Long-Term Activation of Adenosine Monophosphate-Activated Protein Kinase Attenuates Pressure-Overload-Induced Cardiac Hypertrophy

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Abstract   Recent in vitro studies suggest that adenosine monophosphate (AMP)-activated protein kinase (AMPK) exerts inhibitory effects on cardiac hypertrophy. However, it is unclear whether long-term activation of AMPK will affect cardiac hypertrophy in vivo. In these reports, we investigate the in vivo effects of long-term AMPK activation on cardiac hypertrophy and the related molecular mechanisms. To examine the effects of AMPK activation in the development of pressure overload-induced cardiac hypertrophy, we administered 5-aminoimidazole 1 carboxamide ribonucleoside (AICAR, 0.5 mg/g body wt), a specific activator of AMPK, to rats with transaortic constriction (TAC) for 7 weeks. We found that long-term AMPK activation attenuated cardiac hypertrophy, and improved cardiac function in rats subjected to TAC. Furthermore, long-term AMPK activation attenuated protein synthesis, diminished calcineurin-nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) signaling in pressure overload-induced hypertrophic hearts. Our in vitro experiments further proved that activation of AMPK by infection of AdAMPK blocked cardiac hypertrophy and NFAT, NF-κB, and MAPK signal pathways. The present study demonstrates for the first time that pharmacological activation of AMPK inhibits cardiac hypertrophy in through blocking signaling transduction pathways that are involved in cardiac growth. It presents a potential therapy strategy to inhibit pathological cardiac hypertrophy by increasing the activity of AMPK. J. Cell. Biochem. 100: 1086–1099, 2007.   © 2007 Wiley-Liss, Inc.

Key words: AMPK; NFAT; NF-κB; cardiac hypertrophy

Cardiac hypertrophy and heart failure remain the leading causes of death in developed countries. Cardiac hypertrophy is characterized by an increase in myocardial cell size, a higher degree of sarcomeric organization, re-activation of the fetal gene program, and changes in gene transcription and translation resulting in enhanced protein synthesis [Diez et al., 2005; Muslin, 2005]. Although the mechanisms are not well understood, a number of studies have elucidated conceivable mechanisms responsible for the development of cardiac hypertrophy. Several signaling pathways have been shown to be involved in the development of hypertrophic growth, such as mitogen-activated protein kinases (MAPK), nuclear factor κB (NF-κB), and calcineurin/nuclear factor of activated T cells (NFAT) [Dorn and Force, 2005; Brown et al., 2006]. Pharmacological interventions of these signaling pathways have been expected to become promising therapeutic options in treating cardiac hypertrophy and heart failure.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine kinase, which is activated by cellular stresses that deplete ATP and has been identified as a key regulator of cellular energy homeostasis [Chan and Dyck, 2005]. AMPK responds to increases in the AMP/ATP ratio by switching off

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ATP-consuming pathways and switching on energy-producing pathways in an attempt to restore cellular ATP levels. Recent evidence has linked the activation of AMPK with hypertrophic growth in the heart. Pressure overload-induced hypertrophy has been shown to be associated with increased AMPK activity and expression. Recent evidence indicates that AMPK activation inhibits proteins synthesis and therefore, possibly hypertrophy through modulating several key regulators of protein synthesis, such as eukaryotic elongation factor-2 (eEF2), p70S6 kinase, and mammalian target of rapamycin (mTOR) [Proud, 2004]. Taken together, these observations indicate that AMPK may be a key regulator for the development of cardiac hypertrophy. Although much has been learned about the in vitro inhibitory effect of AMPK on cardiac hypertrophic response, the role of long-term activation of AMPK on cardiac hypertrophy in vivo has not been established. Besides, the signaling events upstream and downstream of AMPK in the regulation of cardiac hypertrophy are still unclear. Especially, whether AMPK interferes with other signaling components involved in the regulation of cardiac hypertrophy, such as MAPK, calcineurin/NFAT, and NF-κB, is poorly understood. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) has been studied extensively as an activator of AMPK in different cell types [Reiter et al., 2005]. Once inside the cell, AICAR is phosphorylated by adenosine kinase to ZMP, which mimics AMP and activates AMPK without altering the cellular levels of ATP, ADP, or AMP. Some studies have demonstrated that AICAR inhibits the production of proinflammatory mediators (TNF-α, IL-1β, IL-6, and NO) in primary astrocytes, microglia, and macrophages via inhibition of NF-κB and the C/EBP pathways [Giri et al., 2004]. Others studies showed that AICAR inhibits vascular smooth cell and cancer cell proliferation [Igata et al., 2005; Rattan et al., 2005]. The present study was designed to elucidate these problems through long-term administration of AICAR on animal models of cardiac hypertrophy.

MATERIALS AND METHODS

Materials

AICAR was purchased from Calbiochem (San Diego, CA). Anti-phospho-mTOR (Ser2448), anti-phospho-p70S6K(Thr-421/Ser-424), anti-IKKα, anti-IKKβ, anti-phospho-IκBα, and anti-IκBα bodies were from Cell Signaling Technology (Willmingon, MA). Anti-phospho-Thr172 AMPK and anti-AMPK are from Cell Signaling Technology and anti-phospho-Ser79 ACC and anti-ACC are from Upstate Biotech (Charlottesville, VA). IKK activity kit was from B&D Bioscience, while other antibodies were purchased from Santa Cruz Biotechnology. Unless otherwise indicated, all other chemicals and materials were purchased from Sigma (St. Louis, MO).

Construction of Recombinant Adenovirus

Recombinant adenoviruses expressing either AMPK or green fluorescent protein (GFP) were prepared as previously described [Zou et al., 2002, 2003]. Briefly, DNA encoding residues AMPKα1 1–312 of 1 was used to construct the recombinant adenovirus. The cDNA of $\alpha 1^{312}$ was subcloned into the shuttle vector pAd-Track-CMV. The resultant plasmid was linearized by the restriction endonuclease *PmeI* and cotransformed with the supercoiled adenoviral vector pAd-Easy1 into *Escherichia coli* strain BJ5183. Recombinants were selected by kanamycin resistance and screened by restriction endonuclease digestion. The desired virus (AdAMPK and AdGFP) were amplified, purified by CsCl ultracentrifugation, and stored in 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L $MgCl_2$, and 10% (vol/vol) glycerol at $-70°C$ until used for experiments. The titer of the virus stock was counted by the ratio of GFP-positive cells after serial dilution using HEK293 cells. We found that AdAMPK at a MOI of 100 was sufficient to infect >95% of the cells. After 4 h of incubation, media were removed, and cells were incubated in appropriate media for various periods of time.

Animal Models of Cardiac Hypertrophy

This study as well as procedures involving animals' use and all surgeries were proved by Institutional Animal Care and Use Committee. For the pressure-overload model, we used male Sprague–Dawley rats (8 weeks old, 200–230 g). Transaortic constriction (TAC) was created using a 7–0 suture tied twice around the abdominal aorta and a 35-gauge needle. The needle was then retracted yielding a 70–80% constriction with an outer aortic diameter of approximately 0.3 mm. In the sham-operated group, as surgical and age-matched control, the

same surgical operations were performed as in the above-mentioned rats except the aorta was constricted. After aortic partial coarctation, AICAR-treated animals were subcutaneously injected every morning for 7 weeks with 0.5 mg AICAR/g body wt as previously described. All control animals were injected with a corresponding volume of 0.9% NaCl every day. After 7 weeks of TAC, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and cardiac dimensions and function were analyzed by 15-MHz pulse-wave Doppler echocardiography (Philips, SONOS 5500; The Netherlands). Systolic blood pressure (SBP) and heart rate (HR) was measured as previously [Li et al., 2005; Quan et al., 2004] between 10 and 12 AM in each rat by use of the tail-cuff plethysmograph method (IITC, Blood Pressure Meter, Letica Scientific Instrumentation), after the rats were warm at 37°C for 10 min.

Histological Analysis and Cardiomyocyte Size Measurement

All the hearts were arrested in diastole with KCl (30 mM), followed by perfusion fixation with 10% formalin. Fixed hearts were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin for overall morphology. Mean myocytes diameter was calculated by measuring 100 cells from sections stained with hematoxylin and eosin.

Neurohormonal Factors

Plasma atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), angiotensin II (Ang II), endothelin-1 (ET-1), and renin activity levels were determined with commercially available kits (Phoenix Pharmaceuticals, Inc.).

AMPK Expressions and Activity as Well as ACC Expression and Phosphorylation in Cardiac Tissue

The expressions of phosphorylation of AMPKα (Thr 172), AMPK pan α, and ACC were examined by Western blot. AMPKα1 and α2 activities were determined using procedures described previously [Gonzalez et al., 2004]. Cardiac tissues were homogenized in buffer A (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). The homogenates were incubated with the AMPKα1 and α2 antibody-bound protein A beads for 2 h at 4°C. Immunocomplexes were washed with PBS and suspended in 50 mM Tris buffer (pH 7.5) for AMPK activity assay.

Assay of Calcineurin Activity

Calcineurin activity was determined in lysates of whole ventricular tissues and cardiac myocytes as previously described [Tokudome et al., 2005]. For the phosphatase assay, RII peptide (Sigma) was phosphorylated by protein kinase A (Calbiochem) in the presence of [γ-^{32}P]ATP overnight at 30°C in the presence of 1 mol/L Tris-HCl (pH 7.5), 0.1 mol/L dithiothreitol, and 0.1 mol/L MgCl2. Tissues were homogenized in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.2% NP-40, and protease inhibitor cocktail). After the debris was removed by centrifugation, the supernatant was incubated with phosphorylated RII peptide for 30 min at 30°C. Okadaic acid (500 nmol/L) was added to the reactions to specifically suppress endogenous protein phosphatase PP1 and PP2A. The amount of liberated 32P was determined by the Cherenkov method.

Western Blotting

Fifty micrograms of total cell lysate were used for SDS–PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with primary antibodies at 4°C for 12 h and washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20). It was then incubated with anti-mouse or rat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 37°C for 2 h, and the immune complex was visualized with the ECL system (Santa Cruz Biotechnology).

Northern Blot

All procedures were performed as previously described in detail [Kim et al., 1995]. Whole RNA was extracted from heart tissue with TRIzol reagent according to the manufacturer's instructions (Invitrogen). Twenty micrograms of RNA was run on a 1% agarose/formaldehyde gel at 120 V for 2 h. The RNA was transferred to

a nylon membrane by vacuum for 1.5 h and cross-linked by UV wave. Probes consisting of a 825-bp fragment of rat atrial natriuretic polypeptide (ANP) cDNA and a 347-bp fragment of BNP cDNA were generated by reverse transcription of rat heart mRNA and amplification of the resulting cDNA by the polymerase chain reaction as described previously [Nakagawa et al., 1995; Li et al., 2006], and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3-kb *Pst* I–*Pst* I fragment. The probe was labeled with α-[^{32}P]dCTP using Prime-a-Gene labeling System. The radioactivity was detected by phospholmager using a scanner.

Electrophoretic Mobility Shift Assays (EMSA) and IKK Activity

Nuclear extracts were prepared from cardiac tissue and cardiac myocytes. Synthetic, double-strand oligonucleotide (primers: NF-κB: 5′-TT-GTTACAAGGGACTTTCCGCTGGG GACTTT-CCAGGGAGGCGTGG-3′, NFAT: 5′-CGCCAAA-GACGCCAAAGAGGAAAA TTTGTTTCATA-3′) containing NF-κB and NFAT-binding domain were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and separated from unincorporated [γ-^{32}P] ATP by gel filtration using a Nick column (Pharmacia, Piscataway, NJ). The unlabeled oligonucleotides were used for competition. Nuclear extracts (10 μg) were incubated with 50,000 cpm labeled probe and analyzed by 8% PAGE; then autoradiography was performed. IKK activity was performed as described previously [Li et al., 2005, 2006].

Neonatal Rat Cardiac Myocyte Culture

Neonatal rat cardiomyocytes were prepared as described previously [Li et al., 2005, 2006]: Neonatal rat pups were killed by swift decapitation within the first 1–2 days after birth. Enzymatic and mechanical dissociation of cardiomyocytes was then performed using the Neonatal Cardiomyocyte Isolation System supplied by Worthington Biochemicals. The cells were collected by low-speed centrifugation (1,500 rpm for 10 min at room temperature). The supernatant was discarded and the cell pellet resuspended in DMEM (high glucose) culture medium containing 10% FCS (Hyclone Laboratories). Dispersed cells were preplated for 90 min to remove fibroblasts and other proliferation cells, and unattached cells counted and seeded onto 6-well culture plates at a

density of 3×10^5/well. The media was changed every 48 h beginning the day after seeding. Bromodeoxyuridine (0.1 mM) was added to the culture media for the first 72 h to further minimize contamination from fibroblasts. Using this method, we routinely obtained primary cultures with >95% myocytes, as assessed by microscopic observation of spontaneous contraction and by immunocytochemical staining with an anti cardiac α-myosin heavy chain antibody. After culture 48 h, the cardiac myocytes were infected with AdAMPK for 24 h and subsequently stimulated with 1 μM Ang II.

[^{3}H]-Leucine Incorporation

[^{3}H]-leucine incorporation was measured as described previously [Li et al., 2006]. Briefly, cardiac myocytes were infected with AdAMPK for 24 h and then subsequently stimulated with Ang II and co-incubated with [^{3}H]-leucine (1μCi/mL) for 48 h. At the end of the experiment, the cells were washed with Hanks' solution and scraped off the well, and then treated with 10% trichloroacetic acid at 4°C for 60 min. The precipitates were then dissolved in NaOH (0.4 N). Aliquots were counted with a scintillation counter.

Reporter Assays

Cardiac myocytes were seeded in triplicate in 12-well plates, cells were transfected with 0.2 μg of −1,595 human BNP (hBNP)-luciferase, −2,593 human ANP (hANP)-luciferase (−2,593 to +18 relative to the transcription start site in *hANP* gene, linked to luciferase reporter in pMG-1) [Li et al., 2006] and internal control plasmid DNA using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. After 6 h of exposure to the DNA−LipofectAMINE complex, cells were cultured in medium containing 10% serum for 12 h and then incubated with serum-free medium for 12 h. Cells were infected with AdAMPK for 24 h and then were treated with Ang II for 24 h for ANP and BNP at which time they were harvested to obtain cell extracts for luciferase assays.

Statistical Analysis

Data are expressed as means $\pm$ SD. Measurements in each experiment were expressed as a percentage of the average value for the TAC group. 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

Effect of AICAR on Pressure Overload-Induced Cardiac Hypertrophy

To examine whether AICAR attenuates load-induced cardiac hypertrophy, we injected aortic-banded rat with AICAR or vehicle daily for 7 weeks after surgery. Rats were killed 7 weeks after the operation. The heart weight/body weight (HW/BW) ratio of aortic-banded rat was increased by $34.4 \pm 3.6\%$ compared with vehicle-injected sham-operated rat. In contrast, the HW/BW ratio of AICAR-injected banded rat was increased by only $11.5 \pm 4.4\%$ compared with AICAR-injected sham rat. Thus, AICAR suppressed the load-induced increase in HW/BW ratio by 67% (Table I). AICAR did not affect BW, lung weight, or liver weight (data not shown). Furthermore, injection with AICAR significantly decreased SBP induced by pressure-overload. We also examined the effect of AICAR on cardiac function using echocardiography. AICAR injection significantly decreased LV diastolic and systolic diameters in aortic-banded rat. Cardiac contractility, assessed by fractional shortening, was maintained by AICAR injection (Table I). Thus, AICAR injection was associated with a decrease in chamber size and normal systolic function. The cell area of vehicle-treated aortic-banded rat was increased by $46.7 \pm 6.0\%$ compared with vehicle- treated sham-operated animals. The cell area of hearts from aortic-banded rat

injected with AICAR was decreased by $19.9 \pm 6.2\%$ compared with AICAR-injected sham-operated rat (Fig. 1A,B). Thus, AICAR suppressed the load-induced increase in myocyte cell size by 57%. Collectively, the above data suggest that AICAR attenuated the pressure load-induced increase in heart size primarily through the regulation of myocyte cell size. Plasma ANP, BNP, Ang II, ET-1, and renin activity are established markers of cardiac hypertrophic response. Therefore, we also measured them from blood samples taken while animals were conscious. As shown in Table II, the plasma ANP, BNP, ET-1, and Ang II concentrations as well as renin activity were significantly increased in TAC group and such increase was markedly decreased by AICAR treatment. Cardiac hypertrophy in response to pressure overload is associated with reactivation of "fetal" genes. Therefore, we examined the expression of ANP and BNP in the hearts from rats with TAC. As shown in Figure 1C,D, there were marked elevation of ANP and BNP both at protein and mRNA levels in TAC-induced cardiac hypertrophy. Injection of AICAR significantly inhibited these alterations induced by TAC.

Effect of AICAR on AMPK Activity and Protein Synthesis

Initial experiments were designed to analyze whether AMPK activity is changed upon hypertrophic stimulus or injection of AICAR. Our results illustrated that the enzyme became phosphorylated, and hence stimulated, after rats was subjected to TAC (Fig. 2A,B). Consistent with Western blot results, basal levels of AMPK activity, measured with and without

TABLE I. Echocardiographic Data of Aortic-Banded Rat Injected With AICAR

	Sham	AICAR/Sham	TAC	AICAR/TAC
No. of animals	10	9	8	8
SBP (mmHg)	112 ± 9.4	117 ± 15.6	$154 \pm 7**$	$120 \pm 9.8^{\dagger}$
HR (beats/min)	343 ± 16	343 ± 14	341 ± 12	345 ± 12
HW/BW (mg/g)	3.0 ± 0.2	2.8 ± 1.1	$4.1 \pm 0.4*$	$3.4 \pm 0.2***$
AWT (mm)	1.8 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	$1.5 \pm 0.1***$
LVDD (mm)	6.9 ± 0.3	6.9 ± 0.7	$7.9 \pm 0.5*$	$6.8 \pm 0.5***$
LVSD (mm)	3.5 ± 0.4	3.6 ± 0.4	4.7 ± 0.4	$3.6 \pm 0.4***$
FS (%)	54 ± 3.8	53.5 ± 2.6	$41 \pm 3.6**$	$49 \pm 2.4***$

SBP, systolic blood pressure; HR, heart rate; BW, body weight; HW, heart weight; PWT, posterior wall thickness; LVDD, left ventricular diastolic diameter; LVSD, left ventricular systolic diameter; FS, fractional shortening.
*$P < 0.05$.
**$P < 0.001$ versus sham group.
***$P < 0.05$.
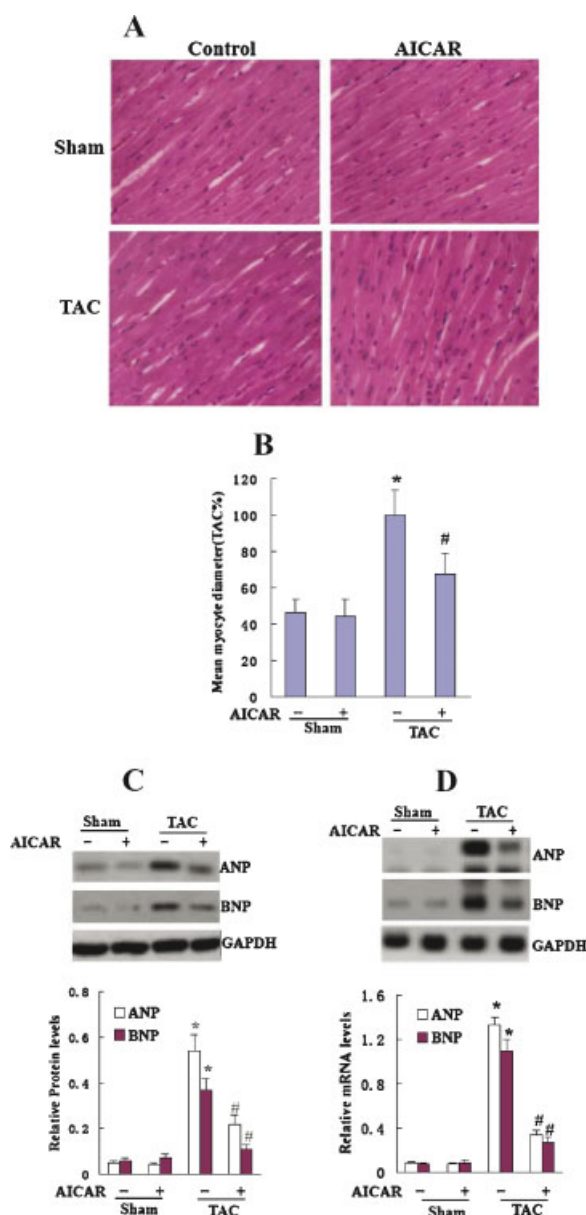$^{\dagger}P < 0.001$ versus vehicle-injected TAC group.

A

B

C

D

Fig. 1. AICAR inhibits cardiac hypertrophy in vivo. AICAR inhibits pressure overload-induced increase mean myocyte diameter. Representative hematoxylin and eosin-stained high magnification (100×) of left ventricles from different groups, Bar, 34 µm (**A**). Mean myocyte diameter was calculated by measuring 100 cells from sections (**B**). AICAR attenuates the upregulation of the expression of ANP and BNP protein and mRNA (**C** and **D**). Data are expressed as mean ± SD from n = 4 animals per group. *P < 0.01 versus sham-operated group, #P < 0.05 versus TAC alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

saturating concentrations of AMP, were very low in sham-operated rats. Moreover, TAC induced the activity of AMPKα2, but not AMPKα1. Interestingly, the increased cardiac tissue AMPKα2 activity became higher com-

pared with TAC group after AICAR treatment (Table III). AICAR also produced a similar stimulation of ACC phosphorylation, but total ACC protein was similar among groups.

To better understand the signaling mechanism by which AMPK regulates protein synthesis during hypertrophic growth, cell lysates from heart tissue were subjected to immunoblot analysis using anti-phospho-eEF2 kinase antibodies. As shown in Figure 2C,D, a remarkable reduction of phosphorylated eEF2 was observed in TAC group, which indicated increased protein synthesis. Treatment of rats subjected to TAC with AICAR almost completely reversed the phosphorylated eEF2 to basal level. The phosphorylation of mTOR and p70S6k, which promote protein synthesis, were measured by using anti-phospho-mTOR and anti-phospho-p70S6K antibodies, respectively. The relative phosphorylation level of them were significantly increased in TAC group, but were almost reversed to normality in AICAR + TAC group (Fig. 2C,D).

Effects of AICAR on MAPK Signaling Pathway

Previous studies demonstrate that MAPK are involved in the regulation of cardiac hypertrophy. In the present study, we examined the effect of AICAR on MAPK activities. As shown in Figure 3, the phosphorylation of extracellular signal-regulated protein kinases (ERKs), JNKs, and p38 MAPK were significantly enhanced in TAC group. Long-term injection of AICAR had different impacts on them during hypertrophic growth. Specifically, AICAR injection resulted in marked decreased ERK1/2 phosphorylation, whereas increased p38 phosphorylation in TAC-induced cardiac hypertrophy (Fig. 3A,B). No significant difference was shown in the phosphorylation of JNKs between TAC group and AICAR + TAC group. The observed effects of AICAR on TAC-mediated MAPKs activation were not due to the changes in total ERK1/2 and p38 protein levels.

Effect of AICAR on NF-κB and Calcineurin–NFAT Signaling Pathway

To investigate whether AMPK affect on NF-κB signaling pathway in pressure load-induced cardiac hypertrophy, nuclear proteins were isolated from heart tissue and subjected to EMSA for assessment of NF-κB-binding activity. As shown in Figure 4A, NF-κB-binding

TABLE II. Plasma Neurohormonal Factors in Aortic-Banded Rats Injected With AICAR

	Sham	AICAR/Sham	TAC	AICAR/TAC
No. of animals	10	9	8	8
ANP(pg/ml)	44 ± 5.2	46 ± 2.6	$307.6 \pm 26^*$	$93 \pm 7.8^{**}$
BNP(pg/ml)	32 ± 4.3	34 ± 1.4	$117.4 \pm 12.6^*$	$45.9 \pm 11.2^{**}$
ET-1(ng/L)	8.9 ± 1.2	8.3 ± 1.7	$24.1 \pm 4.2^*$	$12.7 \pm 2.4^{**}$
Ang II (pg/ml)	43 ± 7.2	39 ± 6.1	$178 \pm 22.1^*$	$62.5 \pm 8.1^{**}$
Renin activity (ng/ml/h)	1.78 ± 0.43	1.83 ± 0.6	$7.19 \pm 1.5^*$	$3.18 \pm 1.2^{**}$

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Ang II, angitensin II; ET-1, endothelin-1.
$^*P < 0.001$ versus sham group.
$^{**}P < 0.001$ versus vehicle-injected TAC group.

activity was low in controls but was significantly increased after aortic constriction. The elevated NF-κB-binding activity induced by TAC was significantly attenuated after AICAR treatment. To determine whether inhibition of

TAC-induced NF-κB activation by AICAR was due to inhibition of IκBα phosphorylation, we examined the cells for IκBα by Western blot. Our data showed that TAC significantly induced the phosphorylation of IκBα, which
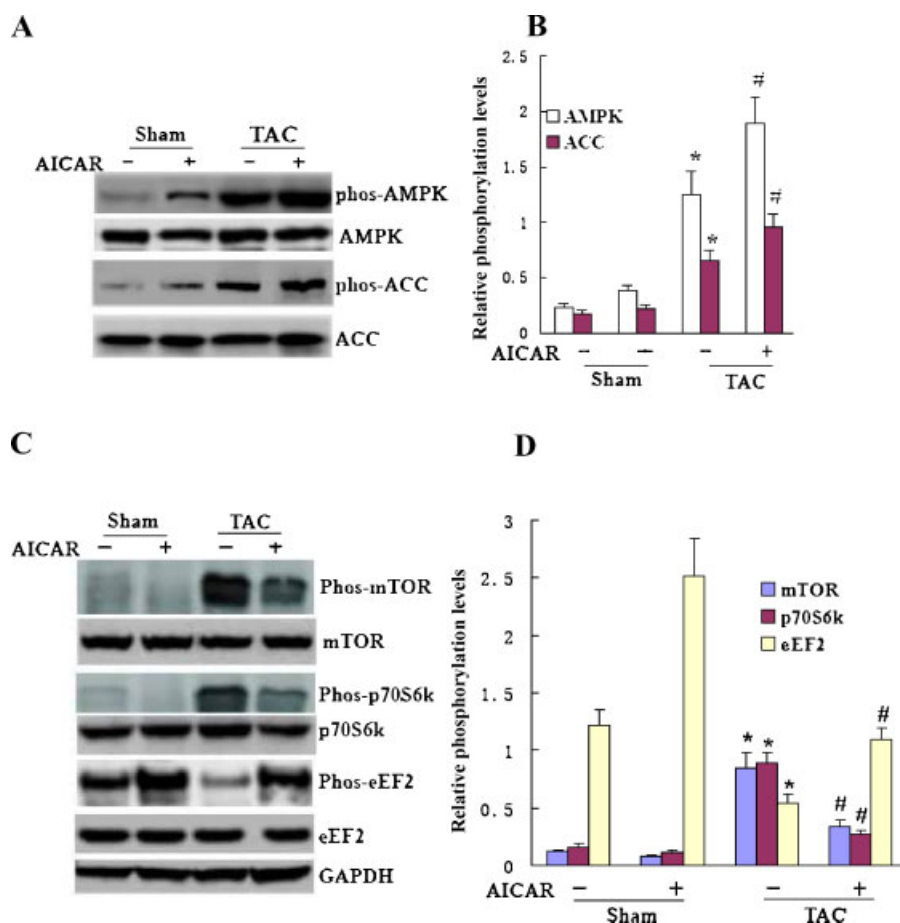

Fig. 2. Effect of AICAR on AMPK activity and protein synthesis pathway. **A**: Effect of AICAR on AMPK and ACC activation in banded or sham-operated animals 7 weeks after surgery. Lysates prepared from different treated-heart tissues and harvested, and Western blot for total and phosphorylated AMPK and ACC. The data presented are representative of a minimum of three separate experiments. **B**: Quantitative densitometry for AMPK and ACC phosphorylation. **C**: Effect of AICAR on mTOR, p70S6K, and eEF2 activation in banded or sham-operated animals 7 weeks after surgery. Lysates prepared from different treated-heart tissues and harvested, and Western blot for total and phosphorylated mTOR, p70S6K, and eEF2. The data presented are representative of a minimum of three separate experiments. **D**: Quantitative densitometry for mTOR, p70S6K, and eEF2 phosphorylation. Data are expressed as mean ± SD from n = 4 animals per group. *P < 0.01 versus sham-operated group, #P < 0.01 versus TAC alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III. AMPK Activity in Aortic-Banded Rats Injected With AICAR

Groups	AMPKα1		AMPKα2	
	0 μM AMP	200 μM AMP	0 μM AMP	200 μM AMP
Sham	7.2 ± 0.7	15.7 ± 1.4	3.6 ± 0.4	9.5 ± 1.1
Sham + AICAR	6.7 ± 0.3	14.9 ± 1.2	4.3 ± 0.5	11.2 ± 0.8
TAC	7.7 ± 0.4	16.5 ± 0.8	$5.2 \pm 0.8^*$	$14.6 \pm 1.5^*$
TAC + AICAR	6.9 ± 0.6	15.2 ± 0.9	$7.4 \pm 0.5^{**,\dagger}$	$19.2 \pm 1.8^{**,\dagger}$

Values are means $\pm$ SD in pmol/min · mg/protein. AMPK, adenosine monophosphate-activated protein kinase.
$^*P < 0.05$.
$^{**}P < 0.001$ versus sham group.
$^{\dagger}P < 0.001$ versus vehicle-injected TAC group.

was almost completely suppressed by AICAR (Fig. 4C). Phosphorylation of IκBα is necessary for its degradation and the subsequent NF-κB activation. We next examined the effects of AICAR on IκBα degradation. The degradation of IκBα was also marked showed in TAC rats and such degradation completely reversed by AICAR injection (Fig. 4C). Because TAC-induced phosphorylation of IκBα is mediated through IKKβ, these results suggested that AICAR must inhibit IKKβ activation. Indeed, as shown in Figure 4B, in immune-complex kinase assays, TAC activated IKKβ and AICAR treatment completely suppressed the activation. Under these conditions, AICAR had no effect on IKKα and IKKβ protein levels. The data also showed that AICAR administration inhibited

NF-κB-mediated gene expression, such as TNF-α, MMP-9, and MCP-1 (Fig. 4D,E).

Accumulating evidences showed that calcineurin is activated and intimately involved in load-induced cardiac hypertrophy. Therefore, we hypothesized that calcineurin–NFAT signaling is involved in the inhibitory effect of AMPK on cardiac hypertrophy. Calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate. After aortic constriction, calcineurin activity was elevated by 35%. In contrast, calcineurin activity was decreased by 26% after injection with AICAR compared with sham-treated rats (Fig. 4F). Moreover, we carried out EMSA to determine NFAT-binding activity. The NFAT-binding activity was increased
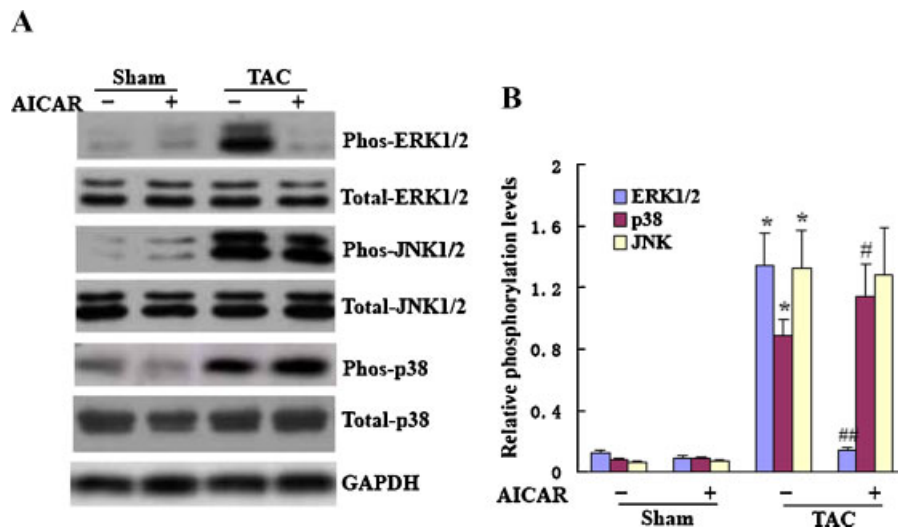

Fig. 3. Effect of AICAR on MAPK activation. **A**: Effect of AICAR on MAPK activation in banded or sham-operated animals 7 weeks after surgery. Lysates prepared from different treated-heart tissues and harvested, and Western blot for total and phosphorylated ERK1/2, JNK1/2, and p38. The data presented are representative of a minimum of three separate experiments. **B**: Quantitative densitometry for MAPKs phosphorylation. Data are expressed as mean $\pm$ SD from n = 4 animals per group. *$P < 0.01$ versus sham-operated group, ##$P < 0.05$, #$P < 0.01$ versus TAC alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

significantly after aortic constriction, while treatment of AICAR reversed it to nearly normal level (Fig. 4G).

AMPK Activation Attenuates Cardiac Hypertrophy Mediated by Ang II In Vitro

To further evaluate the effects of AMPK activation on cardiac hypertrophy, we used cardiac

myocytes. Cardiac myocytes were infected with AdAMPK for 24 h, and then subsequently treated with Ang II for 48 h. Neonatal rat cardiac myocytes treated with 1 μM Ang II displayed a significant 1.8-fold increase in protein synthesis over AdGFP-infected cardiac myocytes (Fig. 5A). However, in the infection of AdAMPK, Ang II-induced protein synthesis was almost completely blocked (Fig. 5A). Further studies demonstrated that AdAMPK infection markedly inhibited the promoter activities of ANP and BNP induced by Ang II compared with AdGFP infection (Fig. 5A).

Consistent with our in vivo results, the in vitro results further showed that AdAMPK infection markedly blocked the activation of NF-κB (Fig. 5B), NFAT (Fig. 5C), and ERK1/2 (Fig. 5D) mediated by Ang II treatment compared with AdGFP infection, indicating that the inhibitory effects of AICAR on cardiac hypertrophy and related signaling pathways is mediated by activation of AMPK.

DISCUSSION

A novel finding in the present study is that long-term activation of AMPK by pharmacological activator AICAR attenuated pressure load-induced cardiac hypertrophy in rats. The cardioprotection effect of AMPK on hypertrophic growth was accompanied by inhibiting protein synthesis and interfering with MAPK
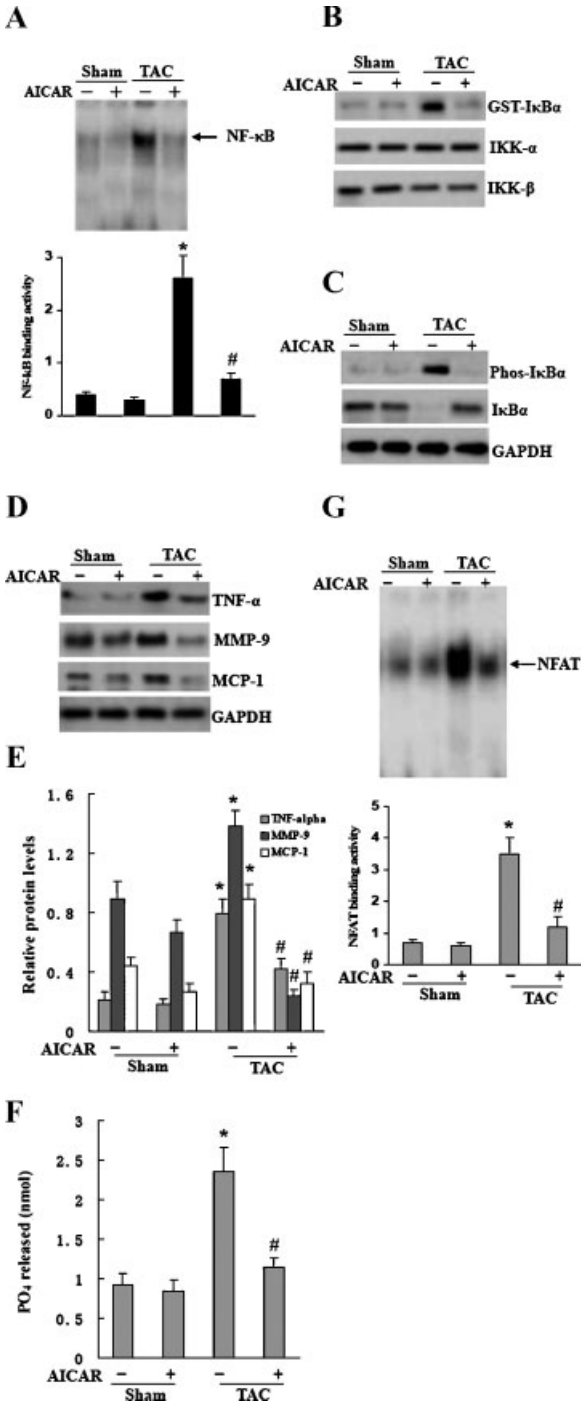
Fig. 4. Effect of AICAR on NF-κB and calcineurin–NFAT signaling pathway. **A**: Representative electrophoretic mobility shift assay of NF-κB activity in myocardium 7 weeks after surgery (**upper**). Optical density in region of NF-κB was quantified with PhosphorImager system (**lower**). **B**: We prepared the cytoplasmic extracts and assayed for IKK by the immunocomplex kinase assay (**upper panel**) and for IKK-α (**middle panel**), and IKK-β (**lower panel**) protein by the Western blot analysis as described in Materials and Methods. **C**: Western blot analysis of the phosphorylation and degradation of IκBα in cytoplasmic fraction of myocardium obtained from different groups. **D**: Inhibition of load-induced proinflammatory cytokine expression by AICAR. **E**: Quantitative results for the expression of proinflammatory cytokine. **F**: Cardiac extracts for the indicated groups were assayed for calcineurin activity, expressed as nmol released phosphate. AICAR-treated rats displayed lower calcineurin activity both under basal conditions and after surgery. **G**: Representative NFAT DNA-binding activity on LV tissue demonstrates significantly increased expression after pressure-overload in rats compared with sham-operated animals. Data are expressed as mean ± SD from n = 4 animals per group. *$P < 0.01$ versus sham-operated group, #$P < 0.01$ versus TAC alone.
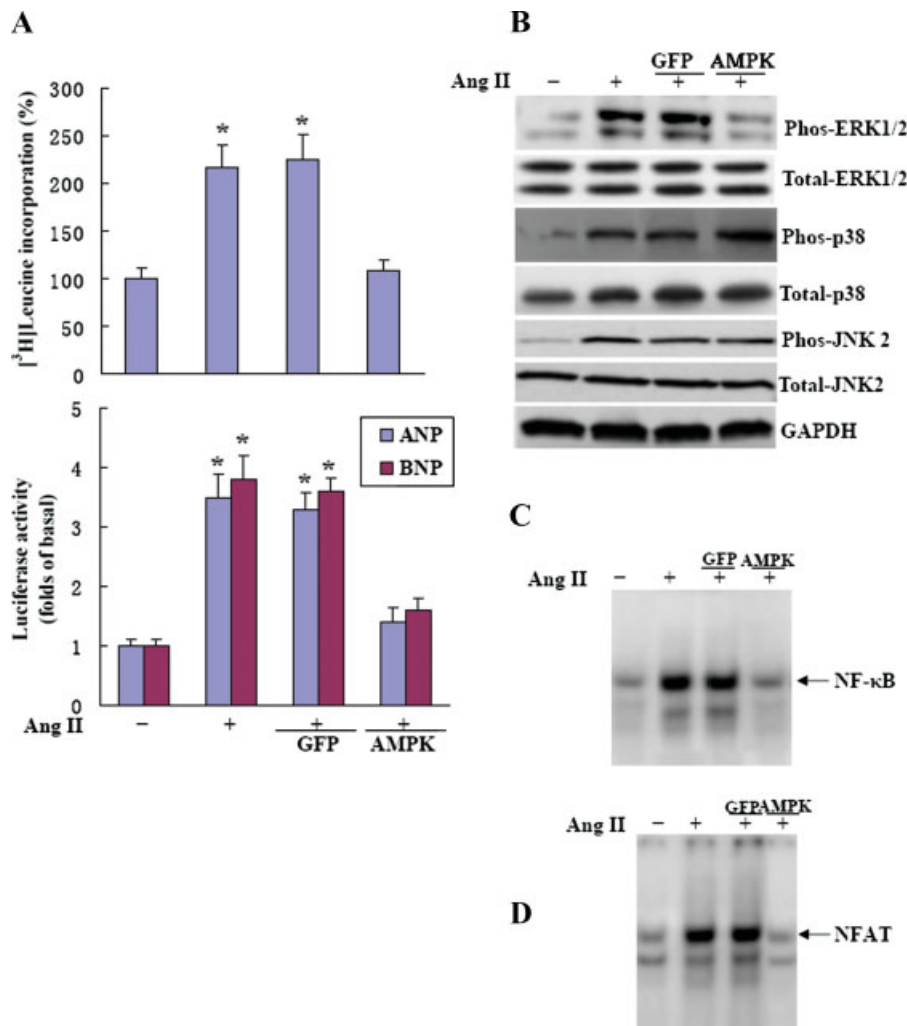
A

B

C

D

Fig. 5. Activation of AMPK inhibits cardiac hypertrophy in vitro. **A**: AdAMPK infection inhibits Ang II-induced protein synthesis measured by [^{3}H] leucine incorporation. Neonatal rat cardiac myocytes cultured in serum-free media were infected with AdAMPK or AdGFP and then incubated with 1 μM Ang II for 48 h. **B**: AdAMPK infection inhibit Ang II-induced ANP and BNP promoter activities. The results were reproducible in three separate experiments. *$P < 0.05$, **$P < 0.01$ versus exposed to AdGFP group. **B–D**: Effect of AdAMPK infection on MAPKs (B), NFAT (C), and NF-κB (D) activation induced by Ang II. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

signaling pathway, and then blocking NF-κB as well as calcineurin–NFAT signaling in vivo and in vitro. These findings suggest that a potential approach for the development of pharmacologic inhibitors of cardiac hypertrophy and heart failure could be targeted at increasing AMPK activity.

Previous studies have indicated that AMPK may be involved in the development of cardiac hypertrophy [Chan et al., 2004; Chan and Dyck, 2005]. For instance, pressure overload-induced hypertrophy has been shown to be associated with increased AMPK activity [Tian et al., 2001]. In addition, AMPK has been linked to

familial Wolff–Parkinson–White syndrome and hypertrophic cardiomyopathy via mutations in *PRKAG2*, the gene encoding the γ_2 subunit of AMPK [Tian et al., 2001]. Chan et al. [2004] reported that activation of AMPK by AICAR blocked the protein synthesis and then blocked cardiac hypertrophy. Although much has been learned about AMPK and protein synthesis and/or hypertrophic growth, the involvement of AMPK in the molecular mechanisms regulating cardiac hypertrophy remains unclear and the role of long-term activation of AMPK on cardiac hypertrophy in vivo has not been established. Our study demonstrated that

long-term activation of AMPK by pharmacological activator AICAR attenuated cardiac hypertrophy in rat hearts subjected to TAC, which is evidenced by decreased HW/BW and mean myocyte diameter morphologically and improved cardiac function. These data are in agreement with those of Chan et al. [2004], but are in contrast to those of Hattori et al. [2006], who reported that AICAR enhanced Ang II-induced hypertrophy of the myocardium through activating ERK1/2. The reason for the discrepant results is not clear at this point and requires further investigation. One important factor may account for this apparent contradiction is the time of treatment. Our system in the current work is 7 weeks, whereas Hattori's system is 7 days. Cardiac hypertrophy is characterized by an enlargement of the cardiac myocytes that often occurs in response to increased hemodynamic load arising from a variety of conditions such as hypertension. These morphological changes are adaptive responses that allow the heart to maintain cardiac output and need a long time to become maladaptive and lead to heart failure. Therefore, duration is important for the treatment cardiac hypertrophy. Unexpectedly, we found that AICAR injections significantly attenuated the increase in blood pressure. Our findings agree with those reported by Buhl et al. [2002]. Our results indicated that preventing the increase of hypertension by AICAR might be just one mechanism for its inhibitory effect on cardiac hypertrophy. No clear insights into the mechanism of antihypertension by AICAR could be provided. The possible mechanisms are involved in evoking endothelium-dependent vasorelaxation [Morrow et al., 2003].

The inhibitory effect of AMPK on hypertrophic growth was mediated by inhibiting protein synthesis. Protein synthesis, as an initiator for cardiac myocyte enlargement, is controlled at the levels of both translation initiation and elongation. Many mediators are involved in the regulation of protein synthesis during cardiac hypertrophic growth, such as mTOR, p70S6k, eEF2, and the tuberous sclerosis complex-2 (TSC2) [Chan and Dyck, 2005]. The role of AMPK in the regulation of protein synthesis during hypertrophic growth has been well examined in previous studies [Bolster et al., 2002; Inoki et al., 2003]. To sum up, AMPK can modulate eEF2 activity via the phosphorylation and activation of eEF2 kinase.

Active eEF2 kinase in turn phosphorylates eEF2, rendering eEF2 inactived and thus inhibiting protein synthesis. Conversely, eEF2 kinase can be inhibited by p70S6 kinase via phosphorylation, thereby allowing eEF2 to enhance peptide chain elongation. In addition to regulating the activity of eEF2 kinase, p70S6 kinase can phosphorylate the ribosomal protein S6, which promotes translation of selected mRNA and contributes to increased protein synthesis. p70S6 kinase itself is phosphorylated and activated by its upstream kinase, known as mTOR. AMPK modulates the activity of mTOR either by direct phosphorylation/inhibition, or by phosphorylation and activation of TSC2. As the TSC1-TSC2 complex functions as a negative regulator of mTOR, the enhancement of TSC1−TSC2 activity also results in the inhibition of mTOR. Therefore, p70S6 kinase cannot be activated by mTOR, and protein synthesis is prevented. These results indicated that the outcome of AMPK activation is the inhibition of protein synthesis and subsequent prevention of cardiac hypertrophy.

To understand the molecular determinants of the hypertrophic response, recent investigation has focused on characterizing intracellular signal transduction pathways in the heart. One of the major systems participating in the transduction of signal from the cell membrane to nuclear and other intracellular targets is MAPK pathway. Involvement of all three classical MAPK pathways has been implicated in the mechanisms of cardiac hypertrophy [Wang et al., 1998; Yue et al., 2000; Behr et al., 2001]. Numerous pathological mediators of cardiac hypertrophy (neurohormones, cytokines, mechanical stretch) have been shown to activate different MAPK pathways. Most of the studies point to the key role of ERK cascade in the mechanisms of hypertrophic response. It was found that in the neonatal rat cardiomyocytes, specific inhibition of ERK pathway reversed the ET-1- and PE-induced cardiac hypertrophy [Yue et al., 2000]. In the adult rat ventricular myocytes, stimulation of alpha1-adrenergic receptors caused hypertrophy dependent on the MEK1/2-ERK1/2 signaling pathway [Xiao et al., 2001]. Consistent with these observations, our present work showed that AMPK activated by AICAR resulted in marked decreased ERK1/2 and increased p38 activity in TAC-induced cardiac hypertrophy. Similar results were showed by using AdAMPK

infection to activation of AMPK. Therefore, it is logical to set up a notion that activation of AMPK may inhibit myocyte hypertrophic growth partly through blocking ERK–MAPK pathway.

However, it is not readily obvious how MAPK would directly regulate these later aspects of the cardiac hypertrophic response. A more reasonable hypothesis is that MAPK signaling initiates the hypertrophic response through a mechanism involving only a handful of direct effectors, transcriptional or otherwise. These immediate downstream targets of MAPK then function as secondary mediators to indirectly coordinate the other necessary aspects of the hypertrophic response. Calcineurin–NFAT signaling has been shown to both affect MAPK as well as being affected by it. Calcineurin, a $Ca^{2+}/$ calmodulin-dependent protein phosphatase, plays important roles in neuronal functions, immune responses, and cell growth. Calcineurin regulates the activity of a number of downstream targets, including the transcription factors NFAT, MEF2, and NF-κB. NFAT, a downstream transcription factor of calcineurin, is directly dephosphorylated by calcineurin resulting in nuclear translocation and promoting inducible gene expression [Molkentin, 2004]. Transgenic mice that overexpressed constitutively active forms of calcineurin and of its downstream transcription factor NFAT showed marked cardiac hypertrophy, whereas calcineurin inhibitors, such as cyclosporin A and FK506, significantly suppressed PE- and Ang II-induced cardiomyocyte hypertrophy in vitro [Wilkins and Molkentin, 2004]. Therefore, we hypothesized that calcineurin–NFAT signaling is also involved in the inhibitory effect of AMPK on cardiac hypertrophy. We detected phosphatase activity and NFAT-binding activity as criteria to monitor calcineurin activity. These assays were in line with increased calcineurin–NFAT activation in the hypertrophied heart. The present study demonstrates that long-term activation of AMPK by AICAR blocked load-induced calcineurin–NFAT pathway, since calcineurin is also regulated by MAPK pathway. Therefore, a possible explain is that activation of AMPK interferes with MAPK pathway and then blocks calcineurin–NFAT pathway. However, the molecular mechanisms which AMPK inhibits calcineurin–NFAT pathway remain unclear at this point and need further investigation.

Although the precise mechanisms by which MAPK/calcineurin–NFAT signaling pathway mediate cardiac hypertrophy remain undetermined, it is of interest that both of them interact with NF-κB, a key transcription factor that may also play an important role in the pathogenesis of cardiac remodeling and heart failure. Recent study also showed that AMPK inhibits fatty acid-induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells [Cacicedo et al., 2004]. Therefore, the inhibitory mechanisms of AICAR on cardiac hypertrophy were examined for its effects on IKKβ/NF-κB pathway. In nearly all instances, the phosphorylation of IκB by IKK triggers the poly-ubiquitination of IκB and the subsequent degradation by 26S proteosome [Nakano, 2004; Papa et al., 2004; Natoli et al., 2005]. Our results showed that among the parameters involved in IKKβ/NF-κB activation, the IKK activity, IκBα degradation, and NF-κB-specific DNA–protein complex formation were blocked by AICAR treatment.

There is also increasing evidence that inflammation and matrix remodeling play important role in cardiac hypertrophy and heart failure. NF-κB regulates expression of a variety of genes involved in inflammation such as TNF-α and MCP-1 as well as extracellular matrix remodeling such as MMP-9. In this study, we also found that AICAR treatment almost completely blocks the expression of MMP-9, TNF-α, and MCP-1. Therefore, the anti-hypertrophic effect of AMPK activation is partly through blocking inflammation and matrix remodeling by suppressing NF-κB signaling. The activity of NF-κB depends on the activity of IKKβ and phosphorylation of IκBα. Previous studies showed that NF-κB activation was regulated by the MAPK pathway through different mechanisms. Since MAPK activation is also regulated by AMPK, we presume that activation of AMPK by AICAR regulates MAPK/calcineurin–NFAT signaling and subsequently influences NF-κB activation. The ultimate outcome is preventing NF-κB translocation to the nucleus where it activates transcription of hypertrophic response target genes.

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