

Original Article

Activation of AMPK inhibits cardiomyocyte hypertrophy by modulating of the FOXO1/MuRF1 signaling pathway *in vitro*

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Aim: To examine the inhibitory effects of adenosine monophosphate-activated protein kinase (AMPK) activation on cardiac hypertrophy *in vitro* and to investigate the underlying molecular mechanisms.

Methods: Cultured neonatal rat cardiomyocytes were treated with the specific AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and the specific AMPK antagonist Compound C, and then stimulated with phenylephrine (PE). The Muscle RING finger 1 (MuRF1)-small interfering RNA (siRNA) was transfected into cardiomyocytes using Lipofectamine 2000. The surface area of cultured cardiomyocytes was measured using planimetry. The protein degradation was determined using high performance liquid chromatography (HPLC). The expression of β -myosin heavy chain (β -MHC) and MuRF1, as well as the phosphorylation levels of AMPK and Forkhead box O 1 (FOXO1), were separately measured using Western blot or real-time polymerase chain reaction.

Results: Activation of AMPK by AICAR 0.5 mmol/L inhibited PE-induced increase in cardiomyocyte area and β -MHC protein expression and PE-induced decrease in protein degradation. Furthermore, AMPK activation increased the activity of transcription factor FOXO1 and up-regulated downstream atrogene MuRF1 mRNA and protein expression. Treatment of hypertrophied cardiomyocytes with Compound C 1 μ mol/L blunted the effects of AMPK on cardiomyocyte hypertrophy and changes to the FOXO1/MuRF1 pathway. The effects of AICAR on cardiomyocyte hypertrophy were also blocked after MuRF1 was silenced by transfection of cardiomyocytes with MuRF1-siRNA.

Conclusion: The present study demonstrates that AMPK activation attenuates cardiomyocyte hypertrophy by modulating the atrophy-related FOXO1/MuRF1 signaling pathway *in vitro*.

Keywords: monophosphate-activated protein kinase (AMPK); Forkhead box O1 (FOXO1); cardiac hypertrophy; MuRF1; ubiquitin-proteasome system; RNA interference; 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR); phenylephrine

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Introduction

Cardiac hypertrophy is an adaptive physiological response to an increase in blood pressure or afterload^[1–3]. However, persistence of cardiac hypertrophy for long periods of time is associated with a significant increase in the risk of sudden death, malignant arrhythmia, and heart failure^[3]. The development of antihypertrophic agents has so far focused on the inhibition of prohypertrophic signals. This inhibition is often unsuccessful in decreasing cell size, probably because

of the overwhelming redundancy of prohypertrophic signaling pathways^[4]. Additional mechanisms must be involved to account for the incomplete inhibition of cardiac hypertrophy, and may include a decrease in protein degradation^[5]. In contrast, activation of atrophy signaling pathways may reverse hypertrophy by increasing proteolysis even in the presence of prohypertrophic signaling in the heart^[4].

The ubiquitin-proteasome system (UPS) is the most important mechanism of proteolysis in cardiac myocytes^[5,6], degrading about 80% of the intracellular proteins^[7]. The role of the UPS in the heart is primarily linked to cardiac atrophy. The strict regulation and specificity that epitomize the UPS are largely dependent on the function of E3 ubiquitin ligases^[8,9], which play a key role in protein loss and muscle atrophy^[7,10].

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Muscle RING finger 1 (MuRF1), a muscle-specific E3 ubiquitin ligase in the ubiquitination process, is proposed to be an archetypal marker for muscle protein degradation^[11], and it functions as a negative regulator of cell size^[12]. A recent study demonstrated MuRF1 to be essential for cardiac atrophy in the setting of therapeutic regression of cardiac hypertrophy^[13].

The Forkhead box O (FOXO) family of transcription factors plays an important role in cellular metabolism, differentiation, and muscle atrophy in mammals^[14, 15]. The FOXO1 transcription factor has been suggested to directly modulate the expression of the MuRF1 protein during muscle atrophy^[16, 17]. Recent studies have indicated that FOXO1 can suppress cardiomyocyte proliferation and blunt cardiac hypertrophy^[15, 18]. Thus, atrophy-related FOXO1/MuRF1 pathways may serve as a target for reversing remodeling of the hypertrophied heart.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine enzyme that participates in the control of fundamental cellular processes such as growth, proliferation, and survival^[19, 20]. Evidence from skeletal muscle suggests that AMPK activation up-regulates the expression of MuRF1^[21-23]. However, the role of AMPK activation on atrophy-related signals in cardiomyocytes has not been established. Recent studies have shown that AMPK activation might inhibit the development of cardiac hypertrophy^[24-26]. However, it is unclear whether the cardioprotective effects of AMPK for cardiac hypertrophy are mediated by the FOXO1/MuRF1 pathway. In the present study, we test the hypothesis that AMPK activation inhibits the hypertrophy induced by phenylephrine in cultured rat cardiomyocytes via modulation of the atrophy-related FOXO1/MuRF1 signaling pathway.

Materials and methods

Drugs and chemicals

Trypsin, collagenase type I, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Chemical Co. Bromodeoxyuridine (BrdU) and PE were purchased from Sigma Chemical Co. Compound C was purchased from Merck Chemical Co and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) was purchased from Cell Signaling Technology. Random primers, dNTPs, M-MLV, and Rnasin were purchased from TaKaRa Bio Inc. The primers and SYBR Green PCR Master Mix for real-time PCR were synthesized by or purchased from Da-an Gene. Small interfering RNAs (siRNAs) were designed and synthesized by Ribobio. Primary antibodies including rabbit anti-rat AMPK, p-AMPK (Thr¹⁷²), FOXO1, and p-FOXO1 (Thr²⁴) were purchased from Cell Signaling Technology; rabbit anti-rat β -myosin heavy chain (β -MHC) and MuRF1 antibodies were purchased from Santa Cruz Biotechnology; and mouse anti-rat GAPDH antibody was obtained from Kangcheng Inc (Shanghai, China). Secondary antibodies including goat anti-rabbit and goat anti-mouse antibodies were purchased from Boshide Inc (Wuhan, China).

Neonatal rat cardiomyocyte culture

Neonatal Sprague-Dawley (SD) rats were obtained from the Experimental Animal Facility of Sun Yat-sen University,

China. Neonatal cardiomyocytes were prepared as described previously^[25]. Briefly, ventricles from one- to three-day-old neonatal SD rats were cut into pieces of approximately 1 mm and treated with 0.125% trypsin and 0.05% collagenase type I. Cell suspensions obtained in this way were pelleted by centrifugation, and then the cells were resuspended with DMEM containing 10% FBS. Subsequently, the cells were cultured at 37°C in an incubator containing 5% CO₂ for one hour to separate out the fibroblasts. The obtained cardiomyocytes were seeded in a six-well plate at a density of 3×10⁵ cells/well. BrdU (0.1 mmol/L) was used to inhibit the proliferation of fibroblasts during the first 48 h. After the cells reached confluence, the cardiomyocytes were transferred to serum-free medium. The cardiomyocytes were treated with AMPK activator AICAR (0.5 mmol/L) for 30 min in the presence and absence of the specific AMPK antagonist Compound C (1 μ mol/L), and subsequently stimulated with PE (10 μ mol/L) for the indicated times.

siRNA transfection

The following sequences were used: for sense MuRF1-siRNA, 5'-GCAUUGUAGAAGCUUCCAA dTdT-3'; for control-siRNA, 5'-UUCUCCGAACGUGUCACGU-3'. Cardiomyocytes were cultured for 48 h and then transfected with MuRF1-siRNA (50 nmol/L final) or control-siRNA (50 nmol/L final) for 24 h using Lipofectamine 2000 (Invitrogen). The ratio of siRNA over Lipofectamine 2000 in a well (6-well cell culture plates) was 5 μ L: 5 μ L. The transfected cells were stimulated with 10 μ mol/L PE or 0.5 mmol/L AICAR for 48 h.

Measurement of the surface area of cardiomyocytes

To determine changes in cell size, the peripheries of cell images captured by a charge-coupled device camera (Olympus, Japan) were traced and analyzed using NIH Image software. For measurements of cell surface area, 100 cells from randomly selected fields in five dishes were measured by planimetry.

Real-time polymerase chain reaction

Total RNA was extracted from cultured cardiomyocytes with Trizol Reagent according to the manufacturer's instructions and quantified by spectrophotometry at 260 nm. Total RNA (1 μ g) was reverse transcribed (RT) into cDNA with the following components: 2 μ L of random primers, 1 μ g of total RNA, 5 μ L of 5×M-MLV buffer, 2 μ L of dNTP mix (10 mmol/L), 0.6 μ L of RNase inhibitor (40 U/ μ L), 1 μ L of M-MLV reverse transcriptase, and RNA-free double distilled H₂O up to 25 μ L. The mixture was incubated first at 42°C for 60 min and then at 99°C for 5 min. The PCR primers sequences were, for GAPDH: 5'-TGGTCTACATGTTCCAGTATGACT-3' (forward) and 5'-CCATTTGATGTTAGCGGGATCTC-3' (reverse) (134 bp); and for MuRF1: 5'-CAGGGAACGACCCGAGTT-3' (forward) and 5'-TCCAGGATGGCGTAGAG-3' (reverse) (127 bp). The real-time PCR conditions were as follows: pre-denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C

for 5 min. Target mRNA levels were quantified by measuring the threshold cycle and comparing it against a calibration curve. The relative level of each mRNA was normalized to the GAPDH gene.

Western blot analysis

Cultured cardiomyocytes were lysed in RIPA buffer containing a mixture of protease inhibitors, and the total protein concentration was determined using the bicinchoninic acid protein assay. Proteins (50 μ g) from cell lysates were electrophoresed by SDS-PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with blocking buffer (1 \times TBS, 0.1% Tween-20, 5% *w/v* nonfat dry milk), washed, and incubated overnight at 4°C with anti-AMPK (1:1000 dilution), anti-p-AMPK (1:1000 dilution), anti- β -MHC (1:1000 dilution), anti-MuRF1 (1:1000 dilution), anti-FOXO1 (1:600 dilution), anti-p-FOXO1 (1:800 dilution), or anti-GAPDH (1:40000 dilution) primary antibodies. The membrane was subsequently washed with TBS-T (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. The immune complex was detected with an enhanced chemiluminescence system (Millipore), exposed to X-ray film, and analyzed using NIH Image software. GAPDH was used as control.

Measurement of myofibrillar protein degradation

N^ε-methylhistidine (3-MH) is an analog of a post-translationally modified and metabolically stable amino acid in actin and myosin^[27]. Previous studies have demonstrated that 3-MH cannot be reutilized for protein synthesis, and therefore, the output of 3-MH has been used as an index of myofibrillar protein degradation^[21, 27, 28]. The concentration of 3-MH was measured as previously described^[27, 28]. Briefly, cardiomyocyte media were collected in six-well plates and analyzed for 3-MH by high performance liquid chromatography (HPLC) after fluorescamine derivation using perchloric acid and heating. The analytes in medium were extracted by protein precipitation using acetonitrile, precolumn derivation with pathaldehyde, and injection within 60 s using ethanolamine as an internal standard.

Statistical analysis

Data are expressed as the mean \pm SEM. Statistical analysis was performed using SPSS 16.0 statistical software. Statistical significance was determined using one-way ANOVA and differences between groups were considered significant when $P < 0.05$.

Results

Effects of AICAR on AMPK activity

To evaluate the effect of AICAR on AMPK activation, cultured cardiomyocytes were first treated with AICAR and Compound C, and subsequently with PE for 6 h. Treatment of cardiomyocytes with AICAR and PE was observed to significantly increase the level of p-AMPK protein ($P < 0.01$, $P < 0.05$

vs control; Figure 1), whereas treatment with Compound C markedly inhibited the phosphorylation of AMPK protein ($P < 0.05$ *vs* control) and reversed the elevated p-AMPK protein level induced by AICAR ($P < 0.01$ *vs* AICAR group). However, these treatments did not affect total AMPK protein expression.

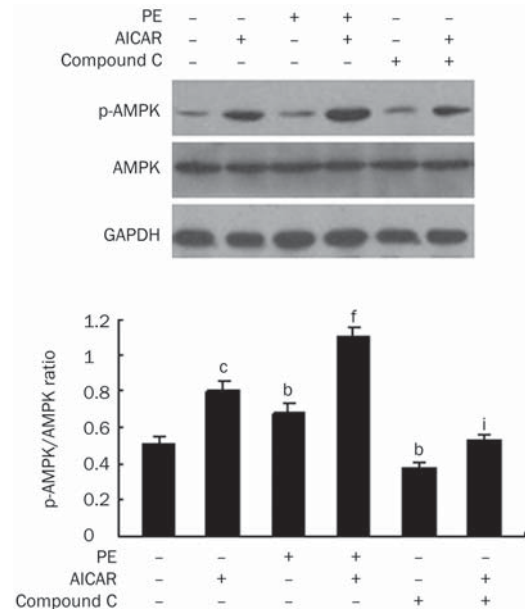


Figure 1. Effect of AICAR on phosphorylation of AMPK. Western blot analysis of p-AMPK in cultured cardiomyocytes. $n = 5$. Mean \pm SEM. ^a $P < 0.05$, ^b $P < 0.01$ *vs* control. ^c $P < 0.01$ *vs* PE group. ^d $P < 0.01$ *vs* AICAR group.

Inhibitory effects of AICAR on cardiomyocyte hypertrophy

To evaluate the effects of AMPK activation on cardiomyocyte hypertrophy, cultured cardiomyocytes were treated with AICAR and Compound C, and subsequently with PE for 48 h. As shown in Figure 2, we found that pretreatment with AICAR significantly reduced the increased cardiomyocyte surface area and β -MHC protein expression induced by PE ($P < 0.01$ *vs* PE group). Further studies demonstrated that the effects of AICAR on cardiomyocyte area and β -MHC protein level seen after 48 h of culture in the presence of PE were markedly blocked after pre-incubation with Compound C ($P < 0.01$ *vs* PE+AICAR group). These results show that treatment with AICAR attenuated PE-induced cardiomyocyte hypertrophy *in vitro*.

Effects of AICAR on MuRF1 expression

To clarify whether ubiquitin ligase MuRF1 is involved in AMPK signaling pathways in PE-induced cardiomyocyte hypertrophy, we tested the effects of AICAR on MuRF1 mRNA and protein expression. Compared with the control group, PE treatment significantly down-regulated MuRF1 mRNA and protein expression levels in cultured cardiomyocytes ($P < 0.01$; Figure 3). However, pretreatment of cells with

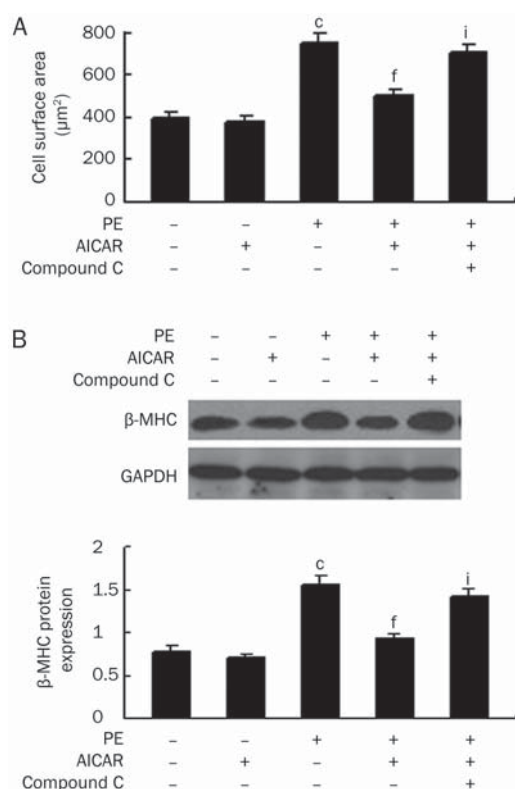


Figure 2. AICAR inhibits cardiac hypertrophy *in vitro*. (A) Effect of AICAR on mean cardiomyocyte surface area in cultured cells after treatment with PE for 48 h. (B) Effect of AICAR on the β-MHC protein level in cultured cells after treatment with PE for 48 h. Western blot analysis of β-MHC protein in cultured cardiomyocytes. *n*=5. Mean±SEM. ^c*P*<0.01 vs control. ^f*P*<0.01 vs PE group. ⁱ*P*<0.01 vs PE+AICAR group.

AICAR markedly reversed the reduced MuRF1 mRNA and protein levels in PE-stimulated cells (*P*<0.01 vs PE group). We also found that blockage of AMPK activity by Compound C eliminated the increase in MuRF1 mRNA and protein expression levels induced by AICAR (*P*<0.01 vs PE+AICAR group).

MuRF1-siRNA blocks the antihypertrophic effects of AMPK

We further examined the change in mean surface area and protein degradation rate of cardiomyocytes after transfecting the cells with MuRF1-siRNA and observed that silencing MuRF1 with siRNA increased the mean surface area and suppressed the protein degradation of cardiomyocytes (*P*<0.01 vs control; Figure 4). AICAR treatment reversed the PE-induced increase in mean surface area and decrease in protein degradation (*P*<0.01 vs PE group), but these effects were significantly blocked after silencing MuRF1 with siRNA (*P*<0.01 vs PE+AICAR group).

Effect of AICAR on FOXO1 transcriptional activity

To determine the effect of AMPK on the activity of the FOXO1 transcription factor in cardiomyocytes, we measured the changes in the phosphorylation level of FOXO1. Compared with the control group, treatment with PE markedly increased

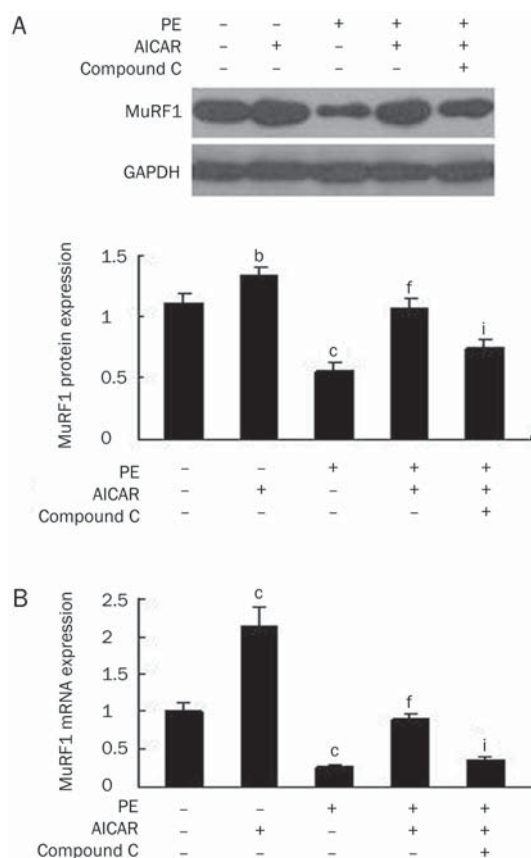


Figure 3. Effect of AICAR on expression of MuRF1. (A) Expression of MuRF1 protein. Western blot analysis of MuRF1 protein in cultured cardiomyocytes. *n*=5. (B) Expression of MuRF1 mRNA: Real-time PCR analysis of MuRF1 mRNA in cultured cardiomyocytes. *n*=5. Mean±SEM. ^b*P*<0.05, ^c*P*<0.01 vs control. ^f*P*<0.01 vs PE group. ⁱ*P*<0.01 vs PE+AICAR group.

the p-FOXO1 level in cultured cardiomyocytes (*P*<0.01; Figure 5). The p-FOXO1 level in cells treated with both AICAR and PE was lower than that in cells treated with PE alone (*P*<0.01). Suppression of AMPK activity with Compound C significantly reversed the decreased p-FOXO1 level induced by AICAR (*P*<0.01 vs PE+AICAR group).

Discussion

The present study elucidates for the first time that the cardio-protective activity of AICAR against hypertrophic growth is accompanied by modulation of the atrophy-related FOXO1/MuRF1 signaling pathway and myofibrillar protein degradation. Previous studies have suggested that AMPK activation inhibits the development of cardiac hypertrophy^[24, 25]. Consistent with these findings, our results here show that activation of AMPK by AICAR significantly suppresses cardiomyocyte hypertrophy as evidenced by decreased cardiomyocyte area, reduced expression of the hypertrophy-associated gene β-MHC, and enhanced protein degradation in PE-stimulated cardiomyocytes. In this study, we have demonstrated that activation of AMPK increases the transcriptional activity of

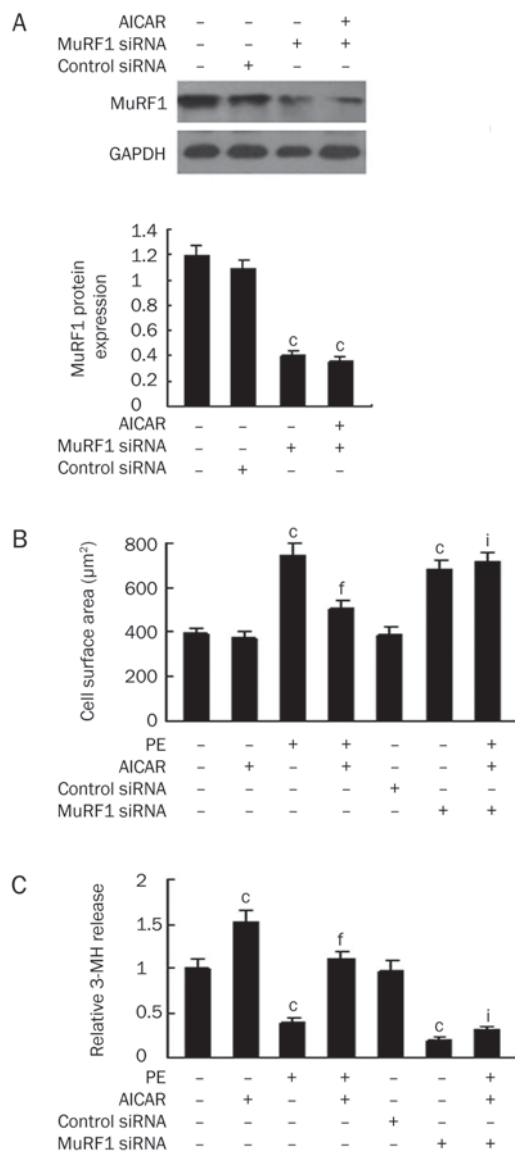


Figure 4. Effects of MuRF1-siRNA on mean surface area and protein degradation in cardiomyocytes. (A) MuRF1-siRNA inhibits the expression of MuRF1 protein. Western blot analysis of MuRF1 protein in cultured cardiomyocytes. $n=5$. (B) Effect of MuRF1-siRNA on cardiomyocyte surface area. $n=5$. (C) Effect of MuRF1-siRNA on 3-MH release in cardiomyocytes. $n=5$. Mean \pm SEM. ^c $P<0.01$ vs control. ^f $P<0.01$ vs PE group. ⁱ $P<0.01$ vs PE+AICAR group.

FOXO1 and enhances the expression of the downstream ubiquitin ligase MuRF1. AICAR's ability to activate the atrogenic regulatory program may partly account for its protective effects on cardiomyocyte hypertrophy and the associated signaling pathway.

The association between AMPK activation and the inhibition of cardiac hypertrophy development has been extensively studied and various mechanisms have been proposed. In the heart, protein turnover is a result of the ordered, regulated, and balanced equilibrium of protein synthesis and degradation^[4]. With both hypertrophic and atrophic remodeling, pro-

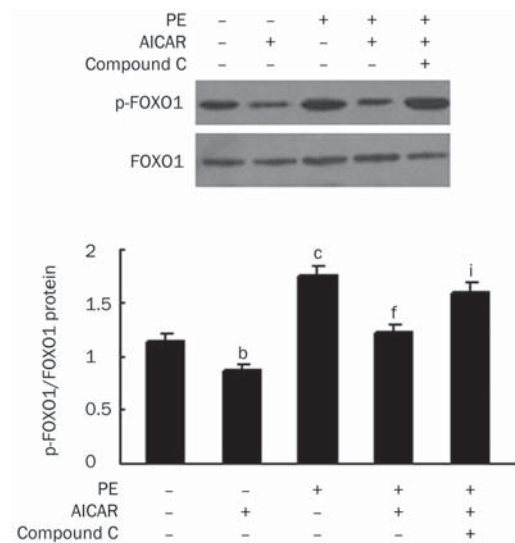


Figure 5. Effect of AICAR on FOXO1 phosphorylation. Western blot analysis of p-FOXO1 in cultured cardiomyocytes. $n=5$. Mean \pm SEM. ^b $P<0.05$, ^c $P<0.01$ vs control. ^f $P<0.01$ vs PE group. ⁱ $P<0.01$ vs PE+AICAR group.

tein turnover is increased in the heart, although relative rates differ. At the cellular level, cardiac hypertrophy is characterized by an enhanced protein accumulation due to an imbalance between protein synthesis and degradation, leading to the addition of contractile myofibers and an increase in cell size. It follows that the reversal of this process must include some mechanism to delete these unnecessary proteins. The major protein degradation pathway involves UPS, which is ubiquitous throughout the body, and degrades ubiquitin-conjugated proteins via the 26S proteasome^[8, 9]. A number of studies have shown that E3 ubiquitin ligase MuRF1 catalyzes the ubiquitylation and breakdown of cardiac structural proteins and plays a pivotal role in cardiac atrophy^[29]. It has also recently been reported that myocardium lacking MuRF1 expression has exaggerated cardiac hypertrophy in response to prohypertrophic stimulation^[13]. More recently, some other reports have demonstrated that MuRF1 inhibits cardiac hypertrophy by degrading sarcomeric proteins that constitute the bulk of cardiomyocyte mass^[30] and interfering with prohypertrophic signaling^[31]. In cardiomyocytes, these processes may determine the balance between prohypertrophic and antihypertrophic signals^[30]. As a sensor of cellular energy status, AMPK responds to an increase in the ratio of AMP to ATP, which can be observed in response to starvation, exercise, and muscle contraction^[20, 32]. AMPK activation can influence the metabolism of cardiomyocytes by increasing glucose transport and glycolysis, as well as by regulating the oxidative phosphorylation of fatty acids^[32]. Recent studies have shown AMPK activation enhances MuRF1 expression in skeletal muscle^[21-23]. However, the relationship between AMPK and MuRF1 expression in the process of cardiac hypertrophy is still unclear. Our results demonstrate that MuRF1 mRNA and protein levels were significantly decreased in hypertrophied cardiomyocytes.

Activation of AMPK with AICAR up-regulated MuRF1 expression and inhibited the cardiomyocyte hypertrophy induced by PE. Compound C, a small reversible inhibitor of AMPK, did not exhibit significant inhibition of several structurally related kinases. Compound C may be used as a selective AMPK inhibitor^[33], although previous investigation has revealed that the application of Compound C may interfere with AICAR uptake itself^[34]. In the present study, inhibition of AMPK with Compound C significantly eliminated the effects of AICAR on MuRF1 expression and cardiomyocyte hypertrophy. We also found that silencing MuRF1 with siRNA abrogated the effect of AICAR to reverse PE-induced cardiomyocyte hypertrophy. These findings demonstrate that the antihypertrophic effects of AICAR may be due to direct or indirect induction of MuRF1 expression in cardiomyocytes.

There is recent evidence to suggest that the expression of MuRF1 is directly regulated by the FOXO1 transcription factor^[35, 36]. To understand how AMPK regulates MuRF1 expression during the process of cardiac hypertrophy, we analyzed the activity of the FOXO1 transcription factor in cardiomyocytes. Our results show that AICAR promotes the transcriptional activity of FOXO1 by inhibiting the phosphorylation of FOXO1 and that this effect was markedly inhibited by Compound C. The increase in MuRF1 mRNA and protein levels was related to the non-phosphorylated form of FOXO1, implying that the modulation of MuRF1 by AMPK is mediated by the FOXO1 transcription factor in cardiomyocytes. Our results therefore suggest that the atrophy-related FOXO1/MuRF1 signaling pathway is associated with the antihypertrophic effects of AMPK in cardiomyocytes. As FOXO1 is dephosphorylated in response to AMPK activation, this finding suggests that some phosphatase is being activated that dephosphorylates FOXO1. However, the precise relationship between AMPK and FOXO1 transcriptional activity requires further investigation.

In summary, our observations suggest that AMPK activation attenuates cardiomyocyte hypertrophy *in vitro*. We conclude that AMPK can mediate cardioprotection against hypertrophy through interfering with the atrophy-related FOXO1/MuRF1 pathway. This paper is highly relevant to the understanding of the effect of AMPK on cardiomyocyte hypertrophy and related molecular mechanisms. Therefore, the present study provides important new insights into the molecular pathways involved that may contribute to our understanding of the effects of AMPK on cardiac hypertrophy.

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Author contributions

Yu-gang DONG, Bao-lin CHEN, and Rong-sen MENG designed the research; Bao-lin CHEN, Yue-dong MA, Zhao-jun XIONG, and Hai-ning WANG performed the research;

Jun-yi ZENG contributed new analytical tools and reagents; Chen LIU analyzed the data; Bao-lin CHEN and Yue-dong MA wrote the paper.

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