed as described previously.\textsuperscript{6,7} TAK1 immunoprecipitates were assayed using His-MKK6 as a substrate, as described previously.\textsuperscript{17}

**Cultured Neonatal Rat Cardiac Myocytes and Fibroblasts**

Primary cultures of cardiac myocytes and fibroblasts were prepared as described.\textsuperscript{12–14} For details, please see the online Data Supplement.

**Results**

**Forced A20 Expression Attenuates Pathological Cardiac Hypertrophy**

To investigate the role of A20 in biomechanical stress in the heart, we performed AB surgery on 8- to 10-week–old TG and wild-type (WT) mice. As shown in Table S1, heart weight/body weight and lung weight/body weight ratios were significantly decreased in TG mice compared with WT mice. Cardiac function was examined by echocardiography after 8 weeks of surgery. The increases in left ventricle chamber dimensions and wall thickness induced by pressure overload were also markedly reduced during both systole and diastole in TG mice compared with WT littermates (Table S1). Gross heart and hematoxylin-eosin staining further confirmed the inhibitory effect of A20 on cardiac remodeling in response to AB (Figure 1). We examined the expression of several cardiac hypertrophy markers in TG and WT mice after AB surgery by Northern blot analysis. Expression levels of atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain were induced to a higher level in WT mice after AB, and such increases were markedly attenuated in TG mice (Figure S1). These results indicate that A20 overexpression in cardiomyocytes decreases the expression of cardiac hypertro-
phy markers atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain and results in attenuated cardiac hypertrophy induced by pressure overload.

**Forced A20 Expression Attenuates Mechanical Stress-Mediated p38/JNK1/2 Signaling**

To examine the molecular mechanisms of A20 on cardiac hypertrophy, we investigated activation of the mitogen-activated protein kinase (MAPK) pathway in our hypertrophic models. We found that the phosphorylated levels of p38, JNK1/2, and ERK1/2 were significantly increased by AB in WT hearts. However, the phosphorylation of p38 and JNK1/2 was almost completely blocked in TG hearts, whereas ERK1/2 activation was similar in the 2 groups after AB (Figure 2). 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 TG mice, as determined by immunoblotting for phosphorylation of AKT (Figure 2). Collectively, these data suggest that A20 overexpression suppresses the activation of p38 and JNK, although it has no effects on ERK1/2 or AKT activation in hearts subjected to AB. In vitro studies further demonstrated that p38 and JNK phosphorylation levels were enhanced after the reduction of A20 expression by RNA interference in response to hypertrophic stimuli. In contrast, p38 and JNK activations were almost completely blocked by increased A20 expression in cultured cardiac myocytes (Figure S2). These findings suggest that p38/JNK signaling was critical to the influence of A20 on cardiac hypertrophy.

**Forced A20 Expression Impairs TAK1 Signaling Involved in Hypertrophy**

Activation of TAK1, an upstream regulator of p38 and JNK, has been shown to participate in cardiac dysfunction after the development of hypertrophy. We, therefore, determined cardiac TAK1 activation by in vitro kinase activity assay. TAK1 activity was markedly increased in response to AB in WT mice (Figure 3). In the AB model, TAK1 activity was increased at 24 hours, peaked after 4 weeks, and then decreased, although the level remained higher than in the sham group. In contrast, the activity of TAK1 in response to AB was significantly abolished in TG hearts (Figure 3), suggesting that A20 overexpression may suppress TAK1 activation. The total protein level of TAK1 was not different among all of the tested groups. Consistent with our in vivo results, in vitro results showed that overexpression of A20 by infection of AdA20 blocked angiotensin (Ang) II–induced TAK1 activation, whereas downregulation of A20 expression by infection with AdsA20 promoted angiotensin II–induced TAK1 activation (Figure S3). To further investigate the molecular mechanisms of the function of A20, we examined the effects of TAK1 activation on p38/JNK and cardiac hypertrophy. Blocking TAK1 activation by dominant-negative TAK1 (AddhTAK1) abrogated angiotensin II–mediated p38/JNK phosphorylation and cardiac hypertrophy, whereas activation of TAK1 by constitutively active TAK1 (AdcaTAK1) augmented these effects, as demonstrated by Western blot, ANF promoter activity, and [3H]-leucine incorporation (Figure S4 and S5). These results indicate that A20 attenuates cardiac hypertrophy by blocking TAK1-dependent JNK/p38 signaling pathways.

**Forced A20 Expression Attenuates Fibrosis In Vivo**

Heart sections were stained with Picrosirius red to detect fibrosis. In both groups, collagen continued to accumulate in the heart after 8 weeks of AB. As shown in Figure 4A, Picrosirius red staining and quantitative analysis showed that increased collagen deposition was significantly attenuated in TG mice. Reduced fibrosis in TG mice may represent increased collagen degradation or decreased collagen synthesis in response to tissue damage. We, therefore, examined the synthesis of collagen by examining the expression of mRNA and protein-encoding connective tissue growth factor, collagen I, collagen III, TGF-β1, and TGF-β3, known to be involved in the proliferation of cardiac fibroblasts and the biosynthesis of extracellular matrix proteins. The results showed that connective tissue growth factor, collagen I, collagen III, TGF-β1, and TGF-β3 mRNA, as well as protein expressions, were significantly lower in TG than in WT mice in response to hypertrophic stimuli (Figures 4B and S6). We then assessed the regulatory role of A20 in Smad cascade activation. TG animals showed suppressed Smad-2 phosphorylation, and almost complete inhibition of Smad-2/3/4 nuclear translocation but negligible effects on Smad-2/3/4 protein expression (Figure S7).
Forced A20 Expression Inhibits Collagen Synthesis Induced by TGF-β1 In Vitro

To confirm our in vivo fibrosis data, we examined the potential antifibrotic effect of A20 by [3H]-proline incorporation assay in cardiac fibroblasts. Cells were infected with AdA20 or AdsiA20 for 24 hours, then serum starved for 24 hours in 0.5% FCS, and subsequently treated with different concentrations of TGF-β1 for 48 hours or with 15 ng/mL of TGF-β1 for the indicated time. TGF-β1 stimulated [3H]-proline incorporation in a time- and dose-dependent manner (data not shown). More importantly, overexpression of A20 by infection of AdA20 inhibited TGF-β1–induced [3H]-proline incorporation, connective tissue growth factor, and collagen I/III protein expression. Conversely, downregulation of A20 expression by infection with AdsiA20 promoted these effects (Figure 5). To further investigate the molecular mechanisms of A20 on fibrosis, we examined the effects of A20 on Smad signaling. Western blot analysis revealed significant phosphorylation of Smad 2 and translocation of Smad 2/3/4 without any significant alterations in Smad 2/3/4 protein expression after TGF-β1 treatment in adenovirus containing green fluorescent protein (AdGFP) and Adsi control groups (Figure S8). AdA20 infection, however, almost completely suppressed Smad 2 phosphorylation, as well as Smad-2/3/4 nuclear translocation, whereas AdsiA20 enhanced these effects (Figure S8).

We then examined the effects of A20 on TGF-β1-induced TAK1 activity. Our further experiments demonstrated that forced expression of A20 significantly blocked TAK1 activity mediated by TGF-β1, whereas decreased A20 expression promoted TAK1 activity in cultured cardiac fibroblasts (Figure S9). Confluent cardiac fibroblasts were infected with AdGFP, AdcaTAK1, or AddnTAK1 and incubated with TGF-β1 for an indicated time. Activation of TAK1 induced a significant increase in collagen synthesis by TGF-β1, whereas blocking TAK1 activity by infection with AddnTAK1 almost completely abrogated the TGF/β1-induced responses (Figure S10). Further-

Figure 3. Forced A20 expression impairs TAK1 signaling involved in hypertrophy. The TAK1 activity and TAK1 protein expression in hearts tissues of mice from indicated groups in WT and TG mice (n = 5). Values are mean ± SEM. * P < 0.01 for difference from WT/AB after AB.

Figure 4. The effects of A20 on fibrosis in vivo. A, Picrosirius red staining on histological sections of the left ventricle (LV) was performed on indicated groups after 8 weeks AB. The magnification of images were ×400. Fibrotic areas from histological sections were quantified using an image-analyzing system (n = 5). * P < 0.01 vs WT/sham. # P < 0.01 vs WT/AB after AB. B, Northern blot analyses of connective tissue growth factor (CTGF), collagen I, collagen III, TGF-β1, and TGF-β3 were performed to determine mRNA expression levels in indicated groups. GAPDH was used as the normalization control. Data represent typical results of 3 different experiments as mean ± SEM (n = 4 to 5 mice per group). * P < 0.01 vs WT/sham. # P < 0.01 vs WT/AB after AB.
more, immunoblot analysis demonstrated that TGF-β1 incubation of cardiac fibroblasts infected with AdcaTAK1 resulted in markedly increased phosphorylation of Smad-2 and nuclear translocation of Smad-2/3/4 in response to TGF-β1. Conversely, infection with AddnTAK1 almost completely blocked these effects (Figure S11).

**A20 Expression Inhibits Apoptosis and Inflammatory Response Induced by AB**

We next examined the effects of A20 on apoptosis by TUNEL assays after 8 weeks of AB. Apoptotic cells were detected in TG and control mice, and the fraction of apoptotic versus total cells was significantly lower in TG mice than in WT mice (Figure 6A). To determine whether TG mice are resistant to apoptotic signals, we examined the cleavage of caspase 3, caspase 8, and caspase 9, as well as that of poly (ADP-ribose) polymerase (PARP). As expected, TG mice displayed a significant delay of cleavage of caspase 3, caspase 8, and caspase 9, as well as PARP degradation in response to AB (Figure 6B). To determine whether expression of A20 prevents the inflammatory responses in the hearts, cytokine induction was characterized by Western blot analyses. TG mice have significantly lower TNF-α, interleukin 6, and monocyte chemoattractant protein 1 protein levels in cardiac tissue after 8 weeks of surgery compared with WT mice (Figure S12). To determine the molecular mechanisms by which A20 attenuated cytokine induction in vivo, we analyzed NF-κB signaling pathways. We detected NF-κB activation, IKKβ, and IκBα phosphorylation, as well as IκBα degradation, clearly after 8 weeks of AB in WT mice. Interestingly, NF-κB activation, IKKβ and IκBα phosphorylation, and IκBα degradation were evidently blocked in TG mice (Figure S13).

**Discussion**

In the present study, we demonstrate that the expression of A20 in the heart protects against cardiac hypertrophy. The cardioprotection of A20 is mediated by interruption of TAK1 activity–dependent signaling pathways (Figure 7). This results in the

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**Figure 5.** The effects of A20 on fibrosis in vitro. The effects of A20 on TGF-β1–induced [³H]-proline incorporation and protein expression of collagen I and collagen III. Cardiac fibroblasts were infected with AdA20, Adsi-control, AdGFP, or AdsiA20 for 24 hours and then incubated with 10 ng/mL of TGF-β1 for an indicated time to observe [³H]-proline incorporation and protein expression. *P<0.01 vs AdGFP+10 ng/mL of TGF-β1 group at the 0 time point. Data represent typical results of 3 different experiments as mean±SEM.

**Figure 6.** The effect of A20 on apoptosis. A, TUNEL–positive cells from histological sections were quantified (n=5). *P<0.01 vs WT/sham, #P<0.01 vs WT/AB after AB. B, Western blot analysis of the cleavage caspase 3, caspase 8, caspase 9, and PARP in response to AB (n=5).
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Figure 7. Proposed model of the process of A20. In the present study we demonstrated that the inhibitory effects of A20 are achieved by blocking 4 proposed signaling pathways. First, activated TAK1 results in activation of p38 and JNK signaling pathways, enhances hypertrophic markers expression, and subsequently leads to cardiac hypertrophy. Second, TAK1 is shown to promote the Smad signaling, increase expression of fibrotic markers, and result in collagen synthesis and fibrosis. Third, activation of TAK1 leads to signaling through the IKKβ/IκBα/NF-κB pathway, promoting proinflammatory cytokine expression and leading to inflammation. Fourth, hypertrophic stimuli also activate apoptotic signaling, ultimately leading to apoptosis. A20 blocks these proposed TAK1-dependent signaling pathways and apoptotic signaling and then protects against cardiac hypertrophy, fibrosis, inflammation, and apoptosis, finally preventing the progression of cardiac remodeling and heart failure.

The MAPK signaling cascade is initiated in cardiac myocytes by activation of G protein–coupled receptors, receptor tyrosine kinases, and stress stimuli.19,20 Once activated, downstream p38, JNKs, and ERKs each phosphorylate a wide array of intracellular targets, including numerous transcription factors, resulting in the reprogramming of cardiac gene expression. A significant finding of the present study is that the increase in JNK and p38 phosphorylation levels in response to hypertrophic stimuli was almost completely blocked in TG mice. The phosphorylations of ERK1/2 and AKT in myocytes were not affected by A20 expression. Further in vitro studies showed that inhibition of A20 expression significantly enhanced the activation of JNK and p38 but not that of ERK1/2 and AKT. Therefore, JNK/p38 signaling was the mediator of influences of A20 on cardiac myocyte growth. Suppression of the JNK/p38 signaling pathway by A20 in the heart attenuates cardiac remodeling. However, there is still some controversy on whether activation of the JNK and p38 MAPK pathway is protective or detrimental. Blocking JNK or p38 signaling by either genetic or pharmacological approaches has previously demonstrated cardioprotective effects.21,22 In contrast, other studies suggest that JNK/p38 signaling may protect against apoptosis.23,24 Another study found that dual JNK/p38 inhibition also leads to increased apoptosis in the heart25; however, this report also shows that the proapoptotic effects of the dual JNK/p38 inhibitor are possibly attributable to suppression of JNK, as opposed to p38 MAPK. These previous studies suggest that, although inhibition/activation of either p38 or JNK pathways produces the same cardiac phenotype, the temporal manifestation of the disease possibly depends on the overall extent of cellular signaling inhibition/activation and especially the upstream molecules of JNK/p38MAPK. This view is further supported by 2 recent studies on mixed-lineage kinase 7 and heat shock protein 20.26,27 Mixed-lineage kinase 7 was reported to activate both JNK and p38 MAPK, and overexpression of mixed-lineage kinase 7 resulted in cardiac hypertrophy and promoted cell death in the heart, indicating that dual activation of JNK/p38 is more catastrophic than either alone. Another study showed that heat shock protein 20 overexpression blocks cardiac hypertrophy and fibrosis through inhibition of the ASK1-p38/JNK cascade.29 These findings suggest that inhibition of the upstream regulator of p38 and JNK may be beneficial in halting cardiac remodeling and the progression of heart failure.

To further investigate the molecular mechanisms by which A20 inhibits cardiac hypertrophy, we examined another protein upstream of p38/JNK, TAK1. TAK1 is an MAPK kinase kinase family member originally identified as a mediator in the TGF-β signaling pathway and can be activated in response to stress stimuli.28 Genetic and biochemical evidence has established TAK1 as a key kinase that mediates the activation of IKK, p38, and JNK by diverse cellular stimuli.29 Recent studies showed that TAK1 is critically important in the cardiac hypertrophic response.30 We found that A20 not only suppressed TAK1 activity in vivo in response to hypertrophic stimuli but also blocked TAK1 activity induced by angiotensin II in vitro. Our in vitro study also showed that a decreased A20 expression level effectively enhanced TAK1 activity resulting from angiotensin II. In addition, our data confirmed that inhibition of TAK1 activity abrogated the activation of JNK/p38, whereas activation of TAK1 activity augmented the phosphorylation of JNK/p38 in response to hypertrophic stimuli in vitro. These findings indicate that A20 attenuates cardiac hypertrophy by blocking TAK1-JNK/p38 signaling.
Cardiac fibrosis is another classic feature of pathological hypertrophy and is characterized by the expansion of the extracellular matrix attributed to the accumulation of collagen. Thus, it is important to understand the mechanisms that stimulate collagen deposition in the heart and define approaches to limit these processes. We found that A20 blocks cardiac fibrosis in vivo and inhibits collagen synthesis in vitro. Our study demonstrated, for the first time, that A20 blocks AB-induced fibrosis in vivo and TGF-β1-induced collagen synthesis in cardiac fibroblasts. In addition, our data suggest, for the first time, that A20 abrogates Smad 2 phosphorylation and Smad 2/3/4 translocation in both cardiac fibroblasts and hypertrophied hearts, thus inhibiting collagen synthesis and fibrosis. There is considerable evidence for synergy between the TAK1-dependent and Smad-dependent TGF-β signaling pathways. TAK1 has been reported to interact with Smad 7 to inhibit TGF-β signaling by a negative feedback mechanism. More recently, TAK1 has been shown to interact with Smads and to inhibit BMP signaling. The relative contributions, however, of TAK-dependent and Smad-dependent pathways to cardiac fibrosis remain undefined. We demonstrated that blocking TAK1 activation led to complete inhibition, whereas activation of TAK1 led to upregulation of collagen synthesis and Smad 2/3/4 activation in vitro. We also showed that TGF-β1-induced collagen synthesis depends on TAK1 signaling, indicating that the inhibitory effects of A20 on fibrosis and collagen synthesis are mediated by blocking TAK1-dependent signaling.


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In the *Hypertension* article by Huang et al (Huang H, Tang QZ, Wang AB, Chen M, Yan L, Liu C, Jiang H, Yang Q, Bian ZY, Bai X, Zhu LH, Wang L, Li H. Tumor Suppressor A20 Protects Against Cardiac Hypertrophy and Fibrosis by Blocking Transforming Growth Factor-β–Activated Kinase 1–Dependent Signaling. *Hypertension*. 2010;56:232–239), corrections have been made to Figure 1A. Parts of the images in Figure 1A were incorrectly shown in the published article. This change affects none of the observations or conclusions made in the article. The correct Figure 1 appears below.

The authors regret the error.
Tumor Suppressor A20 Protects against Cardiac Hypertrophy and Fibrosis through Blocking TAK1-Dependent Signaling

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Running Title: A20 inhibits cardiac hypertrophy

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Methods and Materials

Materials
The antibodies against ERK1/2, P38, JNK, Caspase-3/8/9, phospho-Smad2, TAK1, phospho-p65, IKKα, IKKβ, phospho-IκBα, and IκBα were purchased from Cell Signaling Technology. [3H]-leucine and [3H]-proline were purchased from Amersham. The BCA protein assay kit was purchased from Pierce and the IKK activity kit was obtained from B&D Bioscience. All other antibodies were purchased from Santa Cruz Biotechnology. TGF-β1 was purchased from R&D Systems. Fetal calf serum (FCS) was obtained from Hyclone. Wild type rat A20 cDNA (AdA20) and siA20 adenoviral (AdsiA20) were made as described previously. Cell culture reagents and all other reagents were obtained from Sigma.

Animals, Aortic banding surgery, Blood pressure and Echocardiography
All protocols were approved by institutional guidelines. All surgeries and subsequent analyses were performed in a fashion blinded for genotype. Transgenic mice were produced as described previously. We used 8-10 week-old male mice with cardiac-specific expression of human A20 and their control littermates. Genotyping was performed by polymerase chain reaction (PCR) as described previously. Aortic banding (AB) was performed as described previously. Age- and sex-matched WT and TG mice were anesthetized with isoflurane. A 7.0 nylon suture ligature was tied against a 27-gauge needle at the transverse aorta to produce a 65–70% constriction following removal of the needle. Doppler analysis was performed to ensure that physiologic constriction of the aorta was induced. Hearts and lungs of sacrificed mice were dissected and weighed to compare heart weight/body weight (HW/BW, mg/g) and lung weight/body weight (LW/BW, mg/g) in TG and control mice. A microtip catheter transducer (SPR-839, Millar Instruments, and Houston, Tex) was inserted into the right carotid artery and advanced into the left ventricle under pressure control. 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. 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 M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

Histological analysis and determination of apoptosis
Hearts were excised, placed in 10% potassium chloride solution, 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 hematoxylyn and eosin (H&E) 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 (NIH Image 1.6). The outline of 100 to 200 myocytes was traced in each group. Cell death by apoptosis was evaluated by a TUNEL assay that was performed in sections with use of the CardiaoTACS in situ Apoptosis Detection Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's recommendations. Caspase-3/8/9 activities were also used to examine the effects of A20 on apoptosis.
Western Blot Analysis and Northern blot

All procedures were performed as previously described. Protein extracts from different groups of myocardium (50 µg) were fractionated on a 10% polyacrylamide gel under reducing conditions, transferred to nitrocellulose membranes, and probed with various antibodies. After incubation with a secondary peroxidase-conjugated antibody, signals were visualized by Chemiluminescence kit (Amersham, Sunnyvale, CA). We Northern blot to detect mRNA levels of ANP, BNP, β-MHC, α-MHC, α-skeletal actin, and sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a), as well as fibrosis markers including TGFβ1, TGFβ2, CTGF, Collagen I and Collagen III. Total RNA was extracted from frozen, pulverized mouse tissues using TRIzol (Invitrogen). The detailed information for Northern blot was described in previous work.² We normalized results against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression.

Electrophoretic Mobility Shift Assay, IKK Assay and TAK1 kinase Assay

Electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega, Madison, WI). Nuclear proteins were isolated using our previously method.⁵, ⁶ Protein concentrations were measured by BCA Protein Assay Reagents (PIERCE, Rockford, IL) using bovine serum albumin (BSA) as a standard. To determine the effect of A20 on IKK activation, the IKK assay was performed as described previously.⁵, ⁶ TAK1 immunoprecipitates were assayed using His-MKK6 as substrate as described previously.⁷

Cultured neonatal rat cardiac myocytes and fibroblasts

Primary cultures of cardiac myocytes were prepared as described previously.³, ⁴ 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 6-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), then infected with different adenoviruses followed by Ang II (1 µM) treatment. 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.³ 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 infections, 1×10⁶/well cardiac myocytes or cardiac fibroblasts were cultured in 6-well plates and exposed to 2×10⁸ pfu of each 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. The viruses included AdA20 to overexpress A20, AdsiA20 to downregulate A20 expression, and their respective control viruses AdGFP and Adsi-control, as well as AddnTAK1 to block TAK1 activation, AdcaTAK1 to activate TAK1 and their control AdGFP.

[^3H]-Leucine incorporation and surface area

[^3H]-Leucine incorporation was measured as described previously.⁴, ⁵ Briefly, cardiac myocytes were infected with different adenoviruses for 24 hours and subsequently stimulated with Ang II (1 µM) and coincubated with[^3H]-leucine (2 µCi/mL) for the indicated time. At the end of the experiment, cells were washed with Hanks' solution, scraped off the well, and then treated with 10% trichloroacetic acid at 4°C for 60 minutes. The precipitates were then dissolved in NaOH (1
N) and subsequently counted with a scintillation counter. For surface areas, the cells 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 assay**
Collagen synthesis was evaluated by measuring [\(^3\text{H}\)]-proline incorporation as described previously.\(^3\) In brief, cardiac fibroblasts were infected with different adenoviruses, made quiescent by culturing in 0.1% FCS DMEM for 24 h, and subsequently incubated with TGF-β1 and 5 μCi/ml [\(^3\text{H}\)]-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 solutions and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number.

**Reporter assays**
Cardiac myocytes or cardiac fibroblasts were seeded in triplicate in 6-well plates. Cells were infected with different adenoviruses for 24 hours and then transfected with 0.5 μg of ANF luciferase reporter construct, and internal control plasmid DNA using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. Cardiomyocytes were then treated with Ang II and fibroblasts with TGF-β1. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer's protocol. The luciferase activity was normalized by control plasmid. All experiments were done in triplicate and repeated at least three times.

**Statistical Analysis**
All values are expressed as mean±SEM. Differences between two groups were determined by a Student’s t test. Comparison between groups on Western blotting data was assessed by One-Way ANOVA followed by a Bonferroni correction. A value of \(P<0.05\) was considered statistically significant.
References


Online Supplement Tables

Table S. Echocardiographic and anatomic data showed the effects of A20 on cardiac hypertrophy after 8 weeks aortic banding.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT-sham mice</th>
<th>TG-sham mice</th>
<th>WT-AB mice</th>
<th>TG-AB mice</th>
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<tr>
<td>Number</td>
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<td>n=15</td>
<td>n=13</td>
<td>n=14</td>
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<tr>
<td>BW (g)</td>
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<td>27.6±1.4</td>
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<td>7.87±0.17*</td>
<td>5.42±0.15†*</td>
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<td>LW/BW (mg/g)</td>
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<td>CSA (μm²)</td>
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<td>268±43</td>
<td>449±51*</td>
<td>311±27†*</td>
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<td>SBP (mmHg)</td>
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<td>109.7±1.8</td>
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<td>145.1±5.4*</td>
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<tr>
<td>HR (beats/min)</td>
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<td>498±43</td>
<td>532±47</td>
<td>501±37</td>
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<td>PWT (mm)</td>
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<td>1.20±0.01</td>
<td>2.47±0.04*</td>
<td>1.56±0.03†*</td>
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<td>LVEDD (mm)</td>
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<td>4.02±0.05†*</td>
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<td>FS (%)</td>
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<td>44.8±1.3†*</td>
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</table>

*P<0.01 was obtained for the WT-sham values; † P<0.01 was obtained for the WT-AB values after AB.

HR=heart rate; BW=body weight; HW=heart weight; CSA=cardiomyocyte cross-sectional area; PWT=posterior wall thickness; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; LVSD=left ventricular septum, diastolic; LVPWD=left ventricular posterior wall, diastolic. FS=fractional shortening. SBP=systolic blood pressure; All values are mean ± SEM.
Figure S1. Forced A20 expression attenuates the expression of cardiac hypertrophy markers in vivo.

Total RNA was isolated from hearts of mice of the indicated groups, and expression of ANP, BNP and β-MHC induced by AB were determined by Northern blot analysis. Data represent typical results of 3-4 different experiments as mean±SEM (n=5 mice/per group). *P<0.01 vs WT/sham. #P<0.01 vs WT/AB after AB.
**Figure S2**

**Figure S2.** Forced A20 expression attenuated mechanical stress-mediated activation of stress kinase activation *in vitro.*

ERK1/2, P38, JNK1/2 and AKT activation in cardiomyocytes after infection with different adenovirus. Upper, quantitative results of phosphorylation of P38 and JNK1/2. Bottom, Representative blots of total and phosphorylated ERK1/2, P38, JNK1/2 and AKT. Cardiomyocytes were infected with AdA20, AdGFP, AdsiA20 or Adsi-control for 24 and then incubated with 1 µM Ang II for indicated time. Values are mean±SEM *P<0.01 for difference from AdGFP+Ang II group at zero time point.
Figure S3. Forced A20 expression impairs TAK1 signaling involved in hypertrophy in vitro.
The TAK1 activity and TAK1 protein expression in cardiomyocytes after infection with different adenovirus. Upper, quantitative results. Bottom, Representative blots. Cardiomyocytes were infected with AdA20, AdGFP, and AdsiA20 or Adsi-control for 24 hours and then incubated with 1 µM Ang II for indicated time. Values are mean±SEM *P<0.01 for difference from AdGFP+Ang II group at zero time point.
Figure S4. The effects of TAK1 on the phosphorylation of P38 and JNK1/2 induced by Ang II treatment at time points indicated. Cardiomyocytes were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 1 µM Ang II for indicated time. Values are mean±SEM. *P<0.01 for difference from AdGFP+Ang II group at zero time point.
Figure S5. The effects of TAK1 on the $[^3$H]-Leucine incorporation and ANP promoter activity induced by Ang II treatment at time points indicated. Cardiomyocytes were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 1 µM Ang II for indicated time. Values are mean±SEM *$P<0.01$ for difference from AdGFP+Ang II group at zero time point.
**Figure S6**

Western blot analyses of CTGF, collagen I, collagen III, TGF-β1 and TGF-β3 were performed to determine protein expression levels in indicated groups. GAPDH was used as the normalization control. Data represent typical results of 3 different experiments as mean±SEM (n=4 to 5 mice/group). *P<0.01 vs WT/sham. †P<0.01 vs WT/AB after AB.

**Figure S6. The effects of A20 on the expression of fibrosis markers in vivo.** Western blot analyses of CTGF, collagen I, collagen III, TGF-β1 and TGF-β3 were performed to determine protein expression levels in indicated groups. GAPDH was used as the normalization control. Data represent typical results of 3 different experiments as mean±SEM (n=4 to 5 mice/per group). *P<0.01 vs WT/sham. †P<0.01 vs WT/AB after AB.
Figure S7. The effects of A20 on Smad signaling in vivo.
The effects of A20 on Smad 2 phosphorylation and Smad 2/3/4 protein expression as well as Smad 2/3/4 nuclear translocation in heart tissues in indicated groups. CE: Cytoplasmic extracts; NE: Nuclear extracts.
Figure S8. The effects of A20 on Smad signaling *in vitro*. 
The effects of A20 on Smad 2 phosphorylation and Smad 2/3/4 protein expression as well as Smad 2/3/4 nuclear translocation in cardiac fibroblasts. Cardiac fibroblasts were infected with AdA20, Adsi-control, AdGFP or AdsiA20 for 24 hours, and then incubated with 10 ng/mL TGFβ1 for indicated time.
Figure S9.

The effects of A20 on TGF-β1-induced TAK1 activity in vitro.
The TAK1 activity and TAK1 protein expression in cardiac fibroblasts after infection with different adenovirus. Upper, quantitative results. Bottom, Representative blots. Cardiac fibroblasts were infected with AdA20 or AdGFP, and AdsiA20 or Adsi-control for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM *P<0.01 for difference from AdGFP+ TGFβ1 group at zero time point.
Figure S10. The effects of TAK1 on $[^3]$H-proline incorporation.
Cardiac fibroblasts were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM. *$P<0.01$ for difference from AdGFP+ TGFβ1 group at zero time point.
Figure S11. The effects of TAK1 on Smad signaling \textit{in vitro}.

The effects of TAK1 on Smad 2 phosphorylation as well as Smad 2/3/4 nuclear translocation. Cardiac fibroblasts were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM. *$P<0.01$ for difference from AdGFP+ TGFβ1 group at zero time point.
Figure S12. The effect of A20 on proinflammatory cytokine induction.
Western blot analysis of MCP-1, TNF-α and IL-6 protein expression in the myocardium obtained from indicated groups at 8 weeks AB (n=5). Values are mean±SEM. The results were reproducible in three separate experiments. Each assay was performed in triplicate. *P<0.01 vs WT/sham. †P<0.01 vs WT/AB after AB.
Figure S13. The effect of A20 on NF-κB signaling pathways.
The DNA binding activity of NF-κB and level of IκBα phosphorylation and degradation induced by AB were determined by Western blot. Upper, EMSA results, Bottom, Western blot representative blots.