Cellular repressor of E1A-stimulated genes attenuates cardiac hypertrophy and fibrosis

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Abstract

Cellular repressor of E1A-stimulated genes (CREG) is a secreted glycoprotein of 220 amino acids. It has been proposed that CREG acts as a ligand that enhances differentiation and/or reduces cell proliferation. CREG has been shown previously to attenuate cardiac hypertrophy *in vitro*. However, such a role has not been determined *in vivo*. In the present study, we tested the hypothesis that overexpression of CREG in the murine heart would protect against cardiac hypertrophy and fibrosis *in vivo*. The effects of constitutive human CREG expression on cardiac hypertrophy were investigated using both *in vitro* and *in vivo* models. Cardiac hypertrophy was produced by aortic banding and infusion of angiotensin II in CREG transgenic mice and control animals. The extent of cardiac hypertrophy was quantitated by two-dimensional and M-mode echocardiography as well as by molecular and pathological analyses of heart samples. Constitutive overexpression of human CREG in the murine heart attenuated the hypertrophic response, markedly reduced inflammation. Cardiac function was also preserved in hearts with increased CREG levels in response to hypertrophic stimuli. These beneficial effects were associated with attenuation of the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase 1 (MEK-ERK1)/2-dependent signalling cascade. In addition, CREG expression blocked fibrosis and collagen synthesis through blocking MEK-ERK1/2-dependent Smad 2/3 activation *in vitro* and *in vivo*. Therefore, the expression of CREG improves cardiac functions and inhibits cardiac hypertrophy, inflammation and fibrosis through blocking MEK-ERK1/2-dependent signalling.

Keywords: CREG • cardiac remodelling • ERK1/2 • fibrosis

Introduction

Heart failure is increasing in prevalence and is a debilitating disease with high rates of mortality and morbidity [1]. Cardiac hypertrophy is a common precursor to many forms of heart failure, whose molecular and cellular determinants remain largely unknown [2]. After a period of compensatory adaptation, hypertrophy is associated with functional and histological deterioration of the myocardium, fibrosis, inflammation and altered cardiac gene expression [2, 3].

Studies have demonstrated that adenovirus E1A protein both activates and represses gene expression to promote DNA synthesis and cellular proliferation [4]. In many cell types, the adenovirus E1A protein dramatically alters the transcriptional program of the host cell to stimulate cell division and inhibit differentiation [5]. Investigations into the mechanisms by which E1A activates and represses expression of particular genes have revealed that E1A interacts with several transcriptional regulators of cell proliferation, including the coactivators p300 and CBP, which play important role in cardiac hypertrophy and heart failure [6, 7]. Therefore, E1A is an attractive target for therapeutic intervention to treat or

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prevent cardiac hypertrophy and heart failure. The cellular repressor of E1A-stimulated genes (CREG) is a secreted glycoprotein that has been shown to antagonize transcription activation and cellular transformation induced by the adenovirus E1A oncoprotein [8]. Recent studies showed that CREG transferring to the injured artery inhibited SMC dedifferentiation and proliferation, and reduced neointimal hyperplasia [9, 10]. Xu and colleagues [11] reported that CREG expression is decreased during biomechanical stress in the heart and functions to attenuate cardiac hypertrophy *in vitro*. Despite the potentially significant roles of CREG in attenuating hypertrophic signalling, it has remained unclear whether CREG could regulate cardiac hypertrophy *in vivo*, and whether targeted myocardial overexpression of CREG is cardio-protective. Thus in the present study, our aimed to investigate the role of CREG in cardiac hypertrophy mediated by pressure overload and Angiogensin II (Ang II) and to clarify the related molecular mechanisms.

Methods and materials

Materials

Antibodies for the mitogen-activated protein kinases (MAPKs) and Smads pathways were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-CREG (reactive with mouse or human) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [^{3}H]-Leucine and [^{3}H]-proline were purchased from Amersham. NF-κB-luc, CTGF- and COL1A2-luc report constructs were described previously [12]. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). All other antibodies were purchased from Santa Cruz Biotechnology. Transforming growth factor-β$_1$ (TGF-β$_1$) was purchased from R&D Systems (Minneapolis, MN, USA). Foetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Cell culture reagents and all other reagents were obtained from Sigma (Oakville, ON, Canada).

Animals and animal models

All protocols were approved by the Animal Care and Use Committee of the hospital. Human CREG cDNA construct containing full-length human CREG cDNA (kindly provide by Dr Grace Gill, School of Medicine, Tufts University) was cloned downstream of the cardiac α-myosin heavy chain (α-MHC) promoter. Transgenic (TG) mice were produced by microinjection of the α-MHC-CREG construct into fertilized mouse embryos [friend virus B strain (FVB) background]. Four independent transgenic lines were established and studied. TG mice were identified by PCR analysis of tail genomic DNA. Gene expression levels and cardiac function were analysed in pairs of α-MHC-CREG (TG) and littermate wild-type (WT) male mice ranging in age from 8 to 10 weeks. Aortic banding (AB) was performed as described previously [12]. Doppler analysis was performed to ensure the adequate constriction of the aorta. To confirm the role of CREG in cardiac hypertrophy, the experiments were repeated in an Ang II Infusion model. Ang II (1.4 mg/kg/day and dissolved in 0.9% NaCl) was subcutaneously infused for 4 weeks using an osmotic minipump (Alzet model 2004; Alza Corp, Vacaville,

CA, USA) implanted in each mouse. Saline-infused animals served as infusion controls and were subjected to the same procedures as the experimental animals except for Ang II infusion. The internal diameter and wall thickness of the left ventricle were assessed by echocardiography after surgery or infusion. Hearts and lungs of the killed mice were dissected and weighed to compare heart weight/body weight (HW/BW, mg/g) and lung weight/body weight (LW/BW, mg/g) ratios in different groups.

Echocardiography and blood pressure

Echocardiography was performed by SONOS 5500 ultrasound (Philips Electronics, Amsterdam, Netherlands) with a 15-MHz linear array ultrasound transducer. The left ventricle was assessed in both parasternal long- 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 left ventricle, respectively, was obtained. For measurements of blood pressure and heart rate, a microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle. After stabilization for 15 min., the pressure signals and heart rate were recorded continuously with an ARIA pressure–volume conductance system as described previously [12].

Quantitative real-time RT-PCR and Western blotting

Real-time PCR was used to detect the mRNA expression levels of hypertrophic, fibrotic and inflammatory markers. Total RNA was extracted from frozen, pulverized mouse cardiac tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) and synthesized cDNA using oligo (dT) primers (listed in Table S1) with the Advantage RT-for-PCR kit (BD Biosciences, San Jose, CA, USA). We quantitated PCR amplifications using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and normalized results against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression. For Western blot, cardiac tissue and cultured cardiac myocytes or fibroblasts were lysed in RIPA lysis buffer. Fifty micrograms of cell lysate was used for SDS-PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). Specific protein expression levels were normalized by either the GAPDH protein for total cell lysate and cytosolic proteins, or the Lamin-B1 protein for nuclear proteins on the same nitrocellulose membrane.

Electrophoretic mobility shift assays (EMSA) and histological analysis

For EMSA, nuclear proteins were isolated as described previously [12, 13]. EMSA was performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega, Madison, WI, USA). Synthetic, double-strand oligonucleotides containing NF-κB binding domain were labelled with [γ-^{32}P] ATP using T$_4$ polynucleotide kinase and separated from unincorporated [γ-^{32}P] ATP by gel filtration using a Nick column (Pharmacia, North Peapack, NJ, USA). For histological analysis, hearts were excised, washed with saline solution, and fixed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricle. Several sections of heart (4–5-μm thick) were prepared and stained with haematoxylin and eosin for histopathology or Picrosirius red (PSR) for collagen deposition and then visualized by light microscopy. For
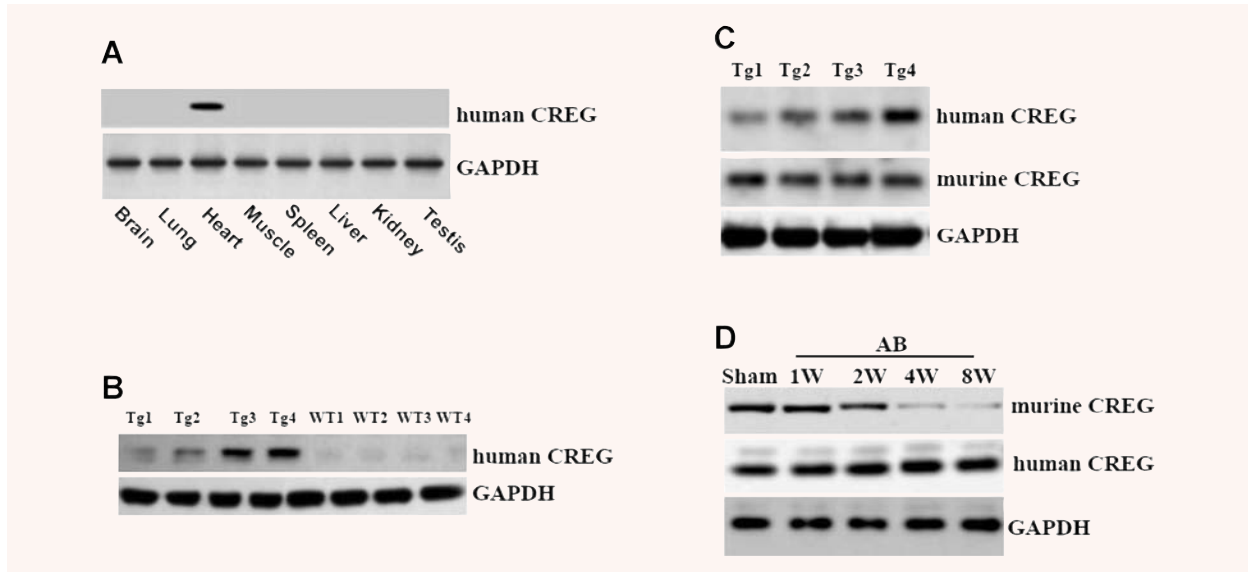
Fig. 1 Characterization of human CREG transgenic (TG) mice. **(A)** Representative Western blot of human CREG protein from different tissue of TG mice as indicated. **(B)** Representative Western blots of human CREG protein in the heart tissue from 4 lines of both TG and WT mice. **(C)** Representative Western blots of TG CREG and endogenous CREG protein levels in the heart from TG mice. **(D)** Representative Western blots of mouse and human CREG protein in the heart tissue from TG mice after aortic banding at time-points indicated.

myocyte cross-sectional area, sections were stained for membranes with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp) and for nuclei with DAPI. Single myocyte was measured with an image quantitative digital analysis system (Image-Pro 4.5, Media Cybernetics, Silver Spring, MD, USA). The outline of 250 myocytes was traced in each section.

Recombinant adenoviral vectors, and cultured neonatal rat cardiac myocytes and fibroblasts

We used replication-defective adenoviral vectors encoding for the entire coding region of human CREG gene under the control of the cytomegalovirus promoter. The same adenoviral vector encoding for the GFP gene (AdEasy XL adenoviral Vector system, Strategene, La Jolla, CA, USA) was used as control. We ordered three independent rat shCREG constructs from SuperArray (Cat. no. KR43991G) and then generated three Ad-shCREG adenovirus, and chose the one that led to a significant decrease in CREG levels for further experiments. Ad-shRNA was used as control. Cardiac myocytes were infected with Ad-CREG and Ad-GFP as well as Ad-shCREG and Ad-shRNA at a multiplicity of infection (MOI) of 100, resulting in 95–100% of cells expressing the transgenes without toxicity. Primary cultures of cardiac myocytes were prepared as described previously [12]. Cardiomyocytes isolated from the hearts of 1- to 2-day old Sprague-Dawley rats (Charles River Laboratories, Senneville, Canada) were seeded at a density of 1×10^6/well onto six-well culture plates in plating medium consisting of F10 medium supplemented with 10% FCS and penicillin/streptomycin. After 48 hrs, the culture medium was replaced with F10 medium containing 0.1% FCS and BrdU (0.1 mM). Cultures of neonatal rat cardiac fibroblasts were prepared as described previously [12]. All

experiments were performed on cells from the first or second passages which were placed in Dulbecco's modified eagle medium (DMEM) medium containing 0.1% FCS for 24 hrs before the experiment. The purity of cardiac fibroblasts in these cultures was greater than 95% as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. For the cell infection, 1×10^6/well cardiac myocytes or cardiac fibroblasts were cultured in six-well plates and exposed to 2×10^8 pfu each of virus in 1 ml of serum-free medium for 24 hrs. The cells were then washed and incubated in serum-containing medium for 24 hrs.

Collagen synthesis assay and reporter assays

Collagen synthesis was evaluated by measuring ^{3}H-proline incorporation as described previously [12]. Briefly, cardiac fibroblasts were infected with different adenoviruses and then made quiescent by cultured in 0.1% FCS DMEM for 24 hrs, and subsequently incubated with TGF-β_1 and 5 μCi/ml ^{3}H-proline for the indicated time. Cells were washed with PBS twice, treated with ice-cold 5% trichloroacetic acid for 1 hr, and washed twice with distilled water. Cells were then lysed with 1 N NaOH solution and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number. For reporter analysis, cardiac myocytes or cardiac fibroblasts were seeded in triplicate in six-well plates. Cells were transfected with 0.5 μg of luciferase reporter constructs, and internal control plasmid DNA using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. After 6 hrs of exposure to the DNA-LipofectAMINE complex, cells were cultured in medium containing

Table 1 Echocardiographic data showed the effects of CREG on cardiac hypertrophy induced by aortic banding model

Parameter	WT-sham mice	TG-sham mice	WT-AB mice	TG-AB mice
Number	$n = 11$	$n = 12$	$n = 9$	$n = 8$
BW, g	25.7 ± 1.5	25.5 ± 2.2	26.8 ± 1.6	26.0 ± 1.2
HW/BW	4.54 ± 0.12	4.47 ± 0.07	7.19 ± 0.11*	5.22 ± 0.09*§
LW/BW	5.33 ± 0.07	5.21 ± 0.12	8.13 ± 0.16*	5.78 ± 0.14§
CSA	258 ± 29.7	263 ± 30.3	442 ± 31.2*	295 ± 22§
SBP, mmHg	115.3 ± 4.2	117.8 ± 3.2	152.0 ± 4.9*	145.0 ± 4.1*
HR, beats/min.	465 ± 21	457 ± 26	473 ± 25	466 ± 29
PWT(mm)	1.22 ± 0.04	1.25 ± 0.03	3.24 ± 0. 02*	1.75 ± 0.03§
LVEDD (mm)	3.63 ± 0.04	3.72 ± 0.05	5.78 ± 0.04*	4.11 ± 0.02§
LVESD (mm)	2.40 ± 0.03	2.38 ± 0.03	3.52 ± 0.04*	2.53 ± 0.04§
IVSd (mm)	0.62 ± 0.02	0.62 ± 0.03	1.65 ± 0.03*	0.91 ± 0.02§
LVPWd (mm)	0.65 ± 0.02	0.66 ± 0.02	1.29 ± 0.04*	0.86 ± 0.02§
FS (%)	53.7 ± 2.2	55.5 ± 4.1	26.3 ± 2.9*	44.2 ± 1.2*§
EF (%)	60.2 ± 3.6	58.7 ± 4.2	30.5 ± 3.4*	47.5 ± 3.1*§
LVEDP (mm Hg)	9.8 ± 0.9	9.6 ± 1.1	17.9 ± 1.3*	12.5 ± 1.5§
Left ventricular dP/dTmax (mm Hg/s)	8813.5 ± 519.5	8877.3 ± 544.1	7003.7 ± 341.9*	8348.7 ± 406.8§
Left ventricular dP/dTmin (mm Hg/s)	−7783.5 ± 581.2	−7822.5 ± 500.6	−6162.8 ± 322.3*	−7481.8 ± 452.7§

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

SBP = systolic blood pressure; 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; IVSd = left ventricular septum, diastolic; LVPWd = left ventricular posterior wall, diastolic. FS = fractional shortening; EF = ejection fraction; LVEDP = left ventricular end-diastolic pressure; All values are mean ± S.E.M.

10% serum for 24 hrs and then incubated with serum-free medium for 12 hrs. Cells were infected with different adenoviral for 24 hrs and then treated with Ang II for cardiac myocytes or TGF-β_1 for fibroblasts. 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 observers in this study were blinded to the actual conditions of the experiment to minimize observer bias. Results were expressed as mean ± S.E.M. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were performed by unpaired Student's t-test. A value of $P < 0.05$ was considered to be significantly different.

Results

Generation of mice with cardiac-specific overexpression of CREG

To determine the role of CREG on cardiac hypertrophy, we generated TG mice with full-length human CREG cDNA under the control of the α-MHC promoter. Four lines of TG mice were confirmed by PCR. These lines were born in a normal Mendelian distribution, thus excluding lethal developmental abnormalities. They also exhibited normal reproductive rate and gender distributions and did not demonstrate any difference in longevity over 56 weeks. All experiments were performed on male mice of 8–10-weeks old. We analysed CREG protein levels in various
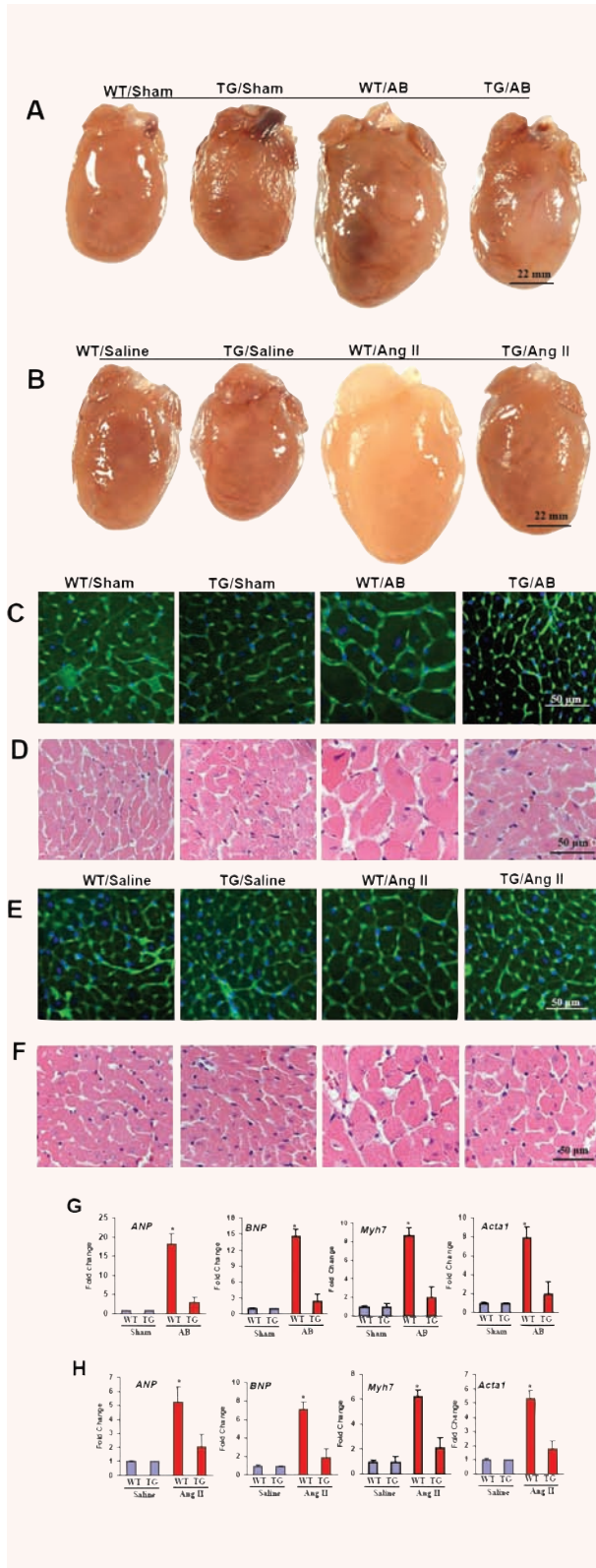
tissues by Western blot analysis using a human-specific anti-CREG antibody. We detected a robust expression of human CREG protein in the hearts of TG mice, but not in other organs (Fig. 1A). Among four established lines of TG mice, the line that expressed the highest levels of the human CREG protein in the heart was used for further experiments (Fig. 1B). Western blot analysis further demonstrated that the endogenous mouse CREG protein expression was not modified by exogenous human CREG gene or protein (Fig. 1C). To investigate whether CREG expression is regulated by pressure overload, WT mice were subjected to AB for different durations. CREG expression in the left ventricle was markedly decreased compared to basal levels after 8 weeks of AB (Fig. 1D). Importantly, the protein levels of TG CREG were not significantly affected by AB and this pattern of expression persisted in hearts for 8 weeks after surgery (Fig. 1D).

The effect of CREG on cardiac hypertrophy *in vivo*

To determine whether cardiac overexpression of CREG antagonized the hypertrophic response to pressure overload, WT littermates and TG mice were subjected to AB surgery or sham operation. TG mice showed significant attenuation of hypertrophy after 8 weeks AB compared to WT littermates, as measured by the ratios of HW/BW, LW/BW and cardiomyocyte cross-sectional area (Table 1). No significant differences were observed in the sham-operated TG and WT mice. Cardiac dilatation, wall thickness and dysfunction were also inhibited or reversed in TG mice, as evidenced by improvements in left ventricular end-systolic diameter, left ventricular end-diastolic diameter, left ventricular posterior wall thickness, left ventricular septum, diastolic and percent fractional shortening (%FS) (Table 1). Gross hearts, haematoxylin and eosin and WGA staining further confirmed the inhibitory effect of CREG on cardiac remodelling after AB (Fig. 2A, C and D). We next examined the potential role of CREG on hypertrophy induced by Ang II infusion. Osmotic minipumps were implanted subcutaneously for a 4-week administration period, followed by cardiac functional assessment. The Ang II-induced increase in HW/BW and LW/BW as well as

Table 2 Echocardiographic data showed the effects of CREG on Cardiac hypertrophy induced by Ang II infusion

Parameter	WT-saline mice	TG-saline mice	WT-Ang II mice	TG-Ang II mice
Number	$n = 9$	$n = 10$	$n = 10$	$n = 9$
BW, g	25.3 ± 1.7	25.1 ± 1.4	25.9 ± 1.2	26.2 ± 1.6
HW/BW	4.58 ± 0.11	4.52 ± 0.03	5.79 ± 0.07*	4.85 ± 0.04§
LW/BW	5.18 ± 0.05	5.16 ± 0.06	6.22 ± 0.05*	5.29 ± 0.07§
CSA	252 ± 24.5	255 ± 25.9	398 ± 30.2*	278 ± 21.4§
SBP, mmHg	117.2 ± 4.7	115.9 ± 4.8	145.2 ± 3.7*	143.7 ± 5.2*
HR, beats/min.	449 ± 28	451 ± 25	468 ± 17	472 ± 31
PWT(mm)	1.23 ± 0.02	1.26 ± 0.04	2.63 ± 0. 03*	1.59 ± 0.04§
LVEDD (mm)	3.65 ± 0.03	3.67 ± 0.04	4.74 ± 0.05*	3.88 ± 0.04§
LVESD (mm)	2.42 ± 0.05	2.41 ± 0.06	3.48 ± 0.02*	2.52 ± 0.03§
IVSd (mm)	0.61 ± 0.03	0.64 ± 0.05	1.35 ± 0.04*	0.85 ± 0.03§
LVPWd (mm)	0.64 ± 0.03	0.65 ± 0.05	1.32 ± 0.05*	0.81 ± 0.03§
FS (%)	55.2 ± 3.1	54.7 ± 2.5	37.6 ± 1.6*	47.1 ± 1.6*§
EF (%)	60.2 ± 3.6	58.7 ± 4.2	30.5 ± 3.4*	47.5 ± 3.1*§
LVEDP (mm Hg)	9.4 ± 1.3	9.7 ± 1.5	16.6 ± 1.7*	11.9 ± 1.6§
Left ventricular dP/dTmax (mm Hg/s)	8983.7 ± 511.8	8879.6 ± 477.8	7029.8 ± 455.5*	8333.9 ± 500.8§
Left ventricular dP/dTmin (mm Hg/s)	−8115.6 ± 431.6	−8289.7 ± 475.3	−6889.7 ± 483.2*	−7884.8 ± 458.2§

*$P < 0.01$ was obtained for the WT-Saline values; §$P < 0.01$ was obtained for the WT-Ang II infusion values after Ang II infusion.

cardiomyocyte cross-sectional area were also attenuated in TG mice compared to WT mice (Table 2). Cardiac-specific overexpression of CREG abrogated Ang II-induced cardiac chamber dilatation and wall thickness in both systole and diastole (Table 2). These findings were confirmed by histological analysis (Fig. 2B, E and F). Atrial natriuretic peptide (*ANP*), B-type natriuretic peptide (*BNP*), myosin heavy polypeptide 7 cardiac muscle β (*Myh7*), and actin α1 skeletal muscle (*Acta1*) are markers for cardiac hypertrophy [13]. To determine whether CREG affected the mRNA expression levels of these markers, we performed real-time PCR. Our results showed that the induction of these hypertrophic genes was severely blunted in TG mice in response to AB and Ang II infusion (Fig. 2G and H). These findings suggest that CREG prevents the development of cardiac hypertrophy induced by pressure overload or Ang II stimulation *in vivo*.

The effect of CREG on MEK-ERK1/2 signalling pathway

To explore the molecular mechanisms through which CREG attenuates the hypertrophic response, we examined the state of activa-

tion of MAPK in TG and WT hearts in the two hypertrophic models. We found that the phosphorylated levels of MEK1, ERK1/2, p38 and JNK1/2 were significantly increased in both AB and Ang II-infused WT hearts. However, the increased level of MEK1/2 and ERK1/2 was almost completely blocked in TG hearts, whereas p38 and JNK1/2 were similarly activated in the WT and TG mice (Fig. 3A and B). Collectively, these data suggest that constitutive expression of CREG blunts the activation of MEK-ERK1/2 signalling, although it has no effect on p38 and JNK activation in hearts subjected to AB or Ang II stimulation.

In order to further examine the role of CREG on MEK-ERK1/2 signalling in the heart, we exposed cultured neonatal rat cardiomyocytes to 1 μm Ang II infected with Ad-CREG or Ad-shCREG. At the beginning, we screened three shCREG and found that no. 3 shCREG markedly inhibited CREG expression in cardiac myocytes (Fig. 3C). Therefore, we chose no. 3 shCREG for the following experiments. Further studies showed that Ang II induced a significant phosphorylation of MEK and ERK1/2 that was almost completely blocked and sustained for all tested time-points by overexpression of CREG, whereas decreased CREG levels by infection of Ad-shCREG resulted in pronounced activation of MEK and ERK1/2 in cardiac myocytes (Fig. 3D). Our
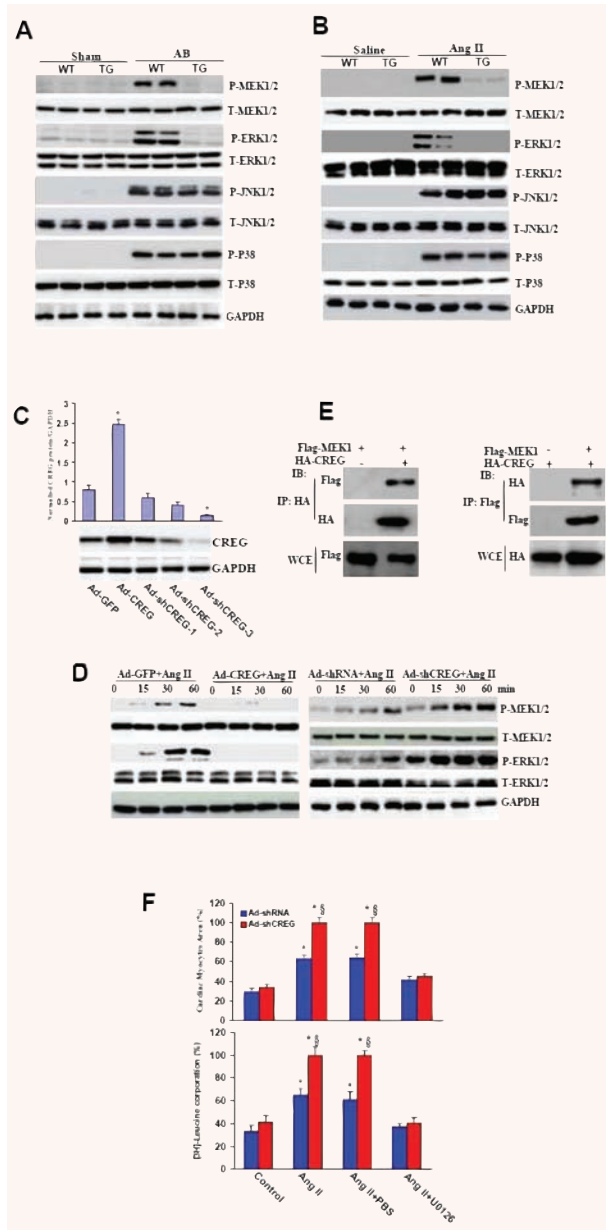
inhibitor U0126 (Fig. 3F). These results suggest that CREG inhibits cardiac hypertrophy through direct inhibition of MEK-ERK1/2 signalling in cardiac myocytes.

The effect of CREG on fibrosis *in vivo* and *in vitro*

Cardiac hypertrophy is associated with increased fibrosis in the myocardium, characterized by the over-production of extracellular matrix proteins [13]. To determine the extent of fibrosis in the heart, paraffin-embedded slides were stained with PSR. Marked interstitial fibrosis was detected in the WT mice subjected to AB or Ang II infusion by PSR staining. The extent of cardiac fibrosis in these two hypertrophic models was, however, remarkably reduced in TG mice (Fig. 4A–E). Subsequent analysis of mRNA expression levels of known mediators of fibrosis including transforming growth factor-β1(*Tgfβ1*), procollagen, type I, α1 (*Col1α1*), procollagen, type III, α1(*Col3α1*) and connective tissue growth factor (*Ctgf*), demonstrated a blunted response in TG mice (Fig. 4F and G). To confirm our *in vivo* data, we examined the effect of CREG on collagen synthesis in isolated cardiac fibroblasts by [^{3}H]proline incorporation assays. Cells were serum-starved for 24 hrs in 1% FCS after infection with Ad-CREG and Ad-shCREG, and then treated with TGF-β₁ (15 ng/ml) for the indicated time. TGF-β₁-stimulated [^{3}H]-proline incorporation was attenuated by infection with Ad-CREG and promoted by infection with Ad-shCREG (Fig. 4H). To further confirm the effects of CREG on collagen synthesis, luciferase assay demonstrated an increase of promoter activities of COL1A2 and CTGF with CREG inhibition, and the converse with CREG overexpression (Fig. 4I).

The effect of CREG on TGF-β/Smad signalling

TGF-β₁ induces collagen synthesis *via* activation of a number of transcription factors, including Smads [14, 15]. To further elucidate

findings suggest that CREG inhibits MEK-ERK1/2 signalling both *in vitro* and *in vivo* in response to hypertrophic stimuli. Naturally, we further investigated whether CREG directly interacts with MEK-ERK signalling. Immunoprecipitation analysis showed that CREG directly interacted with MEK1 (Fig. 3E). In order to examine whether MEK-ERK1/2 signalling has a causative role in CREG-mediated inhibition of cardiac hypertrophy, further *in vitro* experiments were performed. As expected, decreased CREG levels led to pronounced hypertrophy induced by Ang II as assessed by [H^3]-leucine incorporation and surface area measurements, which was strongly blunted by treatment with ERK1/2

Fig. 4 The effects of CREG on fibrosis *in vivo* and *in vitro*. (**A** and **B**) Picrosirius red (PSR) staining on histological sections of the left ventricle was performed on indicated groups 8 weeks after AB or 4 weeks after Ang II infusion. (**C** and **D**) Representative PSR staining of microscopical images of whole heart cross sections. (**E**) Fibrotic areas/whole left ventricle area from histological sections was quantified using an image-analysing system. (**F** and **G**) Real-time PCR analyses of *Tgfβ1*, *Col1α1*, *Col3α1*, *Ctgf* were performed to determine mRNA expression levels in indicated groups. GAPDH was used as the sample loading control. Data represent typical results of 3 different experiments as mean ± S.E.M. (n = 4 to 6 mice/per group). *$P <$ 0.01 was obtained for the WT/sham or WT/saline values. (**H** and **I**) CREG was shown to inhibit TGF-β₁-induced [³H] proline incorporation and the promoter activities of COL1A2 and CTGF. [³H] proline incorporation and luciferase assay were performed as described in 'Materials and methods'. Values are mean ± S.E.M. The results were reproducible in three separate experiments. *$P <$ 0.01 was obtained for Ad-GFP infection group.

the cellular mechanisms underlying the antifibrotic effects of CREG, we assessed the regulatory role of CREG on Smad cascade activation. The increased level of Smad 2 phosphorylation and Smad 2/3 nuclear translocation was attenuated in TG mice in response to hypertrophic stimuli (Fig. 5A and B). We then infected cardiac fibroblasts with Ad-CREG or Ad-shCREG and treated with TGF-β₁. Western blot analyses revealed significant phosphorylation of Smad 2 and translocation of Smad 2/3 without any significant alterations in the expression of Smad 2 protein after TGF-β₁ treatment. Ad-CREG infection, however, almost completely suppressed Smad 2 phosphorylation as well as Smad 2/3 nuclear translocation (Fig. 5C). Importantly, Ad-shCREG infection enhanced TGF-β₁s effects. To further examine the mechanisms involved, we used confluent cardiac fibroblasts infected with Ad-GFP, Ad-caERK1/2, or Ad-dnERK1/2. Activation of ERK1/2 by infection with Ad-caERK1/2 revealed a significant increase in, whereas blocking ERK1/2 activity by Ad-dnERK1/2 infection almost completely abrogated, collagen synthesis and COL1A2 or CTGF promoter activities in response to TGF-β₁ (Fig. 5D). These findings suggest that CREG blocks collagen synthesis by disrupting MEK-ERK1/2-dependent TGF-β-Smad signalling.

The effect of CREG on inflammation *in vivo* and *in vitro*

Activation of inflammatory signalling pathways promotes cardiac hypertrophy and fibrosis, whereas inhibition of inflammation can attenuate these responses [16, 17]. We therefore examined the expression of four representative cytokines IL-6, IL-1β, MCP-1 and TNF-α, after pressure overload or Ang II stimulation. Expression of IL-6, IL-1β, MCP-1 and TNF-α mRNA was significantly elevated after AB or Ang II treatment in WT mice, but not in TG mice (Fig. 6A and B). Further studies demonstrated that
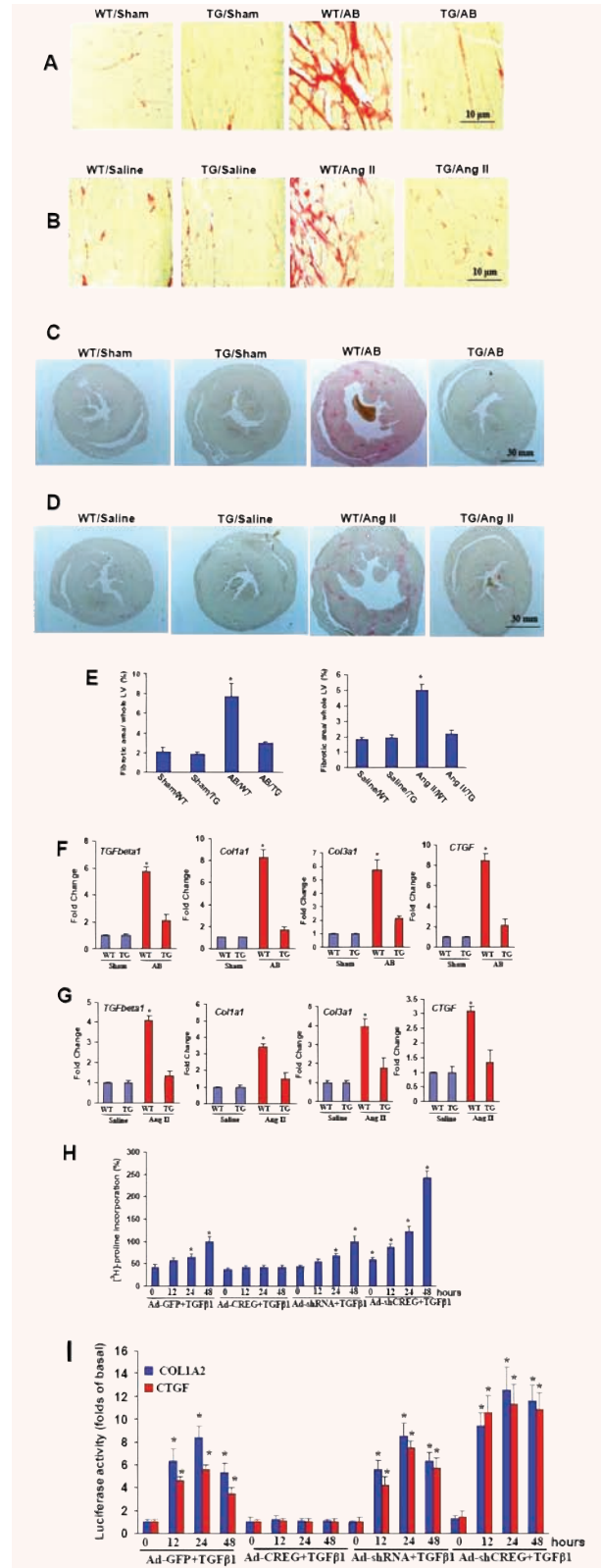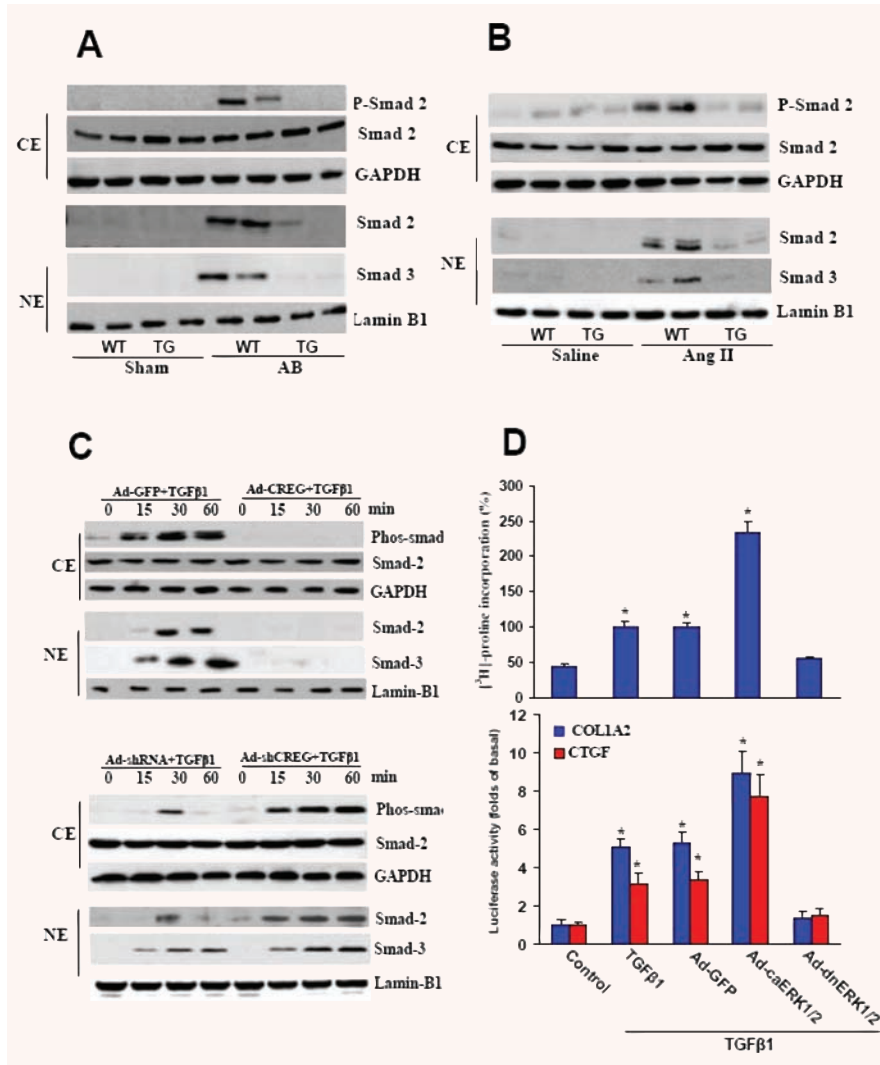
Fig. 5. The effect of CREG on TGF-β/Smad signalling. **(A** and **B)** Representative blots of Smad-2 phosphorylation and Smad-2/3 translocation from indicated groups 8 weeks after AB ($n = 3$) or 4 weeks of Ang II infusion ($n = 4$). The results were reproducible in three separate experiments (CE, cytoplasmic extract; NE, nuclear extract). **(C)** Representative blots of Smad-2 phosphorylation and Smad-2/3 translocation induced by TGF-β₁ in cardiac fibroblasts after infection with different adenovirus. **(D)** The effect of ERK1/2 activation on collagen synthesis along with promoter activities of COL1A2 and CTGF. Cells were infected with or without indicated adenovirus for 24 hrs, and then incubated with 15 ng/ml TGF-β₁ for up to 48 hrs. [³H] proline incorporation and luciferase assay were performed as described in 'Materials and methods'. *$P < 0.01$ was obtained for control group. The results were reproducible in three separate experiments.

8 weeks of AB or 4 weeks of Ang II stimulation increased the phosphorylation of IκBα and IKK-β in the myocardium of WT mice, but not in TG mice (Fig. 6C). To further validate the effects of CREG on NF-κB signalling, we cultured neonatal rat cardiac myocytes for *in vitro* analyses to substantiate our *in vivo* findings. Inhibition of CREG promoted the induction of NF-κB activity, while overexpression of CREG attenuated these effects induced by Ang II (Fig. 6D). These results indicate that CREG blocks NF-κB activity and NF-κB-dependent inflammatory responses in response to hypertrophic stimuli both *in vivo* and *in vitro*. The potential regulation of NF-κB by MAPK signalling prompted us to investigate the functional significance of ERK1/2 on NF-κB activation in response to hypertrophic stimuli. Infection of cardiac myocytes with Ad-caERK1/2 promoted NF-κB activation, whereas infection of Ad-dn-ERK1/2 significantly attenuated NF-κB activation (Fig. 6D). These results indicate that CREG blocks NF-κB

signalling and NF-κB-dependent inflammatory responses partly through disruption of MEK-ERK1/2 signalling.

Discussion

In the present study, we examined the role of CREG in cardiac hypertrophy by using cardiac-specific CREG TG mice. The results demonstrated that elevated levels of CREG protein expression in TG mice profoundly blunt cardiac hypertrophy, chamber dilatation, fibrosis and inflammation *via* disruption of MEK-ERK1/2 signalling following chronic pressure overload or Ang II stimulation.

The mechanism by which CREG mediates its anti-hypertrophic effects remains unclear. Recent studies demonstrated

Fig. 6 The effect of CREG on inflammation *in vivo* and *in vitro*. (**A** and **B**) Real-time PCR analysis of *TNF-α*, *IL-1β*, *IL-6* and *MCP-1* mRNA expression in the myocardium obtained from indicated groups at 8 weeks AB (A, *n* = 4) or 4 weeks Ang II infusion (B, *n* = 6). Each assay was performed in triplicate. **P* < 0.05 for difference from WT/sham values or WT/saline values. (**C**) Western blot analysis of IκBα and IKK-β phosphorylation of the myocardium was obtained from indicated animals at 8 weeks AB (A, *n* = 5) or 4 weeks Ang II infusion (B, *n* = 4). (**D**) The effects of CREG expression and ERK1/2 activation on Ang II-induced NF-κB transcriptional activity. Luciferase assay were performed as described in 'Materials and methods'. Values are mean ± S.E.M. * *P* < 0.01 was obtained for PBS/control group. The results were reproducible in three separate experiments.
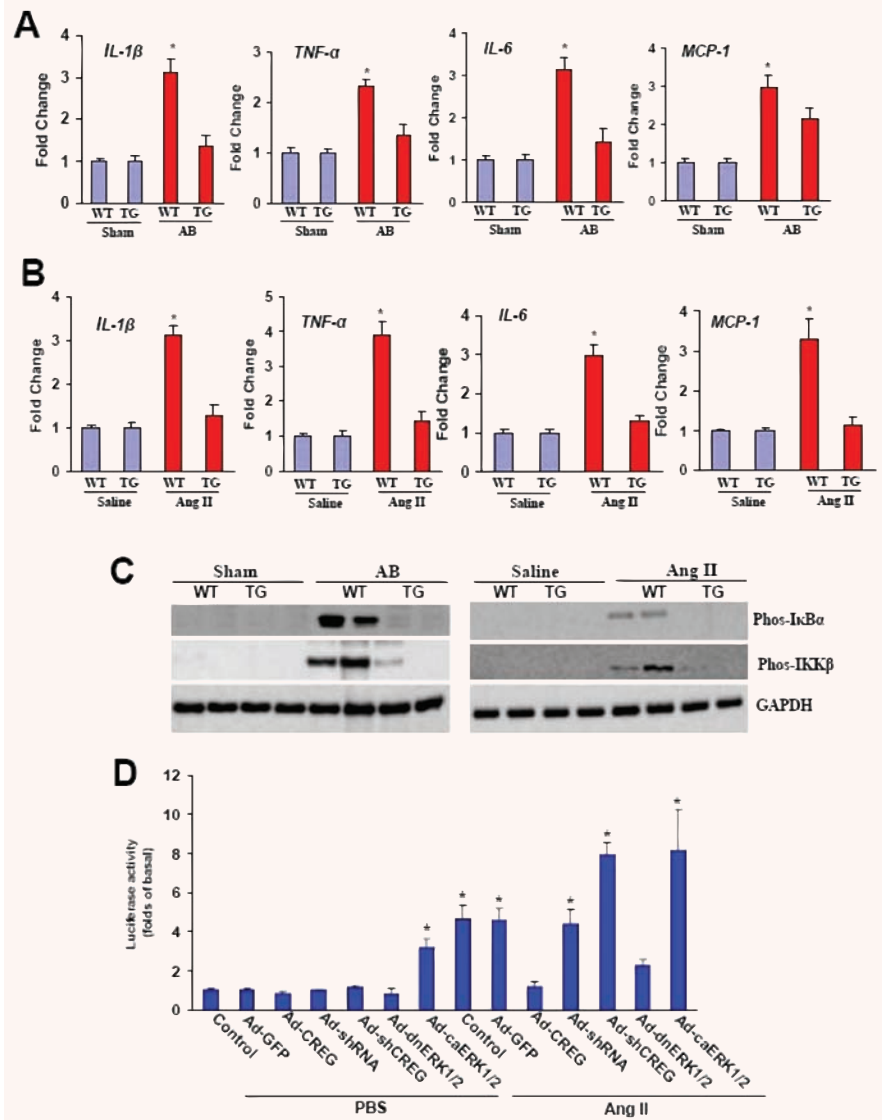
that MAPK signalling pathways play a key role in the progress of cardiac hypertrophy [18, 19]. The MAPK cascade consists of a sequence of successively acting kinases, including p38, JNKs, and ERKs and is initiated in cardiac myocytes by stress stimuli [18, 19]. Once activated, p38, JNKs, and ERKs phosphorylate a wide array of intracellular targets, which include numerous transcription factors resulting in the reprogramming of cardiac gene expression. The role that MEK1-ERK1/2 plays in the regulation of cardiac hypertrophy is an area of ongoing debate [20, 21]. It has been shown that ERK1/2 is activated in cultured neonatal rat cardiomyocytes by agonist stimulation. Activation of MEK1 augmented cardiac hypertrophy in cultured cardiomyocytes while blocking MEK1 attenuated it. Similarly, using the MEK1 inhibitor, U0126, demonstrated that ERKs were required

for the hypertrophic response induced by Ang II and ET-1. However, a number of additional studies have disputed the importance of MEK1-ERK1/2 in the regulation of cardiac hypertrophy, and one study has suggested that ERK activation prevents cardiac hypertrophy. To examine the molecular mechanisms involved in CREG's ability to protect against cardiac hypertrophy, we examined the status of MAPKs signalling in our hypertrophic models. An important finding of this study is that MEK and ERK1/2 activation were almost completely blocked by cardiac expression of human CREG in response to hypertrophic stimuli. However, the phosphorylation of p38, JNK1/2, and Akt was not affected by CREG. Therefore MEK-ERK1/2 signalling was a critical pathway through which CREG influences cardiac growth. Further study demonstrated that CREG can bind directly

to MEK1, disrupt its interactions, and finally block its activation. These findings are consistent with two recent studies showing that CREG inhibits ERK1/2 activation in cardiac myocytes and vascular muscle cells [10, 11]. Therefore, inhibition of MEK-ERK1/2 signalling in the context of the adult heart under stress, such as pressure overload, may provide a therapeutic strategy to regress cardiac hypertrophy, and our data indicated that the inhibitory effects of CREG on cardiac hypertrophy are mediated through MEK-ERK1/2 signalling.

Pathological cardiac hypertrophy is accompanied by interstitial and perivascular fibrosis and approaches to prevent collagen deposition in the heart have been limited to date [22–26]. This study revealed that CREG blocks cardiac fibrosis *in vivo* and inhibits collagen synthesis *in vitro*. Our study is the first to report inhibition of fibrosis and TGF-β_1-induced collagen synthesis in cardiac fibroblasts by CREG. In an attempt to elucidate the mechanisms underlying the inhibitory effect of CREG on fibrosis, we analysed key components of TGF-β_1-Smad signal transduction. Blockade of this signalling pathway was predicted to blunt fibrosis [14, 15]. In line with these notions, our data suggest, for the first time, that CREG abrogates Smad 2 phosphorylation and Smad 2/3 translocation in both cultured cardiac fibroblasts and hypertrophied hearts thus inhibiting collagen synthesis and fibrosis. Recent studies indicate that TGF-β_1/Smad signalling can be regulated by MEK-ERK1/2 signalling [26]. We therefore examined the effects of MEK-ERK1/2 activation on fibrotic signalling and found that blocking MEK-ERK1/2 activation led to significant inhibition, while activation of MEK-ERK1/2 resulted in up-regulation of collagen synthesis and Smad 2/3 signalling, indicating that CREG attenuates fibrosis by blocking MEK-ERK1/2 signalling.

Cardiac hypertrophy and fibrosis may be promoted by activation of inflammatory signalling pathways [27–30]. We observed that the induction of inflammatory cytokines by hypertrophic stimuli was attenuated by cardiac-forced expression of CREG, indicating an important role for CREG in regulating inflammatory response in the heart. One possible mechanism for such a protective effect is that CREG expression directly blocks NF-κB activation, resulting in attenuation of downstream cytokines, including IL-1β, TNF-α, IL-6 and MCP-1, each of which has a κB-binding domain in its promoter site. Our present data suggest that CREG abrogates NF-κB activation by disrupting DNA binding and the phosphorylation of IκBα. By blocking NF-κB signalling, CREG may inhibit the early steps of inflammation and modulate the amplification of multiple cytokine signalling cascades. Therefore, treatment of targeting NF-κB may be a more attractive approach to blocking each respective cytokine individually.

In conclusion, for the first time, we identified the role of CREG in maintaining cardiac contractility and reducing fibrosis and inflammation in response to hypertrophic stimuli both *in vitro* and *in vivo*. The sub-cellular mechanism underlying the protective role of CREG against cardiac hypertrophy has been shown to be *via* inhibiting the MEK-ERK1/2 signalling pathway. Our study provides insights into the mechanisms of cardiac hypertrophy and may be of great value for developing novel treatment strategies for cardiac hypertrophy through targeting CREG signalling pathway.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. A list of the primers used in this study

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