Heart

Novel Role for Caspase-Activated DNase in the Regulation of Pathological Cardiac Hypertrophy

Lu Gao, Kun Huang, Ding-Sheng Jiang, Xiaoxiong Liu, Dan Huang, Hongliang Li, Xiao-Dong Zhang, Kai Huang

Abstract—Caspase-activated DNase (CAD) is a double-strand-specific endonuclease that is responsible for the cleavage of nucleosomal spacer regions and subsequent chromatin condensation during apoptosis. Given that several endonucleases (eg, DNase I, DNase II, and Endog) have been shown to regulate pathological cardiac hypertrophy, we questioned whether CAD, which is critical for the induction of DNA fragmentation, plays a pivotal role in pressure overload–elicited cardiac hypertrophy. A CAD-knockout mouse model was generated and subjected to aortic banding for 8 weeks. The extent of cardiac hypertrophy was evaluated by echocardiography and pathological and molecular analyses. Our results demonstrated that the disruption of CAD attenuated pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. Conversely, transgenic mice with cardiac-specific overexpression of CAD showed an aggravated cardiac hypertrophic response to chronic pressure overload. Mechanistically, we discovered that the expression and activation of mitogen-activated protein kinase–extracellular signal-regulated kinase 1/2 was significantly reduced in the CAD-knockout hearts compared with the control hearts; however, they were greatly increased in the CAD-overexpressing hearts after aortic banding. Similar results were observed in ex vivo cultured neonatal rat cardiomyocytes after treatment with angiotensin II for 48 hours. These data indicate that CAD functions as a necessary modulator of the hypertrophic response by regulating the mitogen-activated protein kinase–extracellular signal-regulated kinase 1/2 signaling pathway in the heart. Our study suggests that CAD might be a novel target for the treatment of pathological cardiac hypertrophy and heart failure. (Hypertension. 2015;65:871-881. DOI: 10.1161/HYPERTENSIONAHA.114.04806.)

Key Words: apoptosis ■ CAD ■ cardiac hypertrophy ■ ERK1/2 ■ MEK1/2

Cardiac hypertrophy is a response of the myocardium to an increased workload and is characterized by an increase in myocardial mass and an accumulation of extracellular matrix.1-3 Although cardiac hypertrophy might initially represent an adaptive response of the myocardium, it frequently progresses to ventricular dilatation and heart failure. Heart failure is a debilitating disease with high morbidity and mortality rates, and its prevalence is increasing.4,5 Although hypertrophy was first described more than a century ago and several studies have identified the signal transduction pathways that promote hypertrophic responses, the mechanisms that regulate these pathways have not been clearly elucidated.6 A better understanding of these regulatory mechanisms in cardiac myocytes might lead to novel strategies for suppressing cardiac hypertrophy.

Caspase-activated DNase (CAD), also known as DNA fragmentation factor 40 kDa, is a double-strand-specific endonuclease responsible for the cleavage of nucleosomal spacer regions and subsequent chromatin condensation during apoptosis.7-11 In growing and nonapoptotic cells, CAD is maintained as an inactive complex combined with its inhibitor (inhibitor of CAD, also known as DNA fragmentation factor-45) and is predominantly localized in the cytoplasm.12-14 When cells are induced to die by apoptotic stimuli, such as Fas ligand or the kinase inhibitor staurosporine, caspases are activated. The activated caspases, predominantly caspase-3, cause CAD to be released from the CAD/inhibitor of CAD complex and subsequently transferred into the nucleus, where it is spontaneously activated.15,16 After activation, unobstructed CAD configures into a scissor-like dimer and cleaves genomic DNA with minimal sequence specificity. This CAD-mediated process is an important mechanism underlying caspase-3-dependent apoptotic nuclear alterations.17-19 CAD not only induces DNA fragmentation during apoptosis but also functions as a prerequisite for inducing cell differentiation.20 The functions of CAD are similar to those of other endonucleases, such as DNase I.
DNase II, and Endog, which are important modulators of apoptosis and cardiac hypertrophy. Investigations have revealed that CAD interacts with several transcriptional regulators of cell proliferation, including HSP70 (heat shock protein 70), HSP40 (heat shock protein 40), and HMGB1 (high mobility group box chromosomal protein 1), which play a critical role in cardiac hypertrophy and heart failure.21–26 These observations ensure that CAD is an attractive target for therapeutic intervention to treat or prevent cardiac hypertrophy and heart failure.

In the present study, we first discovered that CAD was downregulated in heart samples collected from human patients with dilated cardiomyopathy (DCM) and in samples collected from animals after aortic banding (AB). Second, using CAD-knockout (CAD−/−) mice and cardiac-specific transgenic (TG) mice, we demonstrated for the first time that CAD−/− mice are protected from developing cardiac hypertrophy and fibrosis by blocking mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, whereas TG mice with constitutive cardiac expression of human CAD displayed the opposite phenotype in response to AB, suggesting a crucial role for CAD in modulating cardiac remodeling.

Materials and Methods

The animal protocol was approved by the Animal Care and Use Committee of Union Hospital of Huazhong University of Science and Technology. An expanded Methods section is available in the online-only Data Supplement and includes detailed information regarding the following: reagents, human heart samples, experimental animal models and AB surgery, echocardiography and hemodynamic evaluations, histological analysis, cardiomyocyte culture and infection with recombinant adenoviral vectors, assessment of apoptosis, immunofluorescence staining, Western blot analysis, quantitative real-time polymerase chain reaction, and statistical analysis.

Results

CAD Expression Is Downregulated in Human Pathological Hearts and Murine Hypertrophic Hearts

To explore the potential role of CAD in the development of cardiac hypertrophy and heart failure, we first examined whether the CAD expression levels were altered in pathological hearts. Using Western blotting to investigate the correlation between CAD and cardiac hypertrophy, we first examined CAD expression in the left ventricle of patients with DCM undergoing heart transplantation secondary to end-stage heart failure. Our data revealed that the protein levels of CAD were reduced by ≈63%, whereas myosin heavy chain (β-MHC) and atrial natriuretic factor (ANP)—2 markers of hypertrophic hearts—were markedly increased compared with their levels in healthy donor hearts (Figure 1A), which was consistent with the results of human hypertrophic cardiomyopathy and ischemic cardiomyopathy hearts (Figure S1 in the online-only Data Supplement). In an experimental mouse model of AB-induced cardiac hypertrophy (evidenced by elevation of β-MHC and ANP levels), CAD expression was downregulated by ≈48% in mouse hearts 4 weeks after AB and by ≈87% at week 8 after AB compared with its level in sham-operated hearts (Figure 1B). In the cultured neonatal rat cardiomyocytes (NRCMs) treated with angiotensin II (Ang II, 1 μmol/L) or phenylephrine (100 μmol/L) for 48 hours to induce hypertrophy, the CAD protein levels were significantly reduced.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Caspase-activated DNase (CAD) is expressed in the heart and downregulated on exposure to hypertrophic stimuli. **A**, Western blot analysis of β–myosin heavy chain (MHC), atrial natriuretic peptide (ANP), and CAD protein expression in normal and failing hearts collected from human dilated cardiomyopathy (DCM) patients (n=4, ∗P<0.05 vs donor hearts). **B**, Western blot analysis of β-MHC, ANP, and CAD protein expression in an experimental mouse model with aortic banding (AB)-induced cardiac hypertrophy (n=4; ∗P<0.05 vs sham). **C**, Western blot analysis of β-MHC, ANP, and CAD protein expression in neonatal rat cardiomyocytes stimulated by angiotensin (Ang) II (1 μmol/L) and phenylephrine (PE; 100 μmol/L; n=4, ∗P<0.05 vs PBS).
by 55% (Ang II) and 51% (phenylephrine), respectively (Figure 1C). These results indicate that the protein levels of CAD are markedly decreased in human DCM/hypertrophic cardiomyopathy/ischemic cardiomyopathy hearts and in pressure overload-induced hypertrophic mouse hearts as well as in vitro Ang II/phenylephrine-treated cardiomyocytes, suggesting that CAD might be involved in the development of cardiac hypertrophy.

Absence of CAD Attenuates Pressure Overload–Induced Cardiac Hypertrophy

After determining the alterations of CAD levels in diseased hearts, we next sought to examine whether decreased CAD levels in the heart would influence the development of cardiac hypertrophy and failure. We used a global knockout of CAD (CAD−/−) mouse model (RIKEN [Rikagaku Kenkyusho/Institute of Physical and Chemical Research], Figure 2A) and subjected the mice to AB surgery or sham operations for 8 weeks, and CAD−/− mice did not affect blood pressure before or 1 week after AB (Figure S2A). It is important to note that under basal conditions, the CAD−/− mice did not show any pathological/physiological alterations in cardiac structure or function (Table S1). Relative to the AB-treated wild-type (CAD+/+) mice, the myocardial hypertrophic response was significantly blocked in CAD−/− mice after 8 weeks of AB, as shown by direct examination of the gross heart, hema-toxylin–eosin, wheat germ agglutinin–fluorescein isothiocyanate staining, and the cardiomyocyte cross-sectional area (Figure 2B and 2C). The ratios of heart weight/body weight, heart weight/tibia length, and lung weight/body weight were significantly decreased in the CAD−/− mice compared with the CAD+/+ mice after 8 weeks of AB (Figure 2D). The echocardiography measurements indicated that these changes were accompanied by improved cardiac dilation and reduced dysfunction in the CAD−/− versus CAD+/+ mice (Figure 2E, Figure S3A, Figure S4A, and Table S2). Consistent with these morphological alterations, the mRNA levels of several hypertrophy markers, including ANP, brain natriuretic peptide, and β-MHC, were much lower in the CAD−/− mice than those of CAD+/+ mice after 8 weeks of AB (Figure 2F).

Fibrosis is a classical feature of pathological cardiac hypertrophy and is characterized by the accumulation of collagen in the heart. To further define the effect of CAD deficiency on maladaptive cardiac remodeling, we examined the effect of

Figure 2. The absence of caspase-activated DNase (CAD) in the heart attenuates pressure overload–induced hypertrophy. A, Deletion of CAD was confirmed by Western blotting (n=4). CAD+/+ represents wild-type hearts, and CAD−/− represents knockout hearts. B, Histological analyses of the hematoxylin and eosin (H&E) staining and the wheat germ agglutinin (WGA) staining of CAD+/+ and CAD−/− mice 8 weeks after aortic banding (AB) surgery (n=5–6). C, Statistical results for the cardiomyocyte cross-sectional area (CSA, n=100 cells). D, Statistical results for the ratios of heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) in the indicated groups (n=11–14). E, Echocardiographic parameters for CAD+/+ and CAD−/− mice (n=6–10). F, Real-time polymerase chain reaction (PCR) analyses of the hypertrophic markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β–myosin heavy chain (β-MHC) induced by AB in the indicated mice (n=4). G, Picrosirius red staining of histological sections of the left ventricle (LV) in the indicated groups 8 weeks after AB (n=6, scale bar=50 μm). H, Areas of fibrosis in the histological sections were quantified using an image analysis system (n=29–33 fields). I, Real-time PCR analyses of the fibrotic markers (collagen I, collagen III, and connective tissue growth factor [CTGF]) in the indicated mice (n=4). *P<0.05 vs CAD+/+ or Cad+/+ or function (Table S1). Relative to the AB-treated wild-type (CAD+/+) mice, the myocardial hypertrophic response was significantly blocked in CAD−/− mice after 8 weeks of AB, as shown by direct examination of the gross heart, hema-toxylin–eosin, wheat germ agglutinin–fluorescein isothiocyanate staining, and the cardiomyocyte cross-sectional area (Figure 2B and 2C). The ratios of heart weight/body weight, heart weight/tibia length, and lung weight/body weight were significantly decreased in the CAD−/− mice compared with the CAD+/+ mice after 8 weeks of AB (Figure 2D). The echocardiography measurements indicated that these changes were accompanied by improved cardiac dilation and reduced dysfunction in the CAD−/− versus CAD+/+ mice (Figure 2E, Figure S3A, Figure S4A, and Table S2). Consistent with these morphological alterations, the mRNA levels of several hypertrophy markers, including ANP, brain natriuretic peptide, and β–MHC, were much lower in the CAD−/− mice than those of CAD+/+ mice after 8 weeks of AB (Figure 2F).

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CAD on cardiac fibrosis. The extent of fibrosis was quantified based on the collagen volume by visualizing the total amount of collagen present in the interstitial and perivascular spaces. Our results showed that interstitial and perivascular fibrosis were dramatically increased in the CAD+/- hearts subjected to chronic AB, but these types of fibrosis were markedly limited in the CAD−/− hearts (Figure 2G and 2H). Finally, we measured the synthesis of collagen by analyzing the mRNA expression levels of fibrotic markers (eg, connective tissue growth factor, collagen I, and collagen III). Our results consistently revealed a decreased fibrotic response in the CAD−/− mice (Figure 2I). Collectively, these data indicate that the deletion of CAD suppresses pressure overload-induced cardiac remodeling, suggesting that the reduced CAD expression observed in human DCM hearts might play a compensatory role in guarding against the effects of various stress conditions.

**Overexpression of CAD Aggravates Pressure Overload–Induced Cardiac Hypertrophy**

We next addressed whether elevated levels of CAD in the heart had any effect on the development of cardiac hypertrophy and failure. To examine this issue, TG mice with cardiac-specific overexpression of human CAD were generated using the α-MHC promoter (Figure S5A). Four germ lines of CAD TG mice were established and verified by western blotting (Figure S5B). The expression of mouse endogenous CAD was not affected by the overexpression of the human CAD gene (Figure S5B), and transgenic CAD was specifically expressed in the heart (Figure S5C). Under basal conditions, all of the CAD-TG mice were healthy and showed no apparent cardiac morphological or pathological abnormalities (Figure S3B, Figure S4B, and Table S1). We selected transgenic line 1, which expressed the highest levels of CAD, for further experiments. CAD TG mice and their wild-type littermates (referred to as nontransgenic [NTG]) were subjected to AB surgery or a sham operation and examined after 4 weeks. In contrast to the results observed in the CAD−/− mice compared with the NTG mice, the CAD TG mice showed pathological cardiac hypertrophy induced by chronic AB, as evidenced by an increase in cardiomyocyte size (gross heart, hematoxylin–eosin, wheat germ agglutinin–fluorescein isothiocyanate staining, and the cross-sectional area; Figure 3A and 3B) and higher ratios of heart weight/body weight, heart weight/tibia length, and lung weight/body weight (Figure 3C). The cardiac structure and function, as assessed by left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and left ventricular fractional shortening, were

**Figure 3.** The overexpression of caspase-activated DNase (CAD) aggravates pressure overload–induced cardiac hypertrophy. **A,** Histological analyses of hematoxylin and eosin (H&E) staining and the wheat germ agglutinin (WGA) staining of nontransgenic (NTG) and CAD transgenic (TG) mice 4 weeks after aortic banding (AB) surgery (n=5–6). **B,** Statistical results for the cross-sectional area (CSA; n=100 cells). **C,** Statistical results for the ratios of heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) in the indicated groups (n=11–15). **D,** Echocardiographic parameters of NTG and CAD-TG mice after 4 weeks of AB or sham operations (n=6 mice per experimental group). **E,** Real-time polymerase chain reaction (PCR) analyses of the hypertrophy markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β–myosin heavy chain (β–MHC) induced by AB in NTG and CAD TG mice (n=4). **F,** Picrosirius red staining of left ventricular (LV) sections in the indicated groups after 4 weeks of AB (n=6, scale bar=50 μm). **G,** Quantification of the total collagen volume in AB-treated NTG and CAD-TG mice (n=33–36 fields). **H,** Real-time PCR analyses of the fibrotic markers [collagen I, collagen III, and connective tissue growth factor (CTGF)] in the indicated mice (n=4). *P<0.05 vs NTG/sham; #P<0.05 vs NTG/AB. FS indicates fractional shortening; LVEDd, left ventricular end-diastolic diameter; and LVEsd, left ventricular end-systolic diameter.
significantly impaired (Figure 3D and Table S3). Additionally, markers of cardiac hypertrophy (ANP, brain natriuretic peptide, and β-MHC) were dramatically upregulated in the TG mice compared with the NTG mice (Figure 3E). We next assessed the effect of CAD overexpression on AB-triggered cardiac fibrosis. The histological analysis and the analyses of fibrotic markers consistently demonstrated an increased fibrotic response in the AB-operated TG mice compared with the AB-treated NTG mice (Figure 3F–3H). These gain-of-function data indicate that the overexpression of CAD exaggerates cardiac hypertrophy and fibrosis in response to chronic pressure overload.

**CAD Aggravates Angiotensin II–Induced Cardiomyocyte Hypertrophy In Vitro**

We determined the functional contribution of CAD on cardiomyocyte hypertrophy in vitro using primary cultured NRCMs in a well-controlled experimental setting. NRCMs were infected with AdshCAD to knockdown CAD or with AdCAD to overexpress CAD (Figure 4A). Subsequently, these infected cardiomyocytes were exposed to Ang II (1 μmol/L) or to PBS as a control for 48 hours, followed by immunostaining with α-actinin to measure the cell size. Under basal conditions, neither AdshCAD nor AdCAD affected the size of the cultured NRCMs compared with the control AdshRNA and AdGFP cells (Figure 4C and 4D). In response to Ang II-induced cell hypertrophy, AdshCAD significantly reduced the cross-sectional area by 25.2% compared with the AdshRNA-infected controls (Figure 4B and 4C), whereas Ang II-induced cell hypertrophy was greatly enhanced by 30.5% in the AdCAD-treated NRCMs compared with the AdGFP cells (Figure 4B and 4D).

Accordingly, the Ang II-induced expression of hypertrophic hallmarks (ANP and β-MHC) was dramatically suppressed in...
the AdshCAD-infected cardiomyocytes (Figure 4E), whereas it was remarkably enhanced in the AdCAD-infected NRCMs compared with the controls (Figure 4F). Our ex vivo data and in vivo results suggest that the downregulation of CAD mitigates pathological cardiac hypertrophy, whereas the upregulation of CAD promotes pathological cardiac hypertrophy.

Effect of CAD on the Mitogen-Activated Protein Kinase Signaling Pathway

The above results suggest that CAD might aggravate pressure overload–induced cardiac hypertrophy. However, the underlying mechanism by which CAD regulates the hypertrophic response remains unknown. Given that the mitogen-activated protein kinase (MAPK) signaling pathway has been previously shown to play an important role in cardiac hypertrophy, we first examined whether CAD affected the AB-induced MAPK signaling response.27,28 As expected, we observed that MEK1/2, ERK1/2, c-Jun N-terminal kinase 1/2 (JNK1/2), and p38 were significantly phosphorylated in AB mice. However, the phosphorylation of MEK1/2 and ERK1/2 was almost completely inhibited in the CAD−/− hearts compared with the CAD+/+ hearts, whereas JNK1/2 and p38 were not significantly affected (Figure 5A). Conversely, the overexpression of CAD dramatically elevated the levels of MEK1/2 and
ERK1/2 phosphorylation compared with the levels observed in NTGs after AB (Figure 5B). Although AKT signaling plays a crucial role in the regulation of cardiac remodeling, we did not observe any differences in AKT activation between the groups (CAD−/− versus CAD+/+ and TG versus NTG, data not shown). These data suggest that the regulation of CAD in hypertrophy might be mediated by MEK–ERK1/2 rather than by the AKT signaling pathway.

To further confirm the regulatory effect of CAD on MEK–ERK1/2 signaling, we infected NRCMs with AdshCAD, AdshRNA, AdCAD, or AdGFP and exposed the cells to 1 μmol/L Ang II for 48 hours. The western blotting results showed that the Ang II-triggered activation of MEK1/2 and ERK1/2 was significantly attenuated by infection with AdshCAD; however, this activation was markedly increased by infection with AdCAD (Figure 5C and 5D). Additional in vitro experiments were performed to examine whether MEK–ERK1/2 signaling has a causative role in CAD-mediated cardiac hypertrophy. We pretreated NRCMs with U0126 (an MEK inhibitor that prevents ERK1/2 phosphorylation) for 1 hour and then treated the cells with Ang II for 48 hours after infection with AdGFP or AdCAD for 24 hours. As expected, increased CAD levels led to pronounced hypertrophy induced by Ang II as assessed by surface area measurements. This response was strongly blunted in the U0126-treated samples compared with the samples treated with Ang II alone (Figure S6A and S6B). The Ang II-induced expression of hypertrophic hallmarks (ANP and β-MHC) was dramatically suppressed by pretreatment with U0126 (Figure S6C and S6D). These results demonstrate that the detrimental role of CAD in pathological cardiac hypertrophy is largely associated with the regulation of MEK–ERK1/2 signaling. Furthermore, the preinhibition of MEK–ERK1/2 signaling could inhibit the prohypertrophic effects of CAD in pressure-overloaded hearts.

**CAD Regulates Smad Signaling Pathway**

Pathological cardiac hypertrophy is accompanied by interstitial and perivascular fibrosis, and approaches to prevent collagen deposition in the heart have been limited to date. Transforming growth factor-β/Smad is an important signaling pathway involved in the development of cardiac fibrosis. To examine the molecular mechanisms of CAD in collagen deposition, we infected NRCMs with AdshCAD, AdshRNA, AdCAD, or AdGFP and exposed the cells to 1 μmol/L Ang II for 48 hours. The western blotting results showed that the Ang II-triggered activation of MEK1/2 and ERK1/2 was significantly attenuated by infection with AdshCAD; however, this activation was markedly increased by infection with AdCAD (Figure 5C and 5D). Additional in vitro experiments were performed to examine whether MEK–ERK1/2 signaling has a causative role in CAD-mediated cardiac hypertrophy. We pretreated NRCMs with U0126 (an MEK inhibitor that prevents ERK1/2 phosphorylation) for 1 hour and then treated the cells with Ang II for 48 hours after infection with AdGFP or AdCAD for 24 hours. As expected, increased CAD levels led to pronounced hypertrophy induced by Ang II as assessed by surface area measurements. This response was strongly blunted in the U0126-treated samples compared with the samples treated with Ang II alone (Figure S6A and S6B). The Ang II-induced expression of hypertrophic hallmarks (ANP and β-MHC) was dramatically suppressed by pretreatment with U0126 (Figure S6C and S6D). These results demonstrate that the detrimental role of CAD in pathological cardiac hypertrophy is largely associated with the regulation of MEK–ERK1/2 signaling. Furthermore, the preinhibition of MEK–ERK1/2 signaling could inhibit the prohypertrophic effects of CAD in pressure-overloaded hearts.
synthesis, we assessed the regulatory role of CAD on Smad cascade activation. Our results showed that AB-induced increased levels of Smad 2/3 phosphorylation and nuclear translocation were significantly attenuated in CAD−/− mice compared with CAD+/+ hearts (Figure 6A). Importantly, CAD TG mice induced higher levels of Smad 2/3 phosphorylation and nuclear translocation compared with that of NTG control mice (Figure 6B). These findings indicate that the detrimental role of CAD in pathological cardiac hypertrophy is dependent, at least in part associated with Smad signaling pathway.

**Effect of CAD on Apoptosis**

It is well known that apoptosis is involved in pathological cardiac remodeling. Because CAD has been shown to play a vital role in apoptosis, we examined whether the role of CAD in regulating hypertrophy was related to its proapoptotic effect in the AB-induced hypertrophic model. Myocardial tissue sections were subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining to detect apoptotic cells. TUNEL-positive nuclei were present in the control mice subjected to AB, whereas their number was significantly decreased in the CAD−/− mice and markedly increased in the TG mice (Figure 7A and 7B). We examined the signaling pathways related to AB-induced apoptosis in the heart. Caspase-3 is a key mediator of apoptosis, and its activation leads to DNA injury and subsequent apoptotic cell death. The activation and cleavage of caspase-3 induced by AB was modest in the control hearts and rarely observed in the CAD−/− hearts; however, the activity and cleavage of Caspase-3 was increased considerably in the TG hearts. In response to AB, the expression of Bax, which functions as a proapoptotic protein, was increased, whereas the expression of the antiapoptotic protein Bcl-2 was decreased. As predicted, the ratio of Bax to Bcl-2 was remarkably lower in the CAD−/− mice and was significantly higher in the TG mice after AB (Figure 7C).
Moreover, the CAD overexpression-induced aggravation of cardiac remodeling was mitigated by a MEK–ERK1/2 inhibitor, indicating that the MEK–ERK1/2 signaling pathway is critically involved in the prohypertrophic effects of CAD. However, CAD did not affect the phosphorylation of JNK1/2 and p38. Given that the AKT signaling cascade plays a crucial role in the regulation of cardiac remodeling, we examined the status of AKT signaling in our hypertrophic models. We found that CAD did not influence the increased phosphorylation levels of AKT induced by AB. These findings suggest that the detrimental role of CAD in pathological cardiac hypertrophy is at least partially mediated through the MEK–ERK1/2 signaling pathway. However, further experiments are needed to determine the molecular mechanism by which CAD regulates the MEK–ERK1/2 pathway.

Cardiac fibrosis is an additional classic feature of pathological hypertrophy and is characterized by the expansion of the extracellular matrix caused by the accumulation of collagen. Our study demonstrated that CAD ablation significantly inhibits cardiac fibrosis and attenuates the expression of several fibrotic mediators induced by chronic pressure overload, whereas overexpression of CAD augments the fibrotic response. These findings suggest that the detrimental role of CAD in pathological cardiac hypertrophy is dependent, at least in part associated with Smad signaling pathway.

Cardiac myocyte apoptosis plays an important role in the transition of cardiac hypertrophy to heart failure. Our experimental findings revealed a correlation between an increase in the frequency of apoptosis and the extent of cardiac hypertrophy. Consistent with previous reports, the disruption of CAD in the heart markedly decreased the number of apoptotic cells in response to pressure overload. Furthermore, we found that the attenuation of myocardial apoptosis induced by CAD ablation was associated with the increased expression of Bcl-2 and the decreased expression of Bax and caspase-3. The overexpression of CAD significantly increased the number of apoptotic cells subjected to AB in conjunction with lower Bcl-2 expression and increased Bax and caspase-3 expression. The effects of CAD on these signaling molecules might explain the protection from apoptosis observed in the CAD knockout hearts as well as the destructive effects in the CAD TG hearts in pathological cardiac hypertrophy.

In conclusion, this investigation is the first study to define a role for CAD in cardiac hypertrophy. Our findings indicate that knockdown/knockout of CAD mitigates the development of pathological cardiac hypertrophy. Conversely, upregulation of CAD aggravates pressure overload–induced cardiac hypertrophy and heart failure. The mechanism underlying the protective role of CAD in the development of cardiac hypertrophy seems to be related to the MEK–ERK1/2 signaling pathway. These findings provide novel insights into the molecular mechanisms underlying pathological cardiac hypertrophy. Based on these findings, CAD might represent a new therapeutic target for suppressing the onset of cardiac hypertrophy and failure.

Perspectives

The current study provides in vivo and in vitro evidence that CAD, a double-strand-specific endonuclease, functions as
a pivotal regulator in cardiac hypertrophy. The absence of CAD inhibits pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction, whereas the cardiac-specific overexpression of CAD promotes the hypertrophic response by regulation of the MEK–ERK1/2 signaling pathway. These findings suggest that CAD might be a novel therapeutic target for preventing cardiac hypertrophy and heart failure.

Acknowledgments
The caspase-activated DNase knockout mouse model was provided by RIKEN BRC (Rikagaku Kenkyusho/Institute of Physical and Chemical Research BioResource Center) through the National Bio Resources Project of the MEXT (Ministry of Education, Culture, Sports, Science and Technology), Japan. Ya-Fen Lin, Rui Zhang, Xin Zhang, Zhang-Li Li, and Xue-Yong Zhu are gratefully acknowledged for their technical assistance.

Sources of Funding
This work was supported by grants from the National Natural Science Foundation of China (30972425 to K. Huang, 81270306 and 81470375 to X.-D. Zhang, 81000112 to D. Huang).

Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- Caspase-activated DNase (CAD) is downregulated in the heart upon hypertrophic stimuli, and CAD is shown to be expressed in human and mouse heart tissues.
- CAD is a necessary modulator of aortic banding–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction in vivo as well as angiotensin II–induced cardiomyocyte hypertrophy in vitro.
- CAD regulates the development of cardiac hypertrophy by regulating the mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway.

**What Is Relevant?**

- Multiple signaling pathways have been elucidated to contribute to the progression of pathological cardiac hypertrophy.
- The effects and molecular mechanisms of CAD on the development of cardiac hypertrophy are not elucidated.

**Summary**

This study demonstrates that the absence of CAD inhibits pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction, whereas the cardiac-specific overexpression of CAD promotes the hypertrophic response by regulation of the MEK–ERK1/2 signaling pathway. These findings suggest that CAD might be a useful therapeutic target for preventing cardiac hypertrophy and heart failure.
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Hypertension. 2015;65:871-881; originally published online February 2, 2015; doi: 10.1161/HYPERTENSIONAHA.114.04806

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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A Novel Role for Caspase-activated DNase in the Regulation of Pathological Cardiac Hypertrophy

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Running Title: CAD modulates cardiac hypertrophy

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Materials and Methods

Reagents

The following primary antibodies were used in our experiments: Anti-CAD (sc30061), anti-β-MHC (sc53090) and anti-ANP (sc20158) were purchased from Santa Cruz Biotechnology; Anti-ERK1/2 (#4695), anti-phospho-ERK1/2Thr202/Thr204 (#4370), anti-MEK1/2 (#9122), anti-phospho-MEK1/2Ser217/221 (#9154), anti-P38 (#9212), anti-phospho-P38Thr180/Thr182 (#4511), anti-phospho-JNK1/2 (#4668), anti-JNK1/2 (#9258), anti-AKT (#4691), anti-phospho-AKTSer473 (#4060), anti-α-actinin (#3134) and the MEK1/2 Inhibitor U0126 (#9903) were purchased from Cell Signaling Technology; Anti-GAPDH (MB001) was purchased from Bioworld Technology. The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA) were utilized for the visualization of primary antibody binding. Fetal calf serum was purchased from Hyclone (Waltham, MA, USA). The cell culture reagents and all of the other reagents were purchased from Sigma (St. Louis, MO, USA).

Human heart samples

Samples of failing human hearts were collected from the left ventricles of dilated cardiomyopathy (DCM) patients, hypertrophic cardiomyopathy (HCM) patients or ischaemic cardiomyopathy (ICM) patients who were under treatment following heart transplants1-3. Control samples were obtained from the left ventricles of normal heart donors. This study complied with the protocol approved by the Renmin Hospital of Wuhan University Human Research Ethics Committee, and samples were collected after informed consent.

Animals and animal models

All of the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Union Hospital, Huazhong University of Science and Technology. The male C57B/L6J mice used in the experiment were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China), which were imported from Harlan Olac (Bicester, UK) in 1985, and the product license number was SCXK-2014-0004. The CAD knockout mice were purchased from RIKEN Bio Resource Center (stock number: RBRC01723). The original CAD-/- mice were in the 129/Sv background4,5. The CAD-/- mice were crossed with C57BL/6J mice to produce the F1 generation (CAD heterozygous), which was backcrossed with the C57BL/6J strain to obtain the F2 generation (CAD heterozygous) and then further backcrossed to the C57BL/6J background until the F10 generation (CAD heterozygous). F10 generation male and female mice were crossed with each other to produce CAD-/- mice (pure C57BL/6J background). Human CAD cDNA was cloned downstream of the cardiac α-myosin heavy chain.
(α-MHC) promoter. Transgenic mice were generated by microinjecting the α-MHC-CAD construct into fertilized mouse embryos and were identified by PCR analysis of tail genomic DNA. Male CAD knockout mice and their wild-type (WT) littermates, α-MHC-CAD (TG) littermates and non-transgenic (NTG) littermates, aged 8 to 10 weeks (with body weights of 24-27g) were used in these experiments.

Aortic banding (AB) was conducted as described previously. Adequate constriction of the aorta was determined by Doppler analysis. A similar procedure, without constricting the aorta, was performed in the sham-operated group. The wall thickness and internal diameter of the left ventricle (LV) were assessed using echocardiography at the indicated time after surgery. At the end of these procedures, the hearts, lungs and tibiae from the sacrificed mice were harvested, weighed and analyzed to compare the heart weight/body weight (HW/BW, mg/g), lung weight/body weight (LW/BW, mg/g), and heart weight/tibia length (HW/TL, mg/mm) ratios between the KO and WT mice and between the TG and NTG mice.

Echocardiography and hemodynamic evaluations
Echocardiography was performed in mice anesthetized with 1.5% isoflurane using a Mylab 30CV (ESAOTE S. P. A) with a 10-MHz linear-array ultrasound transducer in accordance with previously described methods. The LV dimensions were assessed in the parasternal long-axis and the short-axis views at a frame rate of 120Hz. The LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

For the hemodynamic measurements, a microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle of mice anesthetized with 1.5% isoflurane. After stabilization for 15 min, pressure, volume signals and heart rate were continuously recorded using a Millar Pressure-Volume System (MPVS-400, Millar Instruments, Houston, TX, USA). The results were analyzed with Chart 5.0 software.

Histological analysis
Hearts were excised, immediately placed in a 10% potassium chloride solution to ensure that the hearts were stopped in diastole, washed with a saline solution, and placed in 10% formalin. Subsequently, these hearts were sectioned at 5 µm transversely close to the apex to visualize the left and right ventricles. The sections were stained with hematoxylin-eosin for histopathology or with PSR to evaluate collagen deposition. To determine the myocyte cross sectional area, the sections were stained with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp) for membranes and with DAPI for nuclei. More than 100 myocytes in the sections were outlined in each group. Single myocyte and fibrillar collagen were visualized by microscopy and measured with a quantitative digital image analysis system (Image-Pro Plus, version 6.0).

Cardiomyocyte culture and infection with recombinant adenoviral vectors
Replication-defective adenoviral vectors expressing the full-length CAD gene and a control adenoviral vector expressing the GFP protein were used in this study. The shCAD that led to the greatest decrease in the CAD levels was selected for further experiments, and the shRNA virus was used as a control. We infected cardiac myocytes with AdshRNA, AdshCAD, AdGFP and AdCAD. Cultures of cardiac myocytes were prepared as described in our previous studies with minor alterations. Briefly, PBS containing 0.03% trypsin and 0.04% collagenase type II was used to isolate the cardiomyocytes from 1- to 2-day-old Sprague Dawley rats. After removing fibroblasts by a differential attachment technique, the NRCMs were seeded at a density of 3×10^5 cells per well onto six-well culture plates coated with gelatin in plating medium consisting of DMEM/F12 supplemented with 20% fetal calf serum, BrdU (0.1 mM, to inhibit the proliferation of fibroblasts) and penicillin/streptomycin. These myocytes were then infected with AdCAD and AdshCAD for 12 h with MOIs of 10. The culture medium was then replaced with serum-free medium for 12 h, followed by stimulation with 1 μM Ang II for 48 h.

**Assessment of apoptosis**

Samples were first incubated with a terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP according to the protocol of the ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (S7111, Chemicon, Temecula, CA, USA). Then the paraffin-embedded sections of mouse hearts were stained with α-actinin antibody to make sure the positive cells are cardiomyocyte-specific. At last, the slides were stained with DAPI for 30 min to evaluate the cell nucleus. The numbers of total cells and TUNEL positive cells were counted by Image-Pro plus version, and the percentages of apoptotic cells were calculated by at least three independent individuals in a blinded manner.

**Immunofluorescence staining**

Immunofluorescence staining was performed in the tissue sections or NRCMs with a CAD antibody to determine the expression levels of CAD or with α-actinin antibody to assess the myocyte cross sectional area. Briefly, cardiac myocytes were infected with different adenoviruses for 24 h and subsequently stimulated with 1 μM Ang II for 48 h. The cells were then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, washed three times, permeabilized with 0.1% Triton X-100 in PBS for 40 min, and stained with α-actinin (1:100 dilution) using standard immunofluorescence staining techniques. For the tissue section staining, the procedure was the same as that used for the NRCM staining after the de-paraffinization step (as described above).

**Western blotting and quantitative real-time PCR**

Cardiac tissue and cultured cardiac myocytes were lysed using RIPA buffer, and the protein concentration was determined with a BCA protein assay kit (Pierce). Protein extracts (50 μg) were used for SDS-PAGE (Invitrogen), and the proteins were
transferred to a polyvinylidene fluoride membrane (Millipore) that was incubated with various primary antibodies overnight at 4°C. After incubation with a secondary antibodies for 1 hour at room temperature, the membranes were treated with ECL reagents (170-5061, Bio-Rad) prior to visualization using a Fluor Chem E imager (Cell Biosciences) according to the manufacturer’s instructions. The specific protein expression levels were normalized to GAPDH on the same nitrocellulose membrane.

The total mRNA was extracted from the ventricles and primary cells using the TRIzol reagent (Invitrogen), and cDNA was synthesized using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (Roche). The PCR amplifications were quantified using SYBR Green PCR Master Mix (Applied Biosystems) and normalized to GAPDH gene expression.

**Statistical analysis**

The data are represented as the mean ± SD. Student’s two-tailed t-test was used to compare the means of two groups of samples, and two-way analysis of variance with general linear model procedures using a univariate approach was applied for more than two groups. A value of $P<0.05$ was considered a statistically significant difference. All of the statistical analysis were performed with SPSS software (version 11.0, SPSS Inc.).
Reference


Supplemental Tables

Table S1 Anatomic and Echocardiographic Analysis in 10-Week Old Mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CAD+/+ mice (n=12)</th>
<th>CAD-/- mice (n=11)</th>
<th>NTG mice (n=10)</th>
<th>TG mice (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>26.75±0.44</td>
<td>26.65±0.25</td>
<td>27.22±0.80</td>
<td>27.89±0.82</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.18±0.08</td>
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<td>4.24±0.10</td>
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<td>LW/BW (mg/mg)</td>
<td>5.19±0.12</td>
<td>5.24±0.12</td>
<td>5.22±0.07</td>
<td>5.27±0.20</td>
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<tr>
<td>HW/TL (mg/mm)</td>
<td>6.22±0.14</td>
<td>6.22±0.09</td>
<td>6.35±0.18</td>
<td>6.24±0.09</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>499±12</td>
<td>508±16</td>
<td>497±13</td>
<td>506±9</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.60±0.03</td>
<td>3.70±0.11</td>
<td>3.63±0.12</td>
<td>3.65±0.17</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.06±0.02</td>
<td>2.09±0.06</td>
<td>2.06±0.07</td>
<td>2.07±0.07</td>
</tr>
<tr>
<td>FS (%)</td>
<td>42.1±0.7</td>
<td>41.0±1.3</td>
<td>41.5±1.6</td>
<td>41.3±1.2</td>
</tr>
</tbody>
</table>

BW=body weight; HW=heart weight; LW=lung weight; TL=tibia length; HR=heart rate; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; FS=fractional shortening. All values are presented as means ± SEM.
Table S2 Parameters in CAD-/− and CAD+/+ mice at 8 weeks after sham operation or AB

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham CAD+/+ mice (n=12)</th>
<th>CAD-/− mice (n=14)</th>
<th>AB CAD+/+ mice (n=11)</th>
<th>CAD-/− mice (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>28.46 ± 0.58</td>
<td>27.85 ± 0.53</td>
<td>29.12 ± 0.77</td>
<td>28.96 ± 0.70</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.22 ± 0.06</td>
<td>4.11 ± 0.05</td>
<td>8.33 ± 0.34*</td>
<td>5.01 ± 0.15*†</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>4.97 ± 0.09</td>
<td>5.11 ± 0.13</td>
<td>7.81 ± 0.93*</td>
<td>5.74 ± 0.28*†</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>6.73 ± 0.12</td>
<td>6.24 ± 0.14</td>
<td>13.11 ± 0.30*</td>
<td>7.97 ± 0.22*†</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>523 ± 23</td>
<td>517 ± 21</td>
<td>498 ± 24*</td>
<td>493 ± 10</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.74 ± 0.03</td>
<td>3.57 ± 0.07</td>
<td>5.00 ± 0.10*</td>
<td>4.22 ± 0.05*†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.24 ± 0.07</td>
<td>2.00 ± 0.04</td>
<td>3.78 ± 0.12*</td>
<td>2.84 ± 0.07*†</td>
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<tr>
<td>FS (%)</td>
<td>40.1 ± 2.0</td>
<td>44.4 ± 0.5</td>
<td>24.3 ± 1.0*</td>
<td>33.0 ± 1.0*†</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>114.26 ± 7.90</td>
<td>116.55 ± 6.82</td>
<td>137.47 ± 12.12</td>
<td>156.18 ± 5.50</td>
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<tr>
<td>LVEF (%)</td>
<td>54.2 ± 4.6</td>
<td>57.6 ± 4.9</td>
<td>28.5 ± 2.9*</td>
<td>45.5 ± 3.7*†</td>
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<tr>
<td>dp/dt max (mmHg/sec)</td>
<td>9612.6 ± 382.1</td>
<td>10038.7 ± 749.4</td>
<td>5728.0 ± 264.9*</td>
<td>8019.7 ± 431.6*†</td>
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<tr>
<td>dp/dt min (mmHg/sec)</td>
<td>-8518.8 ± 627.6</td>
<td>-8981.3 ± 747.2</td>
<td>-4688.3 ± 321.0*</td>
<td>-7386.3 ± 444.6*†</td>
</tr>
</tbody>
</table>

LVESP=Left ventricular End-systolic Pressure. LVEF=Left ventricular ejection fraction.
*P<0.05 versus CAD+/− sham operation. †P<0.05 versus CAD+/+ AB after 8 weeks AB.
All values are presented as means ± SEM.
Table S3 Parameters in CAD transgenic mice (TG) and wild type littermates (NTG) at 4 weeks after sham operation or AB.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NTG-Sham mice (n=14)</th>
<th>TG-Sham mice (n=12)</th>
<th>NTG-AB mice (n=11)</th>
<th>TG-AB mice (n=10)</th>
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</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>27.11±0.74</td>
<td>29.35±0.56</td>
<td>28.03±0.43</td>
<td>29.14±0.80</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.09±0.06</td>
<td>4.01±0.06</td>
<td>6.22±0.13*</td>
<td>9.07±0.27*†</td>
</tr>
<tr>
<td>LW/BW (mg/mg)</td>
<td>4.98±0.09</td>
<td>4.98±0.37</td>
<td>6.17±0.51*</td>
<td>9.63±1.41*†</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>6.21±0.13</td>
<td>6.47±0.14</td>
<td>9.68±0.15*</td>
<td>14.39±0.22*†</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>534±23</td>
<td>504±17</td>
<td>524±19</td>
<td>504±11</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.70±0.05</td>
<td>3.85±0.06</td>
<td>4.43±0.06*</td>
<td>5.85±0.20*†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.18±0.07</td>
<td>2.43±0.04</td>
<td>3.20±0.07*</td>
<td>4.70±0.18*†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>40.0±0.4</td>
<td>38.2±0.2</td>
<td>27.7±0.8*</td>
<td>21.0±0.9*†</td>
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<tr>
<td>LVESP (mmHg)</td>
<td>115.69±5.64</td>
<td>108.45±6.56</td>
<td>170.06±9.49*</td>
<td>142.04±4.85</td>
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<tr>
<td>LVEF (%)</td>
<td>58.9±3.2</td>
<td>54.2±3.9</td>
<td>42.5±3.7*</td>
<td>25.8±3.3*†</td>
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<tr>
<td>dp/dt max (mmHg/sec)</td>
<td>10105±529.2</td>
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<td>7618.7±557.0*</td>
<td>5259.4±435.0*†</td>
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<td>dp/dt min (mmHg/sec)</td>
<td>-8695.8±432.7</td>
<td>-8924.3±865.0</td>
<td>-6563.5±434.7*</td>
<td>-4368.0±359.6*†</td>
</tr>
</tbody>
</table>

*P<0.05 versus NTG mice after 4 weeks sham operation. †P<0.05 versus NTG mice after 4 weeks AB operation. All values are means ± SEM.
Supplemental Figures

Figure S1 CAD Expression is decreased in HCM/ICM human hearts

(A) Representative western blots and quantitative results of β-myosin heavy chain (MHC), atrial natriuretic peptide (ANP) and CAD protein expression in normal and failing hearts collected from human hypertrophic cardiomyopathy (HCM) patients (n=4). (B) Western blot analysis of β-MHC, ANP and CAD protein expression in donor hearts and in ischaemic cardiomyopathy (ICM) hearts (n=4). *p<0.05 vs. donor hearts.
Figure S2 The left ventricular end-systolic pressure in mice 1 week after AB
(A) The left ventricular end-systolic pressure in CAD+/+ and CAD-/- mice 1 week after aortic banding (AB). (B) The left ventricular end-systolic pressure in CAD transgenic (TG) and non-transgenic (NTG) mice 1 week after AB. *P<0.05 vs. sham.
Figure S3 Representative echo images of the mice hearts
(A) Representative echo images of the CAD+/- and CAD-/- mice before and after AB.
(B) Representative echo images of the NTG and TG mice before and after AB.
Figure S4 Representative P-V Loop images of the mice hearts

(A) Representative P-V Loop images of the CAD+/+ and CAD-/- mice before and after AB. (B) Representative P-V Loop images of the NTG and TG mice before and after AB.
Figure S5 The expression of CAD transgene mice

(A) Schematic diagram of the construction of transgenic mice with full-length human CAD cDNA under the control of the α-myosin heavy chain promoter. (B) Representative blots for determination of transgenic CAD and endogenous CAD levels expressed in heart tissue from four TG lines and control (NTG) mice (n=4). (C) Representative western blots for the evaluation of transgene expression in different tissues of TG mice as indicated (n=4).
Figure S6 Inhibition of MEK–ERK1/2 signaling rescued abnormalities of CAD

(A) Representative images of cardiomyocytes that were pretreated with U0126 for 1 h and subsequently treated with angiotensin (Ang) II (1 μmol/L) for 48 h after infection with AdGFP or AdCAD for 24 h. (B) Quantitation of the cell surface area (n=50 cells).

(C)and (D) Real-time PCR analysis of the hypertrophy markers ANP and β-MHC; cells were infected with different adenoviruses for 24 h or pretreated with U0126 for 1 h subsequently treated with 1 Mm Ang II for 24 h. (n=4, *P<0.05 vs. AdGFP/PBS; #P<0.05 vs. AdGFP/Ang II).