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Original Article

Hypoxia promotes AMP-activated protein kinase (AMPK) and induces apoptosis in mouse osteoblasts

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Abstract: The hypoxic environment around the fracture site develops post the blood flow disruption and leads to osteoblast cell death and further impairs fracture healing. Hypoxia usually leads to the mitochondrial dysfunction and then results in apoptotic cell death. AMPK is ubiquitously expressed and functions as an intracellular fuel sensor by maintaining energy balance, as is potentially activated by hypoxia, ischemia, and ROS, however, the regulatory role of AMPK in hypoxia-induced apoptosis in osteoblasts and in the fracture healing has not been identified. In present study, we firstly determined the apoptosis induction by hypoxia in mouse osteoblastic MC3T3-E1 cells via examining the apoptotic cells and the activation of apoptosis-related molecules, then investigated the activation of AMPK signaling by hypoxia via analyzing the phosphorylation of AMPKα and ACC1, finally we explored the association of the AMPK activation with the hypoxia-induced apoptosis using loss-of-function strategy. Results demonstrated that hypoxia induced apoptosis in MC3T3-E1 cells and activated the AMPK signaling. And the knockdown of AMPK via chemical treatment or RNA interfering significantly decreased the hypoxia-induced apoptosis in MC3T3-E1 cells. Taken together, present study unveiled the regulatory role of AMPK signaling in the hypoxia-induced osteoblast apoptosis.

Keywords: Hypoxia, AMP-activated protein kinase (AMPK), apoptosis, MC3T3-E1 cells

Introduction

Severe disruption of blood supply and inadequate revascularization significantly contributes to delayed fracture healing or nonunion [1, 2]. The hypoxic environment around the fracture site develops post the blood flow disruption [3] and leads to osteoblast cell death, delayed chondrocyte and osteoblast differentiation, and further impairs fracture healing [4]. Cellular oxygen concentrations are normally maintained within narrow physiological ranges. Lack of oxygen can result in insufficient ATP to maintain essential cellular functions, whereas excess oxygen may result in the generation of damaging [5]. Disrupted blood supply by fracture always results in reduced oxygen tension (pO_2), hypoxia [6, 7], in bone tissues and inhibits fracture healing. Hypoxia of 1-2% O_2 promotes osteoblast cell generation from marrow precursors [8, 9], whereas 0.2% O_2 promotes osteoclastogenesis [10]. Severe hypoxia also impaired multiple cellular processes of osteoblasts and chondrocytes, such as aerobic metabolism [11, 12], collagen synthesis process [13] and the expression of several angiogenic genes [14] during fracture healing. And the severe low PO_2 level also inhibits stem cell maintenance, mobilization, and recruitment to fracture sites [15-19], and reduces the proliferation, mineralization [20, 21] and differentiation [21] of alveolar osteoblasts. Therefore, the hypoxia might be one of key suppressors to fracture healing.

Acute and severe hypoxia leads to the mitochondrial membrane potential decreasing and the release of cytochrome c, and further promotes cell apoptosis [22, 23]. Mitochondrial dysfunction and cytochrome c release, which further triggers caspase-9, as is followed by the activation of apoptosis executioner caspase-3,

then lead to apoptotic cell death. Nitric oxide and hypoxia exacerbate alcohol-induced mitochondrial dysfunction and induces apoptosis in hepatocytes [24]. The hypoxia-induced mitochondrial dysfunction and apoptosis has also been reported in inflammatory arthritis [25], in neuronal cell death [26], in sepsis [27], in retinal ganglion cells [28] and what's more in osteoblasts [29]. Besides the mitochondrial dysfunction, activation of 5'-AMP-activated protein kinase (AMPK) has recently been reported to be implicated in hypoxia-induced cellular apoptosis [30, 31]. AMPK is ubiquitously expressed and functions as an intracellular fuel sensor by maintaining energy balance. Hypoxia, ischemia, and ROS potentially activate AMPK signaling pathway [31-33], which secondarily promotes the hypoxia-induced cellular apoptosis. AMPK has been widely recognized to promote apoptosis in human glioblastoma cells [34], in pancreatic β-cells [35, 36], or in hepatocytes [37], however, the regulatory role of AMPK in hypoxia-induced apoptosis in osteoblasts and in the fracture healing has not been identified.

In present study, we firstly confirmed the apoptosis induction by hypoxia in MC3T3-E1 osteoblast cells, and then investigated the AMPK promotion by hypoxia and identified the regulatory role of AMPK in the hypoxia-induced apoptosis in MC3T3-E1 cells. This study for the first time recognized the implication of AMPK signaling in the hypoxia-induced apoptosis. It implies a novel target to ameliorate the hypoxia-induced injury to osteoblast cells.

Materials and methods

Reagents, cell culture and treatment

α-Minimal essential medium (α-MEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Gibco Laboratories (GIBCO, Rockville, MD, USA). 5-FU, AICAR, DMSO and Compound C (Cmd C) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Mouse osteoblastic MC3T3-E1 cells (cell resource center of Chinese Academy of Medical Sciences) were cultured in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere at 37°C in 5% CO_2. For the AMPK signaling promoting by chemical, Mouse osteoblast MC3T3-E1 cells were inoculated with 0.2 mM AICAR for 24 hours in 5% CO_2. For hypoxia treatment, cells were placed in a hypoxia incubator infused with a gas mixture of 5% CO_2 and nitrogen to obtain 2% oxygen concentration, with or without a supplementing with 50 μg/mL 5-FU in medium for 12, 24 or 48 hours. Oxygen concentration was monitored continuously (Forma 3130; Thermo Scientific, Rockford, IL, USA). To knockdown the AMPKα expression, the AMPKα siRNA or control siRNA (25 or 50 nM; Sangon, Shanghai, China) was transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to abrogate the AMPKα expression in MC3T3-E1 cells.

Apoptosis assay

Apoptosis of MC3T3-E1 cells were examined with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 5×10^5 cells post various treatment were stained with Annexin V-FITC and propidium iodide and then detected by a FACScan flow cytometer (Bio-Rad, Hercules, CA, USA). The results were calculated using the Cell Quest™ Pro software (Bio-Rad, Hercules, CA, USA) and expressed as the percentage of apoptotic cells from the total cells.

Western blot analysis

MC3T3-E1 cells were collected and lyzed with cold lysis buffer (Promega, Madison, WI, USA) containing protease inhibitors (Roche Diagnostics, GmbH, Germany). The lysates were cleared by centrifugation, and the protein concentration was measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA). Then protein samples were separated by a 8-12% gradient SDS-PAGE gel, transferred to PVDF membrane and blocked in 5% skimmed milk. And Rabbit polyclonal antibodies to caspase 3, caspase 8, ACC1, AMPKα, phosphorylated ACC1 (Ser^{89}), phosphorylated AMPKα (Thr^{172}) or β-actin (Cell Signaling Technology, USA) were used to quantify the protein level of each protein. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) and ECL detection systems (Super Signal West Femto; Pierce, Rockford, IL, USA) were used for detection.

ELISA for AMPK activity

The AMPK activity was examined with CycLex® AMPK Kinase Assay Kit (CycLex Co. Ltd.,

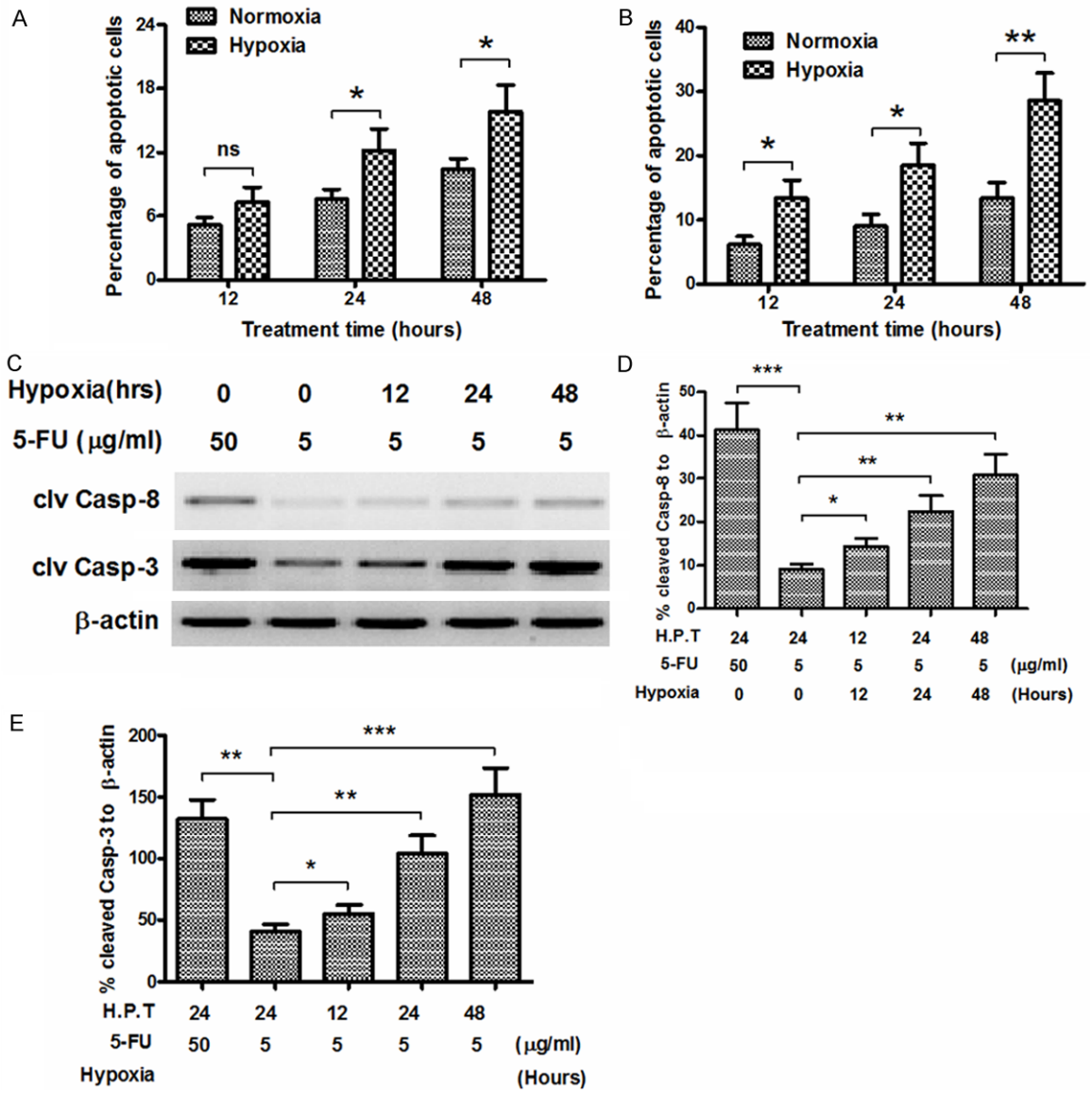

Figure 1. Hypoxia induces apoptosis in mouse osteoblastic MC3T3-E1 cells. A: Apoptotic MC3T3-E1 cells were promoted by hypoxia; B: Hypoxia promoted MC3T3-E1 cell apoptosis, supplementing with 50 μg/mL 5-FU treatment; C: Western blot analysis of cleaved caspase 8 and cleaved caspase 3 in MC3T3-E1 cells post hypoxia and 5-FU treatment; D and E: Relative level of cleaved caspase 8 and cleaved caspase 3 to β-actin in MC3T3-E1 cells post hypoxia and 5-FU treatment. Apoptosis of MC3T3-E1 cells were examined with an annexin V-FITC apoptosis detection kit, and were expressed as the percentage of apoptotic cells to total cells. Clv Casp-8: cleaved caspase 8, Clv Casp-3: cleaved caspase 3. All experiments were performed in triplicate. And statistical significance was showed as **P*<0.05, ***P*<0.01, or ****P*<0.001, ns: no significance.

Nagano, Japan) according to the manufactural mannual. In brief, post being treated with 0.2 mM AICAR for 24 hours or being inoculatd under hypoxia for 12, 24 or 48 hours, MC3T3-E1 cells were directly lysed in ice-cold lysis buffer. Then samples were serially diluted in Kinase buffer and supplemented with phosohorylation substrate, and the amount of phosphorylated substrate was then binded with AS-4C4, an anti-phospho-mouse IgG, which then assayed with anti mouse Ig-G conjugated with horseradish peroxidase and its substrate, by absorbance 450 nm.

Cell viability assay

Cells were seeded in 96-well plates, grew to about 85% confluence, and treated with DMSO (0.1%), 20 μM Cmd C, or normal medium (Blank control) for another 12 or 24 hours. Then cell

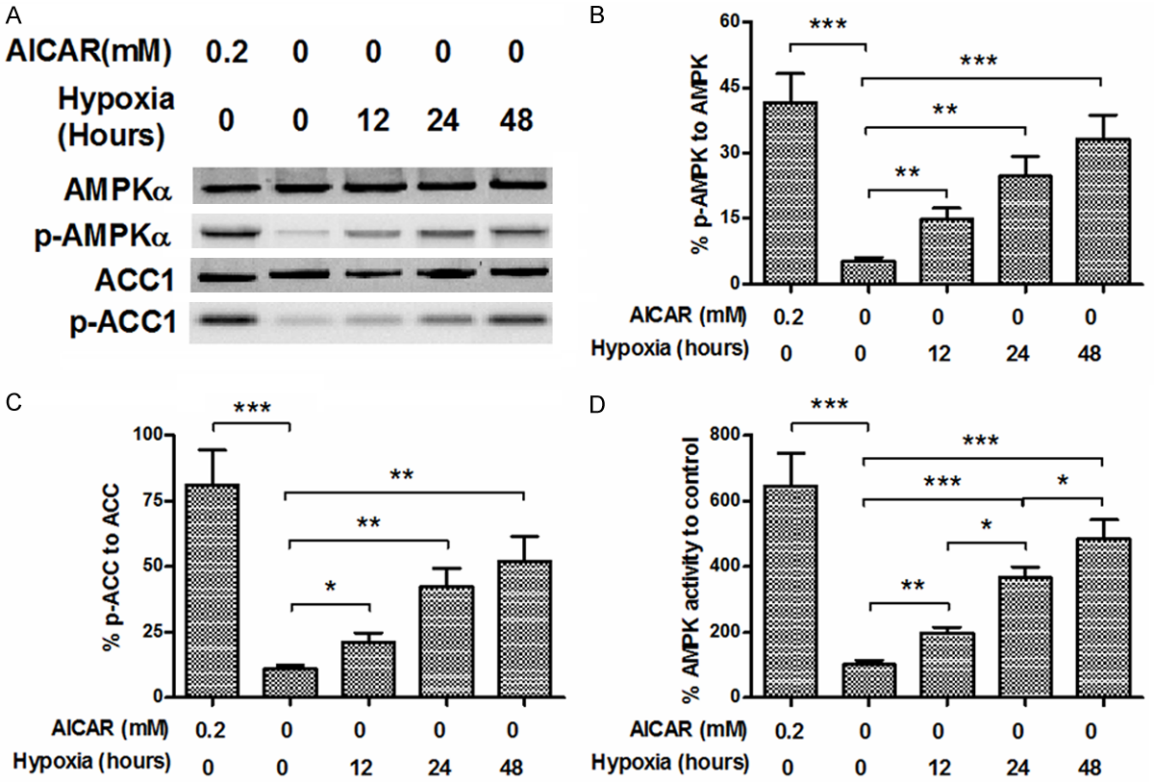

Figure 2. Hypoxia promotes the phosphorylation of AMPKα and ACC1 in MC3T3-E1 cells. (A) Western blot analysis of AMPKα and ACC1 with or without phosphorylation in MC3T3-E1 cells, with 0.2 mM AICAR (activator of AMPKα phosphorylation) treatment, or under hypoxia for 0, 12, 24 or 48 hours; (B and C) Relative level of phosphorylated AMPKα to AMPKα (B) or phosphorylated ACC1 to ACC1 (C) in MC3T3-E1 cells, with AICAR treatment, or with hypoxia for 0, 12, 24 or 48 hours; (D) Relative AMPK activity in MC3T3-E1 cells, with AICAR treatment, or with hypoxia for 0, 12, 24 or 48 hours. All results were expressed as mean ± SEM for three independent experiments. And statistical significance was showed as **P*<0.05, ***P*<0.01, or ****P*<0.001.

viability was measured with MTT assay. The MTT assay was conducted according to the standard protocol. Absorbance was measured at 570 nm with a reference wavelength of 750 nm using a spectrophotometer. In another experiment, cells were transfected with 50 nM AMPKα siRNA or control siRNA and inoculated under hypoxia or normoxia for another 24 hours and measured with the MTT assay.

RNA isolation, reverse transcription, quantitative real-time PCR

Total cellular RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manual and supplemented with Rnase inhibitor (Takara, Tokyo, Japan). The expression of AMPKα in mRNA level was quantified by the real-time RT-PCR method with Takara One Step RT-PCT kit (Takara, Tokyo, Japan). The mRNA samples were amplified using primer sets specific for the genes of AMPKα or β-actin with a Lightcycler 480 II (Roche, Mannheim, Germany). Relative quantification was determined using the ΔΔCt method using β-actin as reference gene [38].

Statistical analysis

Statistical analyses were performed using SPSS19.0 software (IBM SPSS, Armonk, NY, USA). Results were expressed as mean ± SEM, and the difference between two groups in the level of cleaved caspase 3, cleaved caspase 8, AMPKα or phosphorylated AMPKα, ACC1 and phosphorylated ACC1, the percentage of apoptotic or viable cells, was analyzed by Student's t test. A *P* value<0.05 or less was considered statistically significant.

Results

Hypoxia promotes apoptosis in MC3T3-E1 osteoblast cells

Hypoxia has been indicated to induce apoptosis by caspase activation in MC3T3-E1 osteo-

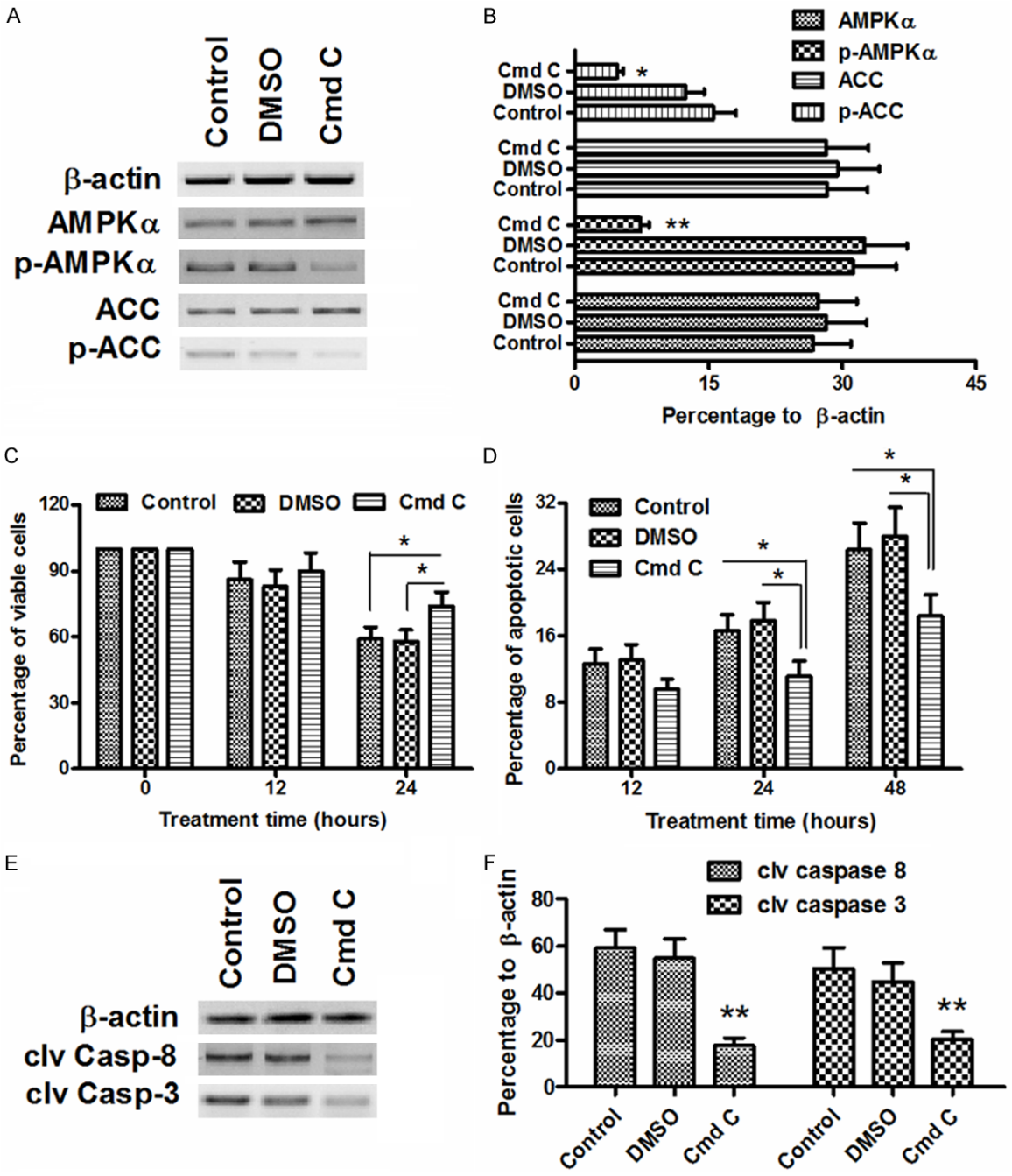

Figure 3. Chemical inhibition of AMPK by Compound C ameliorates the viability decreasing and reduces the apoptosis of MC3T3-E1 cells post hypoxia. A: Western blot analysis of AMPKα and ACC1 with or without phosphorylation in MC3T3-E1 cells under hypoxia, post treatment, for 24 hours, with 20 μM Cmd C (inhitibor of AMPKα phosphorylation), 0.1% DMSO; B: Relative level of AMPKα and ACC1 with or without phosphorylation; C: Viability of MC3T3-E1 cells under hypoxia, post treatment with 20 μM Cmd C or 0.1% DMSO; D: Inhibition of Cmd C on the hypoxia-induced apoptosis of MC3T3-E1 cells; E: Western blotting of cleaved caspase 8 and cleaved caspase 3 in MC3T3-E1 cells under hypoxia, post treatment, for 24 hours, with 20 μM Cmd C; F: Significant reduction of cleaved caspase 8 and cleaved caspase 3 by Cmd C in hypoxia-treated MC3T3-E1 cells. All results were expressed as mean ± SEM for three independent experiments. And statistical significance was showed as $*P<0.05$, or $**P<0.01$.

blasts [39]. To reconfirm the hypoxia-promoted apoptosis in the cells, we used flow cytometric analysis to examine the apoptosis level of MC3T3-E1 cells induced by hypoxia. It was indi-

cated in **Figure 1A** that hypoxia significantly induced MC3T3-E1 cell apoptosis, 24 or 48 hours post treatment ($P<0.05$ respectively). Then we pretreated cells with 50 μg/mL 5-FU before subject to hypoxia, the hypoxia-promoted cell apoptosis was more significant, MC3T3-E1 cells under hypoxia for 12 rather than 24 hours suffered significant apoptosis than cells under normoxia ($P<0.05$), and the difference in 48-hour result was more significant ($P<0.01$; **Figure 1B**). Promoted apoptosis signaling finally catalyzes procaspase 3 to be cleaved into 17 and 12 kDa subunits which execute apoptosis. Therefore, we examined the level of 17 KDa cleaved caspase 3 and its upstream molecule, cleaved caspase 8 by western blot assay. It was shown in **Figure 1C-E** that the activated forms of both caspase 8 and caspase 3 were significantly upregulated in 5-FU-pretreated MC3T3-E1 cells by hypoxia in a time-dependent manner ($P<0.05$ or less).

Hypoxia promotes AMPK signaling and upregulates AMPK activity in MC3T3-E1 osteoblast cells

AMP-activated protein kinase (AMPK) signaling has recently been reported to be promoted by hypoxia [40, 41]. To confirm whether the AMPK signaling is activated by hypoxia in MC3T3-E1 cells, we analyzed the expression and activation of two key molecules, AMPKα and ACC1 (acetyl-CoA carboxylase 1), in AMPK signaling cascades, in hypoxia-treated MC3T3-E1 cells by western blot assay. **Figure 2A** demonstrated that there was no promotion to AMPKα and ACC1 expression in hypoxia-treated MC3T3-E1 cells. Then we analyzed the level of activated forms of both molecules, phosphorylated AMPKα (Thr-172) and phosphorylated ACC1 (Ser-89) in the MC3T3-E1 cells post hypoxia treatment. **Figure 1** demonstrated that the AMPK activator, AICAR, promoted a significantly high level of phosphorylation of AMPKα (Thr-172) and ACC1 (Ser-89) ($P<0.01$ respectively), compared to the control (second column). Moreover, the phosphorylation of both molecules were confirmed in the MC3T3-E1 cells post hypoxia treatment for 12-48 hours ($P<0.05$, $P<0.01$ or $P<0.001$).

To further recognize the promotion of AMPK signaling by hypoxia in MC3T3-E1 cells, we examined the AMPK activity with the AMPK Kinase Assay Kit. Firstly, it was shown that 0.2 mM AICAR significantly promoted the AMPK activity in MC3T3-E1 cells ($P<0.001$) (**Figure 2D**). And the hypoxia upregulated the AMPK activity significantly from 12 hours post hypoxia treatment ($P<0.05$ or $P<0.001$) (**Figure 2D**). And there was a time-dependence of the promotion to AMPK activity by hypoxia, there was significant difference in AMPK activity between 12- and 24-hours treatment, or between 24- and 48-hours treatment ($P<0.05$). Taken together, we confirmed that AMPK signaling was promoted in MC3T3-E1 cells post hypoxia.

Chemical blockage of AMPK signaling inhibits hypoxia-induced apoptosis in MC3T3-E1 osteoblast cells

To investigate the association of AMPK activation with the hypoxia-promoted apoptosis, we applied compound C (Cmd C) to block AMPK activation. Firstly, we analyzed the levels of AMPKα and ACC1, and their activated forms, p-AMPKα and p-ACC1 in hypoxia-treated MC3T3-E1 cells by western blot assay. It was shown in **Figure 3A**, **3B** hypoxia promoted a high level of AMPKα and ACC1 phosphorylation in hypoxia-treated MC3T3-E1 cells post hypoxia treatment for 24 hours (Control and DMSO), whereas the Cmd C-pretreated MC3T3-E1 cells were blocked in the hypoxia-promoted phosphorylation of both molecules ($P<0.05$ or $P<0.01$; **Figure 3B**). Therefore, Cmd C blocked the AMPK signaling activation by hypoxia.

Then we examined the potential role of AMPK signaling blockage by Cmd C in the hypoxia-induced MC3T3-E1 cell apoptosis. MTT assay demonstrated that Cmd C ameliorated the hypoxia-induced cell viability reduction by hypoxia ($P<0.05$; **Figure 3C**). And the hypoxia-induced cell apoptosis was also inhibited by Cmd C treatment ($P<0.05$; **Figure 3D**), compared to DMSO-treatment or nontreatment. To reconfirm the inhibition of Cmd C on the hypoxia-induced apoptosis in MC3T3-E1 cells, we then examined the level of cleaved caspase 3 and cleaved caspase 8 by western blot assay. **Figure 3E** and **3F** demonstrated that both cleaved caspase 3 and caspase 8 were significantly lower in the Cmd C-treated MC3T3-E1 cells under hypoxia (either $P<0.01$). Therefore, the activation of AMPK is implicated in the hypoxia-induced MC3T3-E1 cell apoptosis.

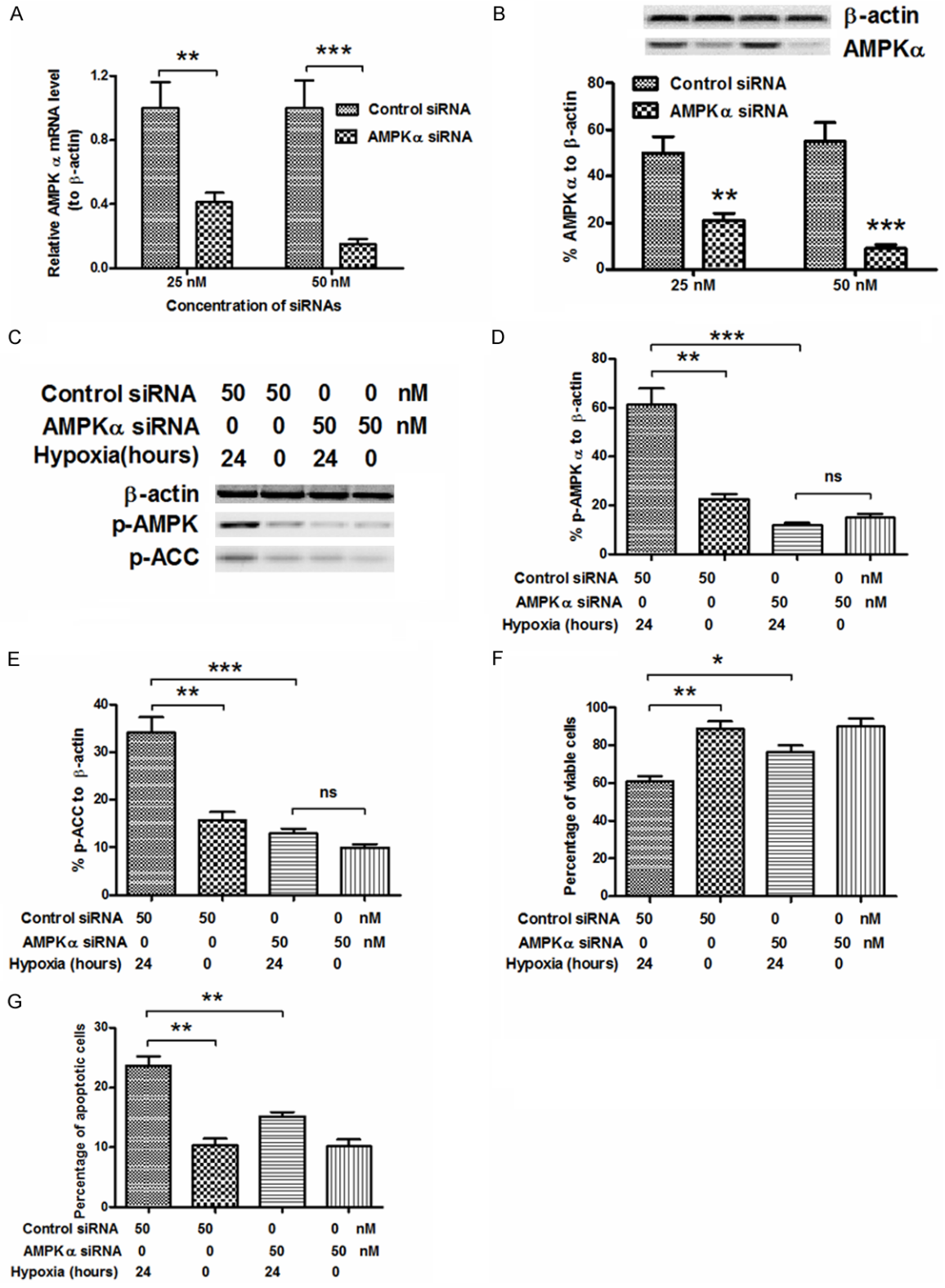

Figure 4. AMPK knockdown by siRNA ameliorates the viability decreasing and reduces the apoptosis of MC3T3-E1 cells post hypoxia. (A) Significant reduction of AMPKα in mRNA level by AMPKα siRNA transfection or control siRNA transfecion, as was examined by RT-qPCR and expressed as relative value to β-actin; (B) Western blot analysis of the AMPKα knockdown in protein level by AMPKα siRNA; (C) Western blot analysis of the phosphorylation of AMPKα and ACC in hypoxia-treated MC3T3-E1 cells post AMPKα siRNA transfection; (D and E) Significant reduction of

hypoxia-induced phosphorylation of AMPKα (D) and ACC (E) in hypoxia-treated MC3T3-E1 cells post AMPKα siRNA transfection; (F) AMPKα siRNA transfection ameliorates the viability reduction of hypoxia-treated MC3T3-E1 cells; (G) AMPKα siRNA transfection reduces the hypoxia-induced apoptosis in MC3T3-E1 cells. All experiments were performed in triplicate. And statistical significance was showed as **P*<0.05, ***P*<0.01, or ****P*<0.001, ns: no significance.

AMPKα knockdown by RNAi inhibits hypoxia-induced apoptosis in MC3T3-E1 osteoblast cells

To reconfirm the association of AMPK activation with the hypoxia-promoted apoptosis, we adopted the RNAi technology to knockdown the AMPKα expression and to further re-evaluate the AMPK activation by hypoxia, then determined the influence of AMPKα knockdown on the hypoxia-induced apoptosis in MC3T3-E1 osteoblast cells. It was demonstrated that the siRNA targeting AMPKα (AMPKα siRNA) transfection for 24 hours significantly reduced both mRNA (**Figure 4A**) and protein (**Figure 4B**) levels of AMPKα (P<0.01 or P<0.001), compared to the control siRNA transfection group. And the phosphorylation of AMPKα and ACC was significantly reduced in hypoxia-treated MC3T3-E1 osteoblast cells post AMPKα siRNA transfection than post siRNA control transfection (P<0.01 or P<0.001; **Figure 4C-E**). Finally, we examined the viability and apoptosis of hypoxia-treated MC3T3-E1 cells post AMPKα siRNA or siRNA control transfection. **Figure 4F** showed that hypoxia (first column) significantly reduced the MC3T3-E1 cell viability (*P*<0.01), whereas this kind of reduction was ameliorated by the AMPKα siRNA transfection (third column) (*P*<0.05). On the other side, **Figure 4G** showed that hypoxia (first column) induced a significant high level of apoptosis in MC3T3-E1 cells (*P*<0.01), whereas the percentage of apoptotic cells was significantly lower in the AMPKα siRNA transfected MC3T3-E1 cells (third column) (*P*<0.01). Taken together, AMPKα knockdown by RNAi inhibits hypoxia-induced apoptosis in MC3T3-E1 osteoblast cells.

Thus, we recognized the AMPK signaling activation by hypoxia in MC3T3-E1 osteoblast cells.

Discussion

AMPK is a heterotrimeric serine/threonine kinase consisting of a catalytic α subunit and two regulatory β and γ subunits [42]. It is a metabolic energy sensor activated by Thr^{172} phosphorylation of the α-subunit, mainly in response to an increase of the AMP/ATP ratio [43]. Activated AMPK metabolically and genetically stimulates the ATP generation, inhibits ATP consumption [23] and regulates mitochondrial biogenesis and function [24]. AMPK is ubiquitously expressed and functions as an intracellular fuel sensor by maintaining energy balance. Hypoxia, ischemia, and ROS are potent activators of AMPK [31-33]. AMPK was rapidly activated *in vitro* by both physiological and pathophysiological low-oxygen conditions, independently of HIF-1 activity, or *in vivo* in AMPKα-null mouse [31]. It implies that HIF-1 and AMPK are components of a concerted cellular response to maintain energy homeostasis in low-oxygen or ischemic-tissue microenvironments. AMPK was also transiently and concentration-dependently activated by H_2O_2 in NIH-3T3 cells [32], indicating that AMPK cascades are highly sensitive to the oxidative stress. Prolonged hypoxia promoted an orchestrated AMPK signaling, which links to mRNA translation and cell growth in part by impinging on the mTOR pathway [44]. And these effects seemed to be mediated by the activation of AMPK and TSC2 in an HIF-independent fashion [45]. Other molecular and cellular pathways have also been recognized to be associated with the AMPK signaling during the hypoxia-induced cellular apoptosis. The activated AMPK has been confirmed to cooperate with deregulation of K+ homeostasis to regulate the hypoxia-induced cellular apoptosis in splenocytes [46].

Hypoxia has been indicated to induce apoptosis by caspase activation in MC3T3-E1 osteoblasts [39]. And in our study, results of apoptotic cells by flow cytometric analysis and of apoptosis-associated molecules by western blot analysis reconfirmed the apoptosis induction by hypoxia in MC3T3-E1 cells, the hypoxia treatment promoted more apoptotic cells and upregulated higher level of caspase 3 cleavage and caspase 8 cleavage. Then we investigated the promotion of AMPK signaling by examining the activation of two key molecules, AMPKα and ACC. And results indicated a significantly high level of phosphorylation of AMPKα (Thr-172) and ACC1 (Ser-89) in the MC3T3-E1 cells post hypoxia treatment in a time-dependent

manner. And a time-dependence of AMPK activity promotion by hypoxia had also been recognized. Therefore, we confirmed in MC3T3-E1 cells the AMPK signaling activation by hypoxia, as has been revealed in other reports [40, 41]. Moreover, present study confirmed that the AMPK signaling activation by hypoxia contributes the hypoxia-promoted apoptosis in MC3T3-E1 osteoblast cells. Both AMPK-inhibitory chemical and siRNA targeting AMPK were confirmed to block the hypoxia-promoted AMPK signaling activation. And the blockage ameliorated the viability reduction of MC3T3-E1 cells, inhibited the hypoxia-induced cell apoptosis. Therefore, the activation of AMPK is implicated in the hypoxia-induced MC3T3-E1 cell apoptosis.

Hypoxia leads to the mitochondrial membrane potential decreasing and the release of cytochrome c, and further promotes cell apoptosis [22, 23]. Mitochondrial dysfunction and cytochrome c release, which further triggers caspase-9, as is followed by the activation of apoptosis executioner caspase-3, then lead to apoptotic cell death. Recently, AMPK has been unveiled to play a diverse role in the molecular mechanism involved in mitochondrial dysfunction [47, 48]. However, it is not clear whether AMPK activation is beneficial or harmful to mitochondrial dysfunction. For example, it was reported that AMPK activation was found in neurons of brain with Alzheimer's disease (AD) or Huntington's disease, suggesting an essential role of AMPK in the neuroprotection [49, 50]. However, abnormal AMPK activation in tangle- and pre-tangle-bearing neurons could promote neurodegeneration, which was suggested as a novel common determinant of tauopathies [51]. In spite of controversary effects of AMPK activation, reports demonstrate that the decline with age in the sensitivity and responsiveness of AMPK has been shown to be associated with many age-associated diseases, including cardiovascular diseases, type 2 diabetes, and metabolic syndrome [52]. And present study has revealed that AMPK activation is implicated in the hypoxia-induced osteoblast cell apoptosis. Based on these recent findings, we suggest that a deeper understanding of the signaling cascade induced by AMPK activation and provide new targets for the treatment of delayed bone fracture healing and other diseases, such as obesity, insulin resistance, cardiovascular disease, and particularly mitochondrial dysfunction related diseases.

In summary, our study unveiled the promotion of AMPK signaling, by upregulating the phosphorylation of two key molecules, AMPKα and ACC in MC3T3-E1 osteoblast cells, in response to hypoxia, and we confirmed that the AMPK signaling activation by hypoxia contributes to the hypoxia-promoted apoptosis in MC3T3-E1 osteoblast cells. Therefore, the activation of AMPK is implicated in the hypoxia-induced MC3T3-E1 cell apoptosis.

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Disclosure of conflict of interest

None.

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