



Published in final edited form as:

*Metabolism*. 2018 June ; 83: 256–270. doi:10.1016/j.metabol.2018.03.004.

## Activation of AMPK inhibits inflammatory response during hypoxia and reoxygenation through modulating JNK-mediated NF- $\kappa$ B pathway

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### Abstract

**Background**—AMP-activated Protein Kinase (AMPK) is a stress-activated kinase that protects against cardiomyocyte injury during ischemia and reperfusion. c-Jun N-terminal kinase (JNK), a mitogen activated protein kinase, is activated by ischemia and reperfusion. NF- $\kappa$ B is an important transcription factor involved in ischemia and reperfusion injury.

**Methods and Results**—The intrinsic activation of AMPK attenuates the inflammation which occurred during ischemia/reperfusion through the modulation of the JNK mediated NF- $\kappa$ B signaling pathway. Rat cardiac myoblast H9c2 cells were subjected to hypoxia and/or reoxygenation to investigate the signal transduction that occurred during myocardial ischemia/reperfusion. Mitochondrial function was measured by the Seahorse XF24 V7 PS system. Hypoxia treatment triggered AMPK activation in H9c2 cells in a time dependent manner. The inhibition of hypoxic AMPK activation through a pharmacological approach (Compound C) or siRNA knockdown of AMPK  $\alpha$  catalytic subunits caused dramatic augmentation in JNK activation, inflammatory NF- $\kappa$ B phosphorylation, and apoptosis during hypoxia and reoxygenation. Inhibition of AMPK activation significantly impaired mitochondrial function and increased the generation of reactive oxygen species (ROS) during hypoxia and reoxygenation. In contrast, pharmacological activation of AMPK by metformin significantly inhibited mitochondrial

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### Author Contributions

Design and conduct of the study: XC, XL, WZ, JH, BX, BL, WZ, CC, TR, and JL. Data collection and analysis: XC, XL, CC, TR, and JL. Data interpretation: XC, XL, ZW, CC, TR, and JL. Manuscript writing: XC, CC, TR, and JL.

### Conflict of Interest

None.

permeability transition pore (mPTP) opening and ROS generation. Moreover, AMPK activation significantly attenuated the JNK-NF- $\kappa$ B signaling cascade and inhibited mRNA and protein levels of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, during hypoxia/reoxygenation in H9c2 cells. Intriguingly, both pharmacologic inhibition of JNK by JNK-IN-8 and siRNA knockdown of JNK signaling pathway attenuated NF- $\kappa$ B phosphorylation and apoptosis but did not affect AMPK activation in response to hypoxia and reoxygenation.

**Conclusions**—AMPK activation modulates JNK-NF- $\kappa$ B signaling cascade during hypoxia and reoxygenation stress conditions. Cardiac AMPK activation plays a critical role in maintaining mitochondrial function and inhibiting the inflammatory response caused by ischemic insults.

## Keywords

AMP-activated protein kinase; inflammation; hypoxia and reoxygenation

## 1. Introduction

Cardiovascular disease, the main cause of death globally, was responsible for more than 17.3 million deaths in 2013 [1], and that number is anticipated to reach more than 23.6 million by 2030 [2]. Among these deaths, those due to ischemic heart disease increased by an approximated 41.7% from 1990 to 2013 [3]. Myocardial ischemia is the lack of blood flow to the myocardium, resulting in insufficient oxygen, glucose, and other nutrients which can support mitochondrial oxidative phosphorylation. Perfusion after ischemia is an important therapeutic strategy, but damage also occurs in the myocardium due to reperfusion, termed “reperfusion-injury” [4]. Myocardial ischemia/reperfusion (I/R) injury is a complex pathophysiological process in which calcium is overloaded, mitochondrial permeability transition pores open, and reactive oxygen species (ROS) are excessively produced, leading to mitochondrial dysfunction and cell death [5]. Many harmful inflammatory cytokines are released during I/R, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , and IL-6 [6].

AMP-activated protein kinase (AMPK) is an energy sensor that regulates the intracellular ATP to AMP ratio. It is regarded as a regulator of cardiac metabolism and is activated when the cellular energy state is low [7]. AMPK has many ways to increase ATP production and decrease ATP consumption. It can increase glucose and fatty acid uptake, enhance glycolysis and fatty acid oxidation, and inhibit glycogen and protein synthesis [8]. There is evidence that AMPK activation can modulate glucose and fatty acid metabolism via the phosphorylation of the downstream acetyl-CoA carboxylase (ACC) in order to protect the heart from ischemic damage caused by I/R [9–11]. During I/R, excessive ROS production can topple naturally occurring antioxidant systems, and excessive ROS can cause the mitochondrial permeability transition pore (mPTP) to open and activate harmful signaling [12]. Mitochondrial dysfunction during I/R is mainly caused by the opening of the mPTP [13]. Chronic energy deficiency stimulates AMPK in order to facilitate muscle mitochondrial biogenesis [14], but whether the activated AMPK can protect mitochondrial function during I/R remains uncertain.

The c-Jun N-terminal protein kinase (JNK) is a mitogen activated protein kinase (MAPK) family member that modulates multiple cellular functions, including proliferation,

differentiation, and apoptosis [15]. Chronic AMPK activation decreased JNK activation via hydrogen peroxide in endothelial cells [16]. Interestingly, metformin, as an AMPK agonist used for the treatment of Diabetes has been recently recognized for protecting cardiomyocytes from high glucose and hypoxia/reoxygenation-induced injury via modulating the JNK signaling pathway [17]. The Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) is a nuclear transcription factor that can regulate gene expression, which is very important to apoptosis and inflammation signaling in many diseases, including ischemic pathology [18]. Inactive NF- $\kappa$ B is located in the cytoplasm, where it is limited by the I $\kappa$ B family of proteins, including I $\kappa$ B- $\alpha$ . When NF- $\kappa$ B is activated, I $\kappa$ B- $\alpha$  is phosphorylated by I $\kappa$ B kinase (IKK) followed by degradation, leading to the translocation of NF- $\kappa$ B subunits from the cytoplasm to the nucleus. Previous studies have shown that the NF- $\kappa$ B subunit p65 is associated with I/R injury in a liver model, due to increased inflammation [19]. Moreover, the components of the mitogen-activated protein kinase (MAPK) signaling pathway, which include p38 MAPK, extracellular signal-regulated kinase (ERK) and JNK, also participate in inflammation and are considered to be upstream factors of NF- $\kappa$ B [20]. We hypothesize the AMPK signaling pathway could protect mitochondrial function under ischemic conditions and modulate the inflammatory response through ROS-mediated JNK signaling.

## 2. Methods

### 2.1. Cell Culture and reagents

Rat cardiac myoblast H9c2 cells (ATCC CRL-1446<sup>TM</sup>) were cultured in high glucose (4.5 g/L) DMEM (Corning Cellgro) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco. Life Technologies) and antibiotics (100 U/mL penicillin and 100  $\mu$ g/mL streptomycin) (ATCC). The cells were maintained in a humidified incubator with 95% air and 5% CO<sub>2</sub> at 37°C. Cells were grown in 100 mm plates and sub-cultured when they reached 70–80% confluence. AMPK inhibitor Compound C (Dorsomorphin) and JNK inhibitor JNK-IN-8 were purchased from Sigma-Aldrich Co.; metformin was obtained from MP Biomedicals LLC.

### 2.2. Hypoxia/Reoxygenation

To establish an I/R model *in vitro*, a H/R cell model was used in our study. Before different treatments were given to H9c2 cells, the culture medium would be changed to serum-free no glucose DMEM (Gibco Life Technologies) when the H9c2 cells were grown to about 80% confluence. The cells were placed in a hypoxia incubation chamber, and normal air was replaced with 95% N<sub>2</sub> and 5% CO<sub>2</sub>. The cells were cultured in hypoxic conditions for 15 hours. Following hypoxia, the cells were removed from the hypoxia chamber, and the medium was replaced with culture medium. Cells were kept in a normoxic incubator for 1 hour. For each evaluation, all experiments were conducted in triplicate.

### 2.3. Transient transfection with small interfering RNA (siRNA)

To knockdown the endogenous AMPK and JNK, H9c2 cells were transiently transfected with 100 nM mouse siRNAs targeting AMPK $\alpha$ 1/ $\alpha$ 2, JNK1/2, or with the non-silencing

control siRNA (scram) (Ambion) using Lipofectamine™ RNAiMAX (Invitrogen) in culture medium without antibiotics according to the manufacturer's recommendations.

#### 2.4. Mitochondrial respiration measurements

Mitochondrial oxygen consumption rate (OCR) was assessed with a Seahorse XFe24 Analyzer (Seahorse Bioscience). H9c2 cells (20,000/well) were seeded into 24 wells of Seahorse XF24 V7 PS cell culture microplates (Seahorse Bioscience). After treatment, the medium was replaced with XF Assay Medium (Seahorse Bioscience), supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM D-glucose. After placing the cell culture microplate into a 37°C non-CO2 incubator for 1 hour, OCR was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Measurements were taken as the cells were incubated sequentially under four conditions: (1) basal levels were measured with no additives; (2) oligomycin (1 μM) was added to reversibly inhibit ATP synthase and OXPHOS, showing glycolysis alone; (3) FCCP (2 μM), a mitochondrial uncoupler, was added to induce maximal respiration; and (4) Rotenone/antimycin A (0.5 μM), a Complex I inhibitor and mitochondrial poison, was added to end the reaction. The Seahorse software was used to plot the results. OCR was normalized to cell protein concentration per well.

#### 2.5. Reactive oxygen species (ROS) measurement

To examine the generation of ROS, the ROS-ID™ Total ROS Detection kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA) was used according to the manufacturer's instructions. H9c2 cells were seeded in a 96-well plate with a clear bottom and reached 70~80% confluence. Fluorescent signals were measured through the bottom of the cell culture plates at an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Spectramax M2 plate reader)

#### 2.6. Flow cytometric measurement of the mitochondrial permeability transition pore

The MitoProbe transition pore assay kit (Invitrogen) and the MACS flow cytometry (Miltenyi Biotec) was used to study the opening of the mitochondrial transition pore (mPTP) in H9c2 cells. This kit provides a more direct method of measuring mPTP opening than assays relying on mitochondrial membrane potential alone. In brief,  $1 \times 10^6$  cells were loaded with the acetoxymethyl ester of calcein dye, calcein AM, which passively diffused into the cells and accumulated in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleaved the acetoxymethyl esters to liberate the very polar fluorescent dye calcein. The fluorescence from cytosolic calcein was quenched by the addition of CoCl<sub>2</sub> (cobalt chloride), while the fluorescence from the mitochondrial calcein was maintained. However, opening the mPTP instigated the release of mitochondrial calcein to the cytosol where the signal was quenched by CoCl<sub>2</sub>, leading to the dramatic reduction of calcein fluorescence.

#### 2.7. mRNA analysis of pro-inflammatory cytokines

mRNA levels were quantified using the Real-Time PCR <sup>ΔT</sup> method with the CFX96 platform (Bio-Rad Laboratories, Inc.). Total RNA was purified using the Pure Link RNA

mini kit (Ambion) according to the manufacturer's instructions. cDNA was synthesized with the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative PCR reactions were performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All primers were synthesized by Integrated DNA Technology (IDT, Canada). The primer sequences for mRNA analysis are: TNF- $\alpha$ , forward: 5'-CGTGTTTCATCCGTTCTCTACC-3'; reverse: 5'-GCAATCCAGGCCACTACTT-3'; IL-6, forward: 5'-CCGTTTCTACCTGGAGTTTGT-3'; reverse: 5'-GTTTGCCGAGTAGACCTCATAG-3'; 18S rRNA: forward: 5'-TTGATTAAGTCCCTGCCCTTTGT-3'; reverse: 5'-CGATCCGAGGGCCTAACTA-3'. Expression of 18S rRNA was used to normalize gene expression. Quantification of gene expression was carried out using the CFX manager software (Bio-Rad Laboratories, Inc.).

## 2.8. Pro-inflammatory cytokines

Pro-inflammatory cytokines TNF- $\alpha$  and IL-6 were estimated using ELISA kits (R&D Systems and BD Biosciences, U.S.A. respectively) and analyzed through an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.).

## 2.9. Immunoblotting

Immunoblots were performed as previously described [21, 22]. Rabbit antibodies p-AMPK $\alpha$  (Thr<sup>172</sup>) (#2535), AMPK $\alpha$  (#5831), p-Acetyl-CoA carboxylase (ACC) (Ser<sup>79</sup>) (#3661), ACC (#3676), p-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>) (#4668), JNK (#9252), p-c-Jun (Ser<sup>73</sup>) (#3270), c-Jun (#9165), p-p65 (Ser536) (#3033), p65 (#8242) and  $\beta$ -tubulin (#2146) were all obtained from Cell Signaling Technology (Danvers, MA, USA).

## 2.10. Statistical analysis

All data was acquired from independent experiments and represented as the mean  $\pm$  SEM. The comparisons between two groups were analyzed using the non-parametric Mann-Whitney test using GraphPad Prism 5.0 (GraphPad Software, Inc.). A value of  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. AMPK and JNK signaling in response to hypoxia and reoxygenation

In order to characterize the roles of AMPK and JNK signaling pathways in response to hypoxic stress, the H9c2 myoblast cells were treated with 95% nitrogen (N<sub>2</sub>) and 5% carbon dioxide (CO<sub>2</sub>) for different time points; the results demonstrated that hypoxia treatment can trigger AMPK activation by stimulating phosphorylation of the catalytic  $\alpha$  subunit at Thr<sup>172</sup> in a time dependent manner (Figure 1A). The peak activation time of AMPK due to hypoxic stress in the H9c2 cells was 15 hours. Interestingly, JNK signaling was significantly inhibited during hypoxia (Figure 1B), and was augmented by hypoxia and reoxygenation (H/R), especially at 15 hrs hypoxia followed by 1 hour reoxygenation (Figure 1B). Conversely, AMPK signaling was activated during hypoxia and attenuated during reoxygenation following hypoxia (Figures 1A and 1B).

### 3.2. Inhibition of AMPK augmented JNK activation by hypoxia and reoxygenation

There is evidence that inactivation of AMPK increases JNK signaling during reperfusion after cardiac ischemia [23]. In order to determine whether there is a feedback loop relationship between AMPK signaling and JNK activation in response to hypoxic stress, AMPK specific inhibitor compound C [24] and JNK specific inhibitor JNK-IN-8 [25] were used to characterize the relationship between AMPK and JNK signaling pathways in response to hypoxia with or without reoxygenation treatments. The results showed that hypoxia triggered phosphorylation of AMPK and downstream Acetyl CoA carboxylase (ACC) but not JNK phosphorylation (Figure 2A); compound C pretreatment significantly inhibited hypoxia-triggered AMPK phosphorylation, while JNK inhibitor JNK-IN-8 did not have any effect on hypoxic AMPK activation (Figure 2A). JNK signaling pathways were activated, as shown by phosphorylation of JNK and downstream c-Jun during H/R; intriguingly, AMPK inhibitor compound C significantly augmented JNK phosphorylation during H/R (Figure 2A), and JNK-IN-8 treatment completely blocked JNK phosphorylation induced by H/R (Figure 2A).

Furthermore, the genetic approach with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA was used to avoid the non-specific effects of the pharmacological approach regarding the relationship between AMPK and JNK signaling pathways during H/R stress conditions. The immunoblotting results demonstrated that AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knocked down AMPK $\alpha$  protein levels and attenuated the phosphorylation of AMPK and downstream ACC induced by hypoxia in H9c2 cells (Figure 2B). JNK1/2 siRNA clearly knocked down JNK protein levels of H9c2 myoblast cells and attenuated the phosphorylation of JNK and downstream c-Jun by H/R (Figure 2B). Intriguingly, the AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knockdown of AMPK $\alpha$  significantly augmented activation of JNK caused by H/R, as shown by phosphorylation of JNK and downstream c-Jun (Figure 2B), but the JNK1/2 siRNA knockdown of JNK1/2 did not have any effect on AMPK signaling triggered by hypoxia, as shown by phosphorylation of AMPK and downstream ACC (Figure 2B).

### 3.3. AMPK plays a critical role in mitochondrial respiration during hypoxic stress conditions

In order to better understand how AMPK activation modulates the JNK signaling pathway during hypoxic stress conditions, the mitochondrial oxidative capacity of H9c2 myoblast cells was evaluated by Seahorse analyzer with different treatments under normoxia or H/R conditions. The mitochondrial functions of H9c2 myoblast cells were measured with the Seahorse XF Cell Mito Stress Test under normoxia or H/R (Figure 3A). The results demonstrated that the oxygen consumption rate (OCR) of mitochondria in H9c2 cells was significantly decreased during H/R as compared to normoxia conditions (Figures 3A and 3B). Interestingly, AMPK inhibitor compound C did not affect mitochondrial functions of H9c2 under normoxia conditions, as shown by lack of changes in basal respiration, maximal respiration, spare respiratory capacity, and ATP production (Figure 3B), but compound C treatment significantly exacerbated mitochondrial dysfunctions during H/R, as exhibited by decreased basal respiration, decreased maximal respiration, decreased spare capacity, and lower ATP production (Figure 3B). However, JNK signaling inhibitor JNK-IN-8 did not show any effects on mitochondrial functions of H9c2 myoblast cells under normoxia or H/R

conditions (Figures 3A and 3B). In addition, the genetic approaches with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA to treat H9c2 myoblast cells showed that both AMPK and JNK signaling pathways did not affect mitochondrial functions of H9c2 cells, as shown by unchanged basal respiration, maximal respiration, spare capacity, and ATP production under normoxia conditions (Figures 3C and 3D). However, lack of AMPK signaling significantly exacerbated mitochondrial dysfunction during H/R conditions, while JNK signaling did not make any contributions to the mitochondrial dysfunction caused by H/R (Figures 3C and 3D).

#### **3.4. AMPK modulates hypoxic stress-mediated mitochondrial reactive oxygen species (ROS) production**

The mitochondria are a major source for generation of ROS, especially under stress conditions [26]. In order to characterize the role of AMPK signaling in hypoxic stress induced ROS generation, the amount of ROS generated under different conditions was measured with the ROS-ID™ Total ROS Detection kit. The results demonstrated that AMPK inhibition by compound C significantly augmented ROS production of H9c2 cells during H/R (Figure 4A), while JNK signaling blocked by JNK-IN-8 appears to not affect ROS generation under both normoxia and H/R conditions (Figure 4A). The genetic approaches with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA also showed that the AMPK signaling pathway plays a critical role in hypoxic stress mediated ROS production of H9c2 myoblast cells, as AMPK $\alpha$  knockdown by AMPK $\alpha$ 1/ $\alpha$ 2 siRNA augmented ROS production by H/R (Figure 4B), while JNK signaling did not have any effect on mitochondrial ROS generation under both normoxia and H/R conditions, as JNK1/2 knockdown by JNK1/2 siRNA did not affect ROS production of H9c2 cells (Figures 4A and 4B).

#### **3.5. AMPK limits mitochondrial permeability transition pore opening by hypoxic insults**

There is evidence that myocardial ischemia and reperfusion induces opening of the mitochondrial permeability transition pore (mPTP), which can decrease mitochondrial respiration and result in cardiomyocyte death [27]. The opening of mPTP in H9c2 myoblast cells was measured with the MitoProbe transition pore assay kit. The results demonstrated that mPTP opening in H9c2 cells was increased in response to H/R as compared to normoxia conditions (Figure 4C). AMPK signaling inhibitor compound C augmented the mPTP opening by H/R, while JNK inhibitor JNK-IN-8 did not show this augmentation effect in H9c2 cells under H/R conditions (Figure 4C). The genetic approach with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA also demonstrated that AMPK $\alpha$  knockdown enhanced the hypoxic stress-induced mPTP opening, while JNK1/2 knockdown did not show significant effect on mPTP opening of H9c2 cells under both normoxia and hypoxic stress conditions (Figure 4D).

#### **3.6. AMPK inhibits hypoxic stress-mediated inflammatory response via modulating NF- $\kappa$ B activity**

There are several reports that intracellular redox alterations leading to ROS generation could trigger inflammation [26, 28]. In order to determine whether AMPK and JNK signaling pathways play a role in the hypoxic stress-mediated inflammatory response, the inflammation-related signaling pathway NF- $\kappa$ B was observed in H9c2 myoblast cells with

or without AMPK/JNK antagonist under normoxia and H/R conditions. The immunoblotting data showed that H/R stimulated NF- $\kappa$ B signaling, demonstrated by an increased phosphorylation of p65 at Ser<sup>276</sup> (Figure 5A), and AMPK inhibitor compound C treatment significantly augmented NF- $\kappa$ B activation by H/R (Figure 5A). Intriguingly, JNK inhibitor JNK-IN-8 can attenuate the hypoxic stress induced NF- $\kappa$ B activation (Figure 5A). The genetic approach with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA also showed that AMPK $\alpha$  knockdown can enhance hypoxic stress-triggered NF- $\kappa$ B activation, but JNK1/2 knockdown abolished the NF- $\kappa$ B activation caused by H/R (Figure 5B). We further measured the mRNA and protein levels of two pro-inflammatory cytokines, TNF- $\alpha$  and IL-6, by performing real-time RT-PCR and ELISA measurements, respectively. The results demonstrated that H/R induced up-regulation of TNF- $\alpha$  and IL-6 mRNA and increased the secretion of TNF- $\alpha$  and IL-6 to the medium (Figures 5C and 5D). AMPK inhibitor treatment significantly augmented this up-regulation, while JNK inhibitor JNK-IN-8 significantly inhibited the up-regulation of both TNF $\alpha$  and IL-6 by H/R (Figure 5C). The genetic approach with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA showed that AMPK $\alpha$  knockdown enhanced hypoxic stress-mediated up-regulation of TNF $\alpha$  and IL-6 while JNK1/2 knockdown inhibited the up-regulation by H/R (Figure 5D).

### 3.7. AMPK protects H9c2 cells from apoptosis induced by hypoxia and reoxygenation

In order to determine whether the AMPK regulated inflammatory response is involved in apoptosis during H/R, H9c2 myoblast cells were treated with AMPK inhibitor compound C under normoxia or H/R conditions. The fluorescence imaging data showed that H/R caused an upregulation of apoptosis in H9c2 cells as compared to normoxia conditions (Figures 6). AMPK inhibitor compound C treatment can significantly augment apoptosis of H9c2 by H/R, but JNK inhibitor JNK-IN-8 attenuated this apoptosis (Figures 6A and 6C). The genetic approach with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA showed that AMPK $\alpha$  knockdown enhanced apoptosis of H9c2 cells by H/R, but JNK1/2 knockdown inhibited the hypoxic stress-triggered apoptosis of H9c2 cells (Figures 6B and 6D).

### 3.8. Activation of AMPK by metformin protects cells from hypoxic insults

Lastly, we tested the protective effects of AMPK agonist metformin on hypoxic injury in H9c2 myoblast cells. Metformin (5 mM) treatment triggered AMPK activation as shown by phosphorylation of the AMPK  $\alpha$  subunit at Thr<sup>172</sup> under normoxia and H/R conditions (Figure 7A). Interestingly, metformin treatment can attenuate JNK activation by H/R, as demonstrated by the phosphorylation of JNK (Figure 7B). Additionally, metformin inhibited hypoxic stress-triggered NF- $\kappa$ B activation, as shown by phosphorylation of p65 at Ser<sup>276</sup> (Figure 7B). The level of mPTP opening was decreased by metformin treatment during H/R (Figure 7C). Moreover, metformin treatment significantly inhibited ROS production in H9c2 cells by H/R (Figure 7D), and the up-regulation of levels of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 caused by H/R was inhibited (Figures 7E).

## 4. Discussion

There is evidence suggesting a potential link between metabolic disorders and inflammation, both of which are associated with abnormal cytokine production [29]. AMPK is a



heterotrimeric enzyme composed of a catalytic  $\alpha$  subunit tethered to the regulatory  $\beta$  and  $\gamma$  subunits and is an important regulator of cardiac energy homeostasis [11]. This serine/threonine kinase is activated by ATP depletion through phosphorylation at its Thr<sup>172</sup> residue by upstream kinases such as liver kinase B1 (LKB1) [26]. AMPK responds to increases in the AMP/ATP ratio by switching off energy-consuming pathways and stimulating ATP-producing pathways such as fatty acid  $\beta$ -oxidation and glycolysis [26]. A principal event of AMPK activity is the phosphorylation-mediated inhibition of acetyl-CoA carboxylase (ACC), resulting in the down-regulation of malonyl-CoA levels. Since malonyl-CoA is an inhibitor of carnitine palmitoyltransferase I (CPT-1), which is a mitochondrial enzyme responsible for the formation of acyl carnitine by catalyzing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to I-carnitine, AMPK activation will result in an increase in energy production [11]. AMPK activation has been reported to protect the heart against ischemic injury [9], and these cardioprotective effects stem from its capacity to improve both glucose and fatty acid metabolism [21, 30].

The ischemia and reperfusion (I/R) injury in the heart has been recognized to induce the release of many cytokines and chemokines, resulting in cell apoptosis and death [31, 32]. There is accumulating evidence that an inflammatory response occurs during human myocardial ischemia/reperfusion injury. Moreover, AMPK activation has been shown as a promising pharmacologic approach to limit organ injury caused by ischemia/reperfusion before transplantation [33]. In order to investigate the signaling mechanisms associated with the inflammatory response of ischemic stress, the H9c2 cardiomyoblast cell culture model is used to determine the signaling events which occur during hypoxia/reoxygenation and reveal potential molecular targets to limit cardiac injury during ischemia/reperfusion. These reports were checked in our study, during which we found that hypoxia/reoxygenation, a model of I/R injury, induced cardiac myoblast H9c2 cell apoptosis and ROS production. Our study also showed that the levels of pro-inflammation cytokines IL-6 and TNF- $\alpha$  were increased in H/R. The AMPK signaling pathway plays a critical role in controlling mPTP opening of H9c2 cell mitochondria under H/R, since AMPK inhibitor and AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knockdown both augment mPTP opening and ROS production in H9c2 myoblast cells under H/R. However, the JNK signaling pathway did not make contributions to hypoxic stress-mediated mPTP opening and ROS generation in the H9c2 cells. Although, JNK signaling is involved in mediating the inflammatory response during H/R, as both JNK inhibitor and JNK1/2 siRNA knockdown JNK1/2 treatment reduced NF- $\kappa$ B activation and mRNA levels of proinflammatory cytokines TNF $\alpha$  and IL-6.

Interestingly, AMPK signaling appears to modulate JNK activation in response to H/R, since both AMPK inhibitor and AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knockdown AMPK $\alpha$  significantly augmented JNK activation while AMPK agonist metformin can reduce the hypoxic stress-induced JNK activation and downstream NF- $\kappa$ B activation and inflammatory response by up-regulation of TNF $\alpha$  and IL-6 mRNA. NF- $\kappa$ B is an important transcription factor. It has function in the processes of inflammation and apoptosis induced by I/R injury [34, 35]. It was verified that the NF- $\kappa$ B subunit p65 is activated during I/R injury in the rat steatotic liver, resulting in inflammation and necrosis [19]. Accordingly, the suppression of NF- $\kappa$ B by its inhibitor BAY 11-7802 or the inhibition of I $\kappa$ B phosphorylation has been proven to reduce the inflammation and apoptosis induced by myocardial or cerebral I/R injury [36].

Our results showed that the inflammatory response-related NF- $\kappa$ B signaling was activated during H/R in H9c2 myoblast cells, which leads to the upregulation of mRNA levels of the targets of NF- $\kappa$ B inflammation-related genes such as TNF- $\alpha$  and IL-6. It is well known that c-Jun N-terminal protein kinase (JNK) induces NF- $\kappa$ B activation in response to a variety of stress stimuli [35, 37]. The response of AMPK, JNK and NF- $\kappa$ B signaling pathways to ischemia and/or reperfusion was also observed in the *in vivo* regional myocardial ischemia and reperfusion in mouse hearts by ligation of the left anterior descending coronary artery (Suppl. Fig. 1). Our study also suggests that AMPK plays a critical role in limiting H9c2 cell apoptosis caused by H/R, since both AMPK inhibitor compound C and AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knockdown AMPK $\alpha$  can significantly enhance the apoptosis occurring under H/R conditions. In contrast, both JNK inhibitor JNK-IN-8 and JNK1/2 siRNA knockdown JNK1/2 can reduce activation of the NF- $\kappa$ B and apoptosis in H9c2 cells under H/R conditions. The study demonstrated that hypoxic AMPK activation controls mPTP opening and ROS generation that triggers the JNK signaling pathway; in other words, JNK activation is a downstream event of mPTP opening and ROS generation by hypoxic stress. Therefore, the inhibition of the JNK signaling pathway did not affect mPTP opening and ROS generation in the H9c2 cells. AMPK regulation of the JNK signaling cascade could make a critical contribution to limiting cell damage by hypoxic stress via modulating mitochondrial homeostasis and inflammatory response.

Finally, a well-known AMPK activator, metformin, was used to test whether pharmacological intervention could be applied to prevent H9c2 myoblast cells from hypoxic injury. Metformin can activate the AMPK signaling pathway in H9c2 cells under both normoxia and H/R conditions. Metformin inhibited mPTP opening and ROS generation caused by H/R. Moreover, metformin treatment attenuates JNK-NF- $\kappa$ B signaling and inhibits mRNA levels of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 during H/R.

In conclusion, the results indicate that intrinsic AMPK plays a critical role in maintaining mitochondrial integrity during hypoxic stress conditions; thus, AMPK can control ROS production and the JNK-NF- $\kappa$ B signaling cascade for inflammatory response and can limit cell damage under H/R conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Funding

These studies were supported by American Diabetes Association 1-17-IBS-296, NIH R21AG044820, R01AG049835, P01HL051971, and P20GM104357, the Taishan Scholar Program of Shandong Province (tshw201502046), and 2016 Yantai Shuangbai Scholar Program.

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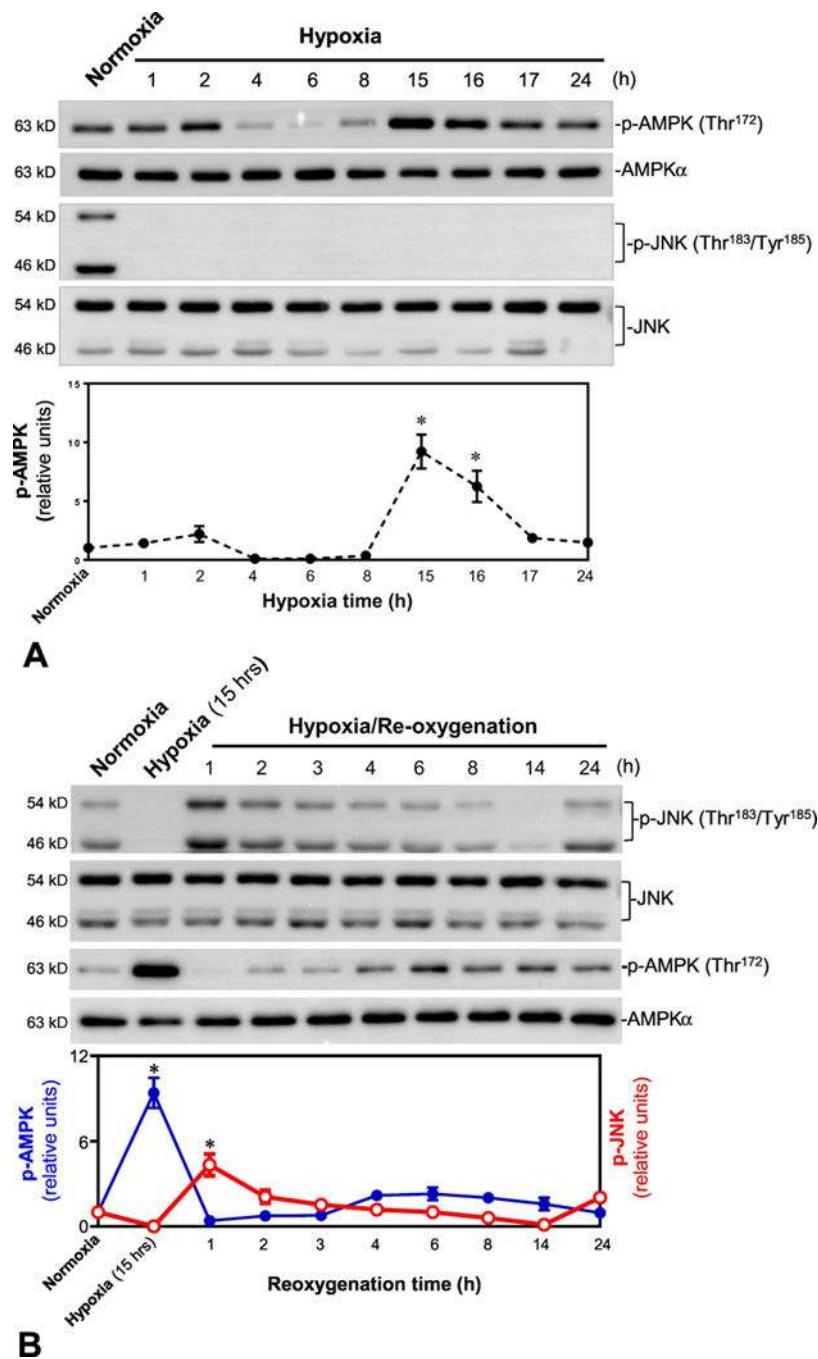
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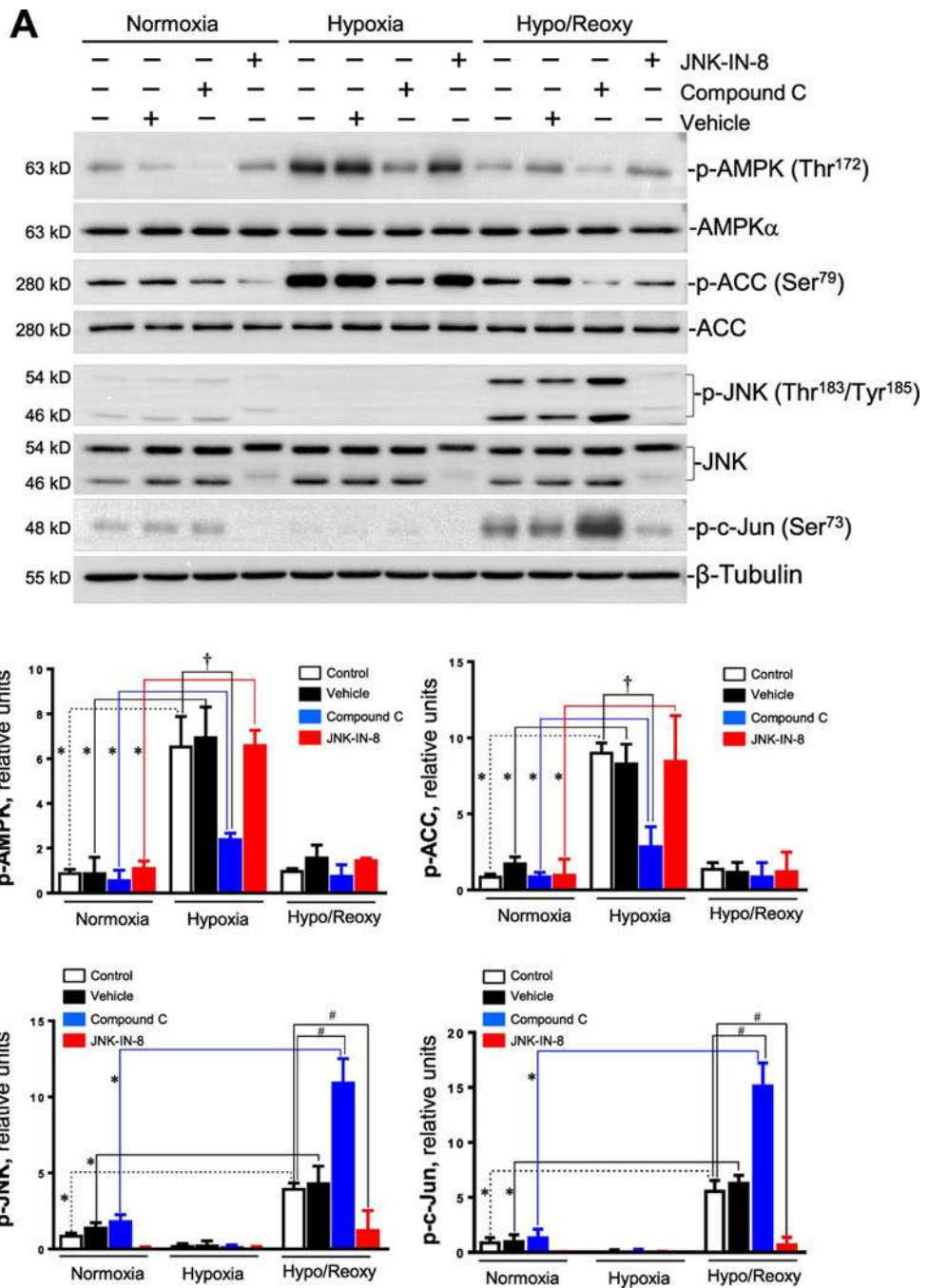
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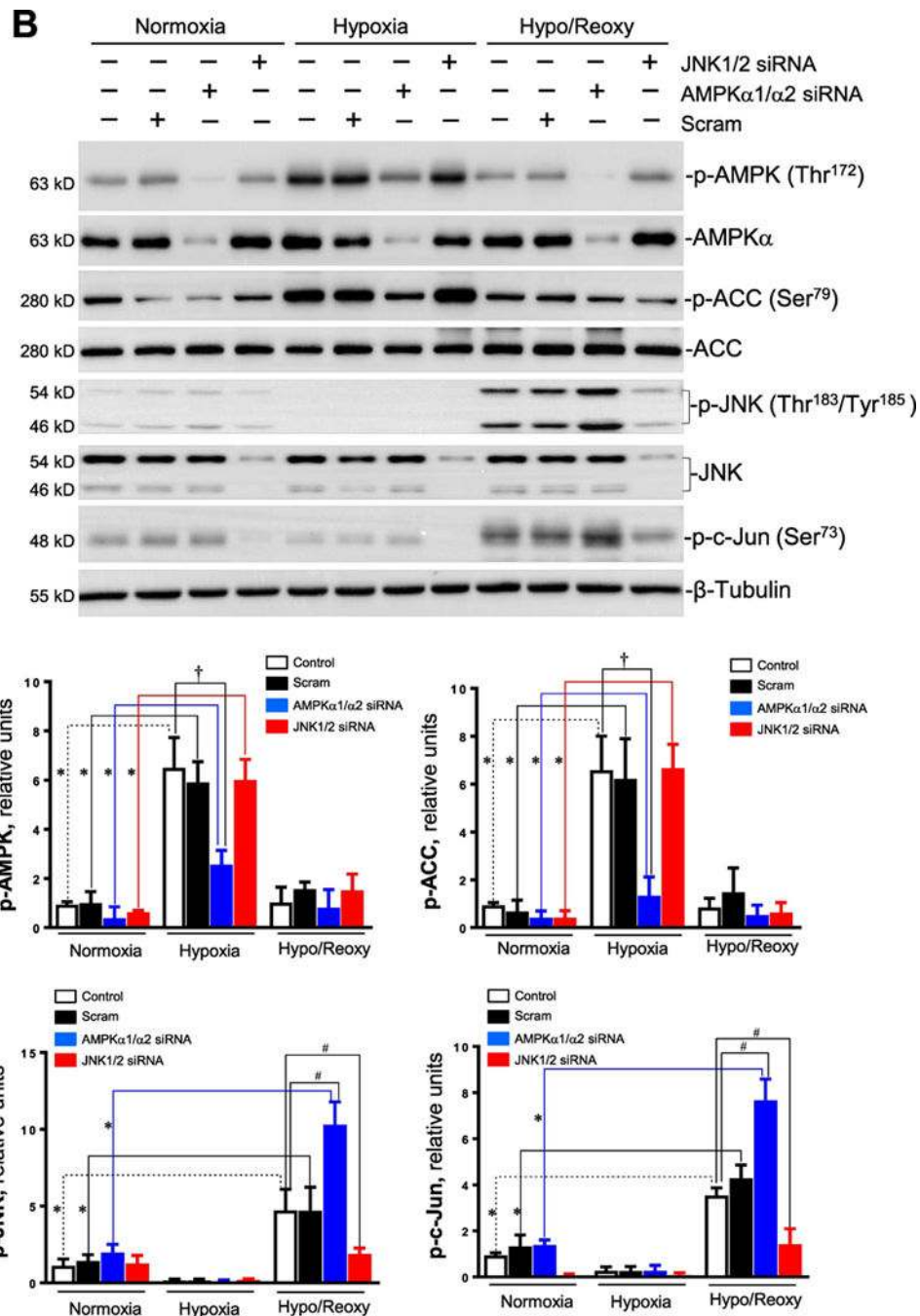
### Highlights

- AMPK activation modulates the c-Jun N protein kinase (JNK) signaling pathway under hypoxic stress conditions.
- The opening of the mitochondrial permeability transition pore (mPTP) is controlled by AMPK in response to hypoxia and reoxygenation.
- Pharmacological AMPK activation could inhibit the inflammatory response during hypoxia and reoxygenation.



**Figure 1.** The kinetics of AMPK activation and JNK activation in response to hypoxia with or without reoxygenation in H9c2 myoblast cells (A) Immunoblotting measured the levels of p-AMPK and AMPK $\alpha$  protein expression in normoxia or hypoxia conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia. (B) Immunoblotting measured the levels of p-AMPK, AMPK $\alpha$ , p-JNK, and JNK protein expression in normoxia, hypoxia or hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia.

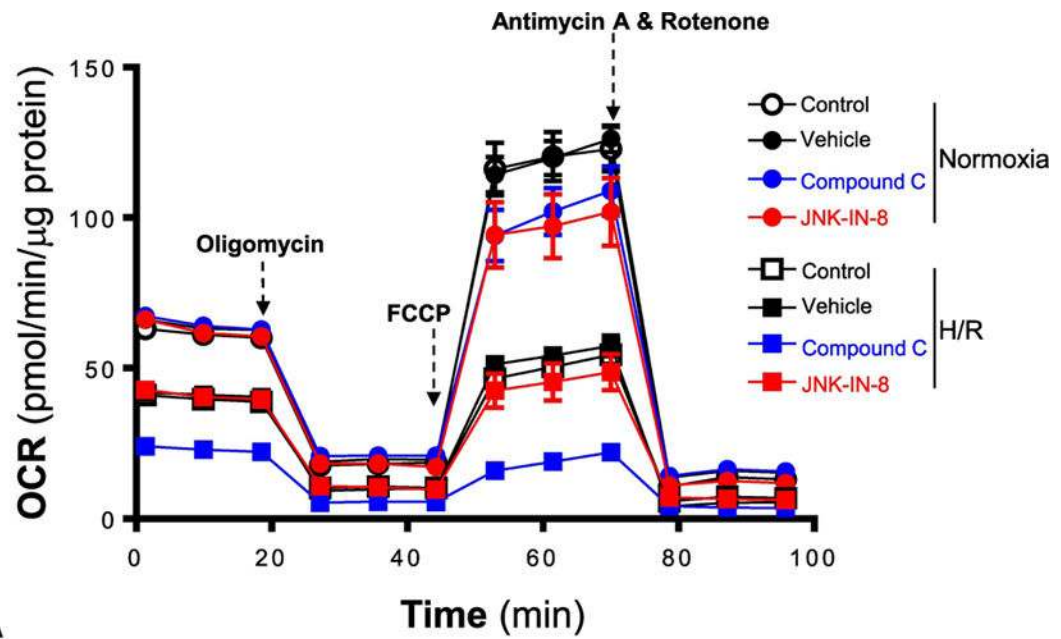




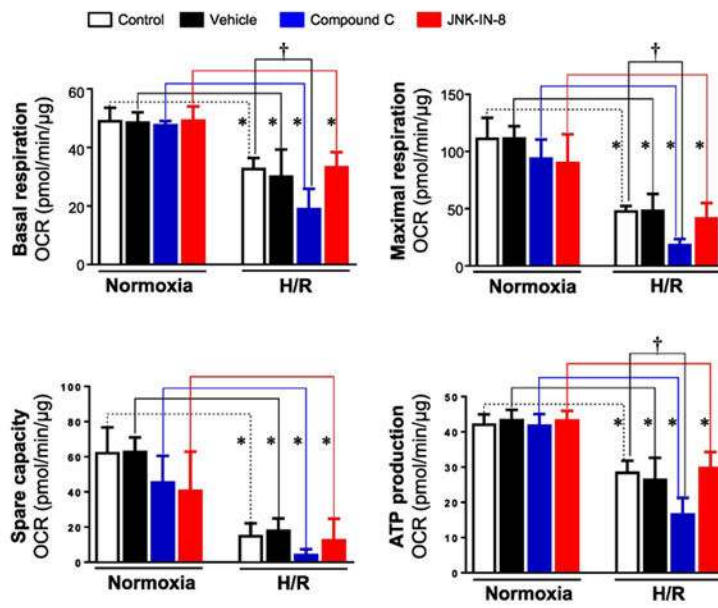
**Figure 2. AMPK inactivation augments JNK activation during hypoxia and reoxygenation** (A) H9c2 cells were treated with compound C (10  $\mu$ M) and JNK-IN-8 (1  $\mu$ M) under normoxia, hypoxia and hypoxia/reoxygenation. Upper: Immunoblotting showed phosphorylation of AMPK and downstream ACC, and phosphorylation of JNK and downstream c-Jun in H9c2 cells with or without AMPK inhibitor compound C and JNK inhibitor JNK-IN-8 under normoxia, hypoxia, and hypoxia/reoxygenation conditions; Lower: quantitative analysis of the relative levels of p-AMPK, p-ACC, p-JNK, and p-c-Jun in H9c2 cells with or without AMPK inhibitor or JNK inhibitor under normoxia, hypoxia,



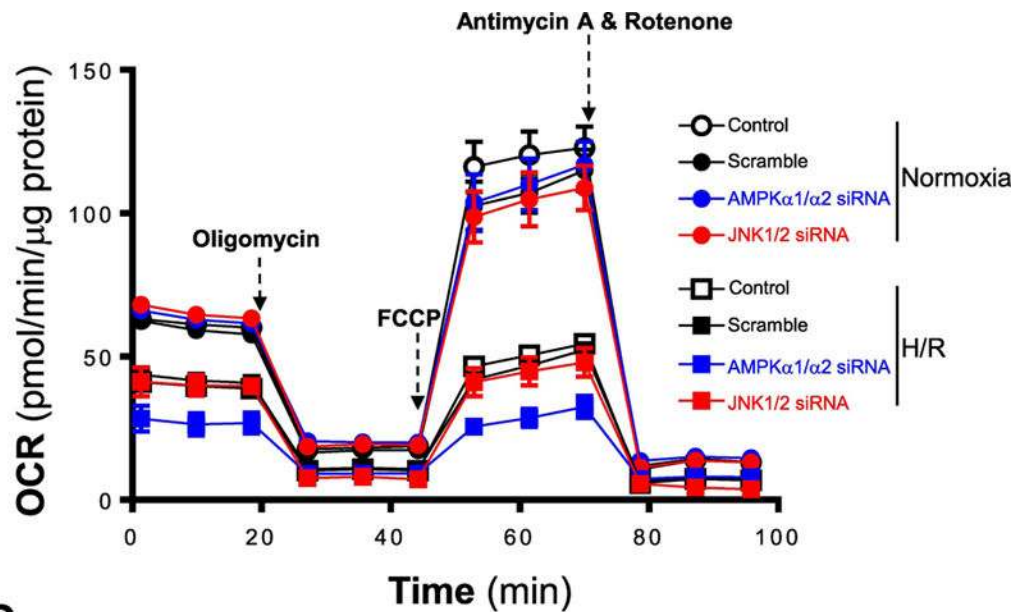
and hypoxia/reoxygenation. Values are mean  $\pm$  SEM. N=4, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypoxia vehicle; #p<0.05 vs. hypo/reoxy vehicle. (B) AMPK $\alpha$ 1/ $\alpha$ 2 siRNA knockdown AMPK $\alpha$  catalytic subunit and JNK1/2 siRNA knockdown JNK1/2 in the H9c2 myoblast cells under normoxia, hypoxia, and hypoxia/reoxygenation. Upper: Immunoblotting showed phosphorylation of AMPK and downstream ACC, and phosphorylation of JNK and downstream c-Jun in H9c2 cells with or without AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA under normoxia, hypoxia, and hypoxia/reoxygenation conditions; Lower: quantitative analysis of the relative levels of p-AMPK, p-ACC, p-JNK, and p-c-Jun in H9c2 cells with or without AMPK $\alpha$ 1/ $\alpha$ 2 siRNA and JNK1/2 siRNA under normoxia, hypoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia, repectively; †p<0.05 vs. hypoxia scram; #p<0.05 vs. hypo/reoxy scram.



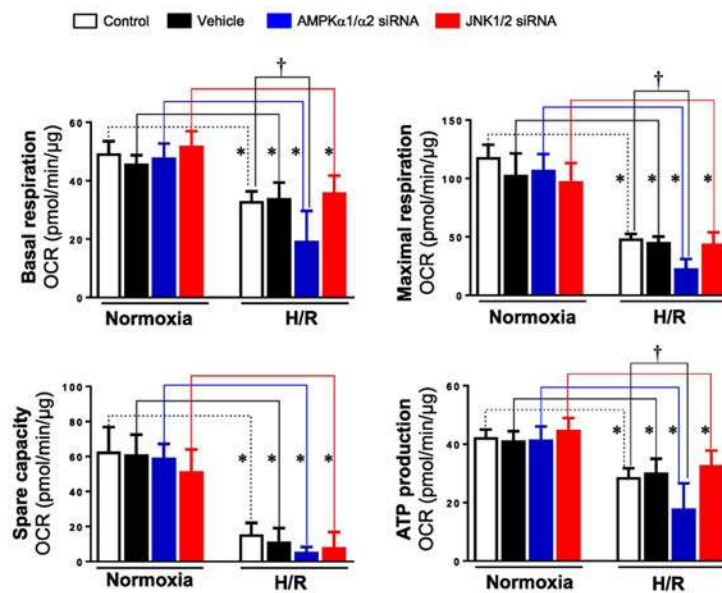
**A**



**B**



C



D

### Figure 3. AMPK plays a role in maintaining mitochondrial integrity during hypoxia and reoxygenation

(A) Mitochondrial respiration measurements of oxygen consumption rate (OCR) were performed with a Seahorse metabolic analyzer. Oligomycin (1  $\mu$ M), FCCCP (2  $\mu$ M), and rotenone (0.5  $\mu$ M) combined with antimycin (0.5  $\mu$ M) were added sequentially to H9c2 cells treated with or without compound C (10  $\mu$ M) and JNK-IN-8 (1  $\mu$ M) under normoxia and hypoxia/reoxygenation conditions. (B) Quantitative analysis of mitochondrial function parameters (basal respiration, maximal respiration, spare capacity, and ATP production) are shown in the bar charts. Values are mean  $\pm$  SEM. N=5, \*p<0.05 vs. normoxia, respectively;

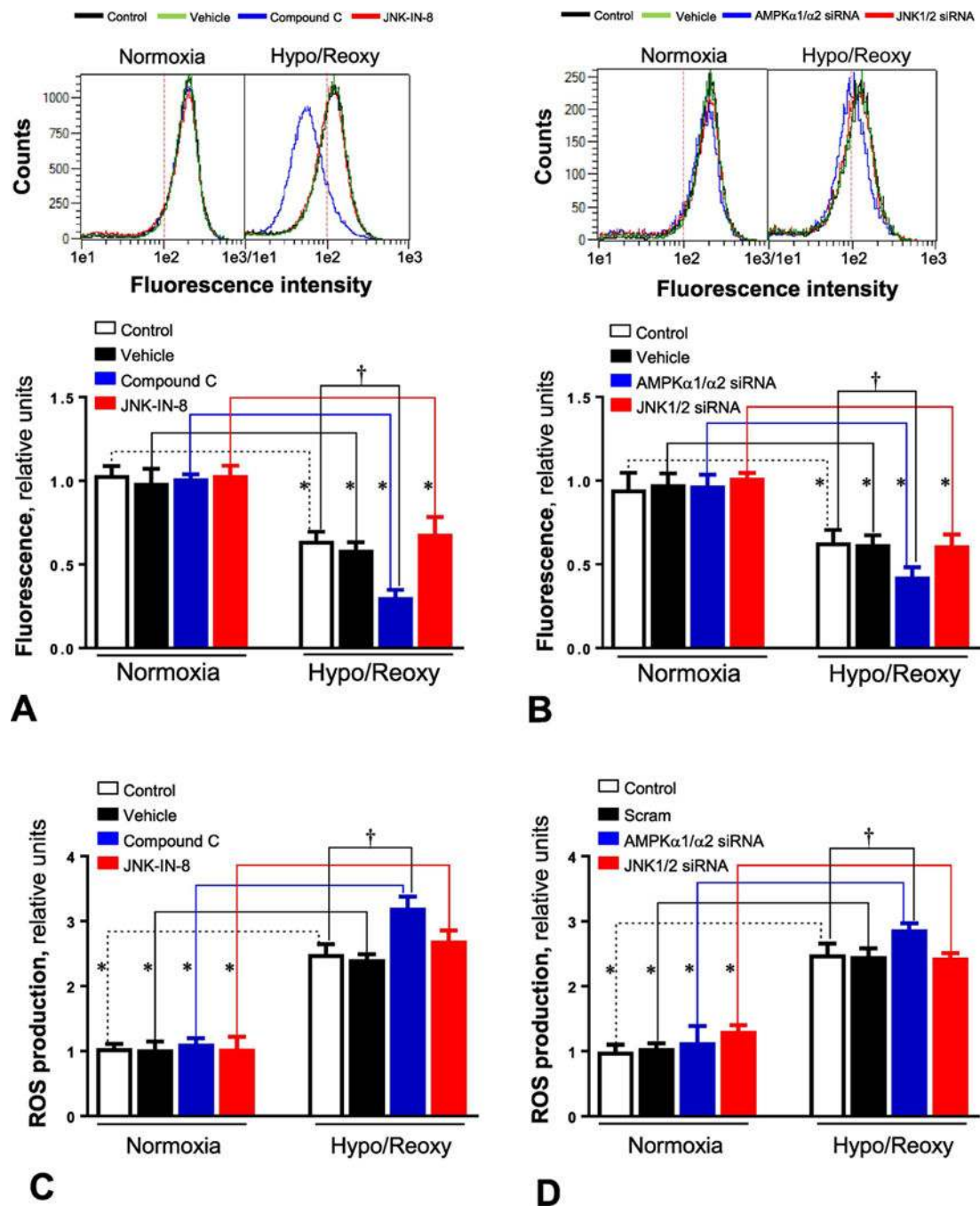
† $p < 0.05$  vs. hypo/reoxy vehicle. (C) Mitochondrial respiration measurements of OCR were performed with a Seahorse metabolic analyzer. Oligomycin (1  $\mu\text{M}$ ), FCCP (2  $\mu\text{M}$ ), and rotenone (0.5  $\mu\text{M}$ ) combined with antimycin (0.5  $\mu\text{M}$ ) were added sequentially to H9c2 cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. (D) Quantitative analysis of mitochondrial function parameters (basal respiration, maximal respiration, spare capacity, and ATP production) are shown in the bar charts. Values are mean  $\pm$  SEM. N=5, \* $p < 0.05$  vs. normoxia, respectively; † $p < 0.05$  vs. hypo/reoxy scram.

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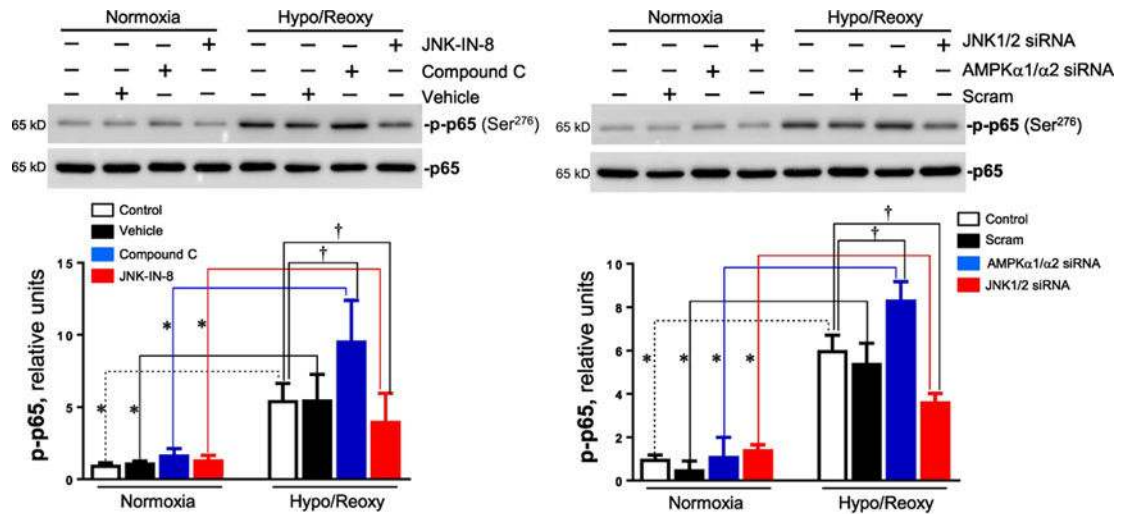
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**Figure 4. AMPK regulates opening of mitochondrial permeability transition pore (mPTP) and production of reactive oxygen species (ROS) during hypoxia and reoxygenation**

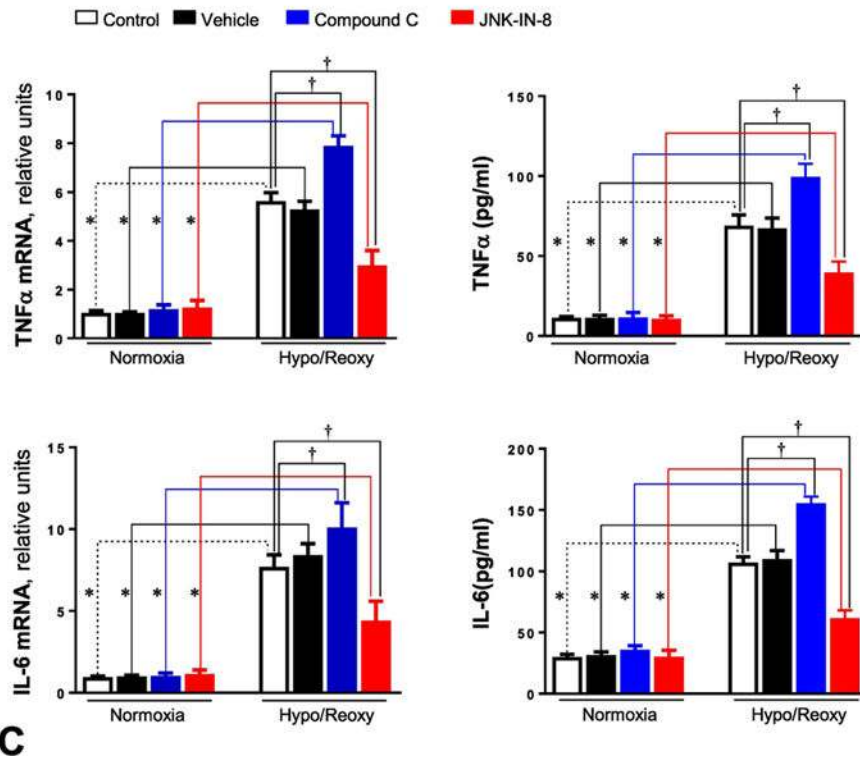
(A) H9c2 myoblast cells were treated with AMPK inhibitor compound C (10  $\mu$ M) or JNK inhibitor JNK-IN-8 (1  $\mu$ M) under normoxia and hypoxia/reoxygenation conditions. The opening of mitochondrial permeability transition pore (mPTP) was measured with the MitoProbe transition pore assay kit. Values are mean  $\pm$  SEM. N=5, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs. hypo/reoxy vehicle. (B) The mPTP opening in H9c2 myoblast cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=5, \* $p$ <0.05 vs. normoxia,

respectively; † $p < 0.05$  vs. hypo/reoxy scram. (C) The levels of reactive oxygen species (ROS) in H9c2 cells treated with compound C (10  $\mu\text{M}$ ) or JNK-IN-8 (1  $\mu\text{M}$ ) under normoxia or hypoxia and reoxygenation conditions. Values are mean  $\pm$  SEM.  $N=6$ , \* $p < 0.05$  vs. normoxia, respectively; † $p < 0.05$  vs. hypo/reoxy vehicle. (D) The ROS production in H9c2 cells treated with AMPK $\alpha 1/\alpha 2$  siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM.  $N=6$ , \* $p < 0.05$  vs. normoxia, respectively; † $p < 0.05$  vs. hypo/reoxy scram.

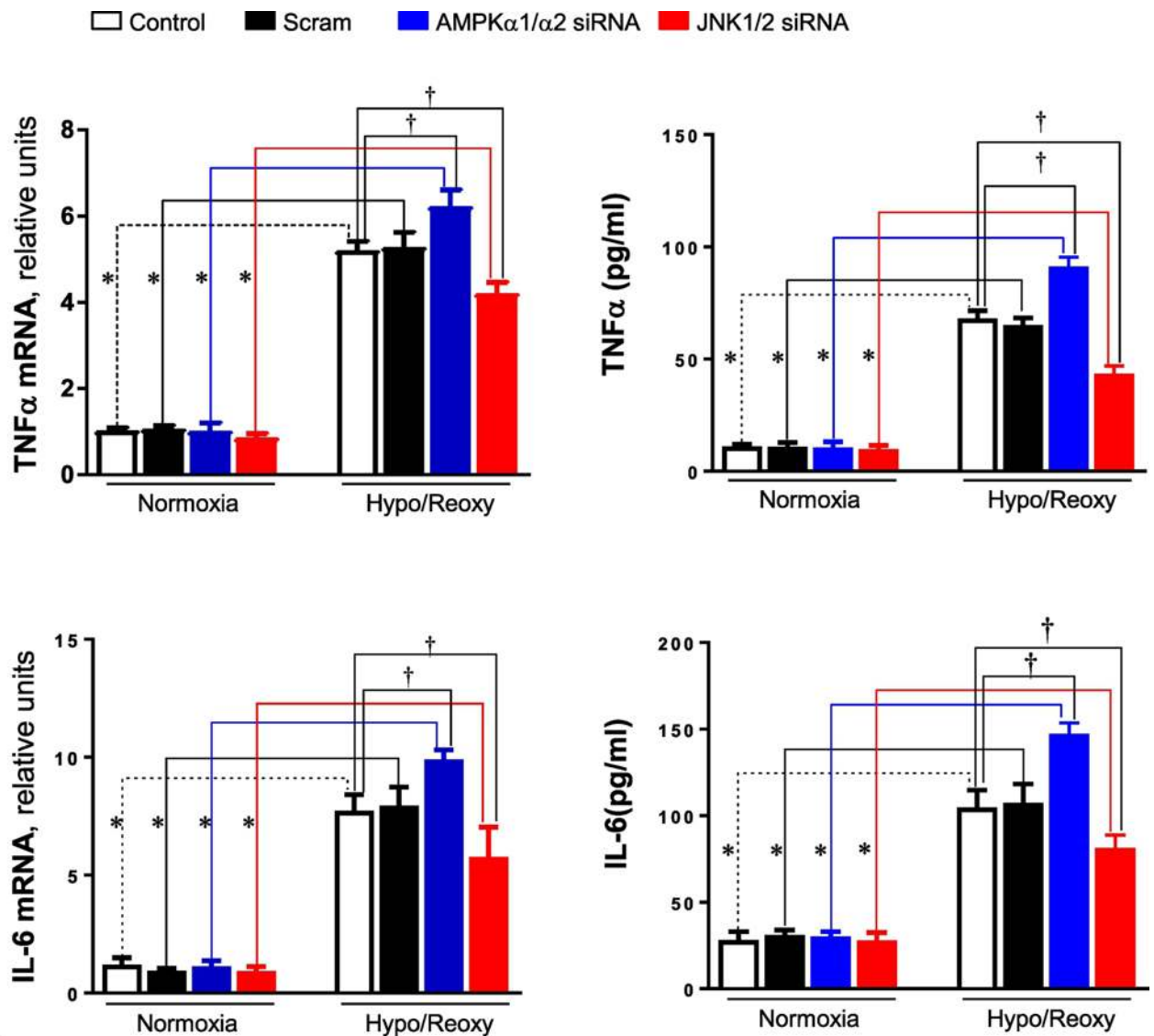


**A**

**B**



**C**



D

**Figure 5. AMPK modulates inflammatory NF- $\kappa$ B signaling activation and proinflammatory cytokine expression during hypoxia and reoxygenation**

(A) Immunoblotting measured the levels of p-p65 and p65 protein expression in H9c2 myoblast cells treated with compound C (10  $\mu$ M) or JNK-IN-8 (1  $\mu$ M) under normoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypo/reoxy vehicle. (B) Immunoblotting measured the levels of p-p65 and p65 protein expression in H9c2 myoblast cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypo/reoxy scram. (C) The total RNA was isolated and subjected to real time RT-PCR to analyze the products of pro-inflammatory cytokines, TNF- $\alpha$  and IL-6. 18S rRNA was used as an internal control. Real-time mRNA levels of TNF $\alpha$  and IL-6 were measured in H9c2 cells treated with compound C (10  $\mu$ M) or JNK-IN-8 (1  $\mu$ M) under normoxia and hypoxia/reoxygenation conditions. The



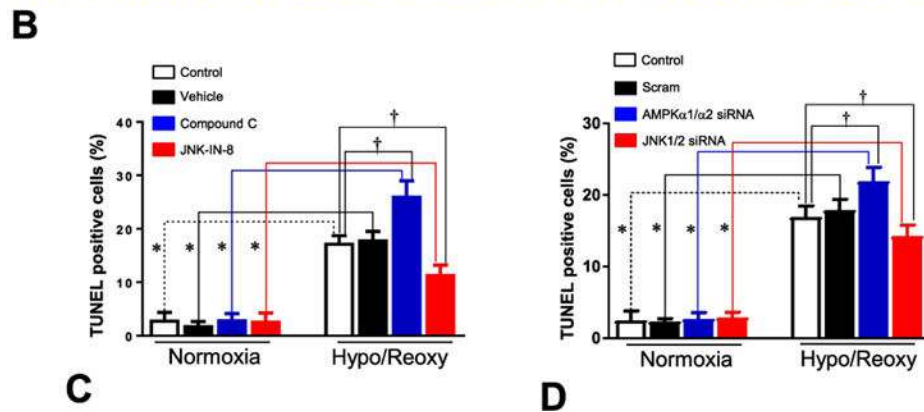
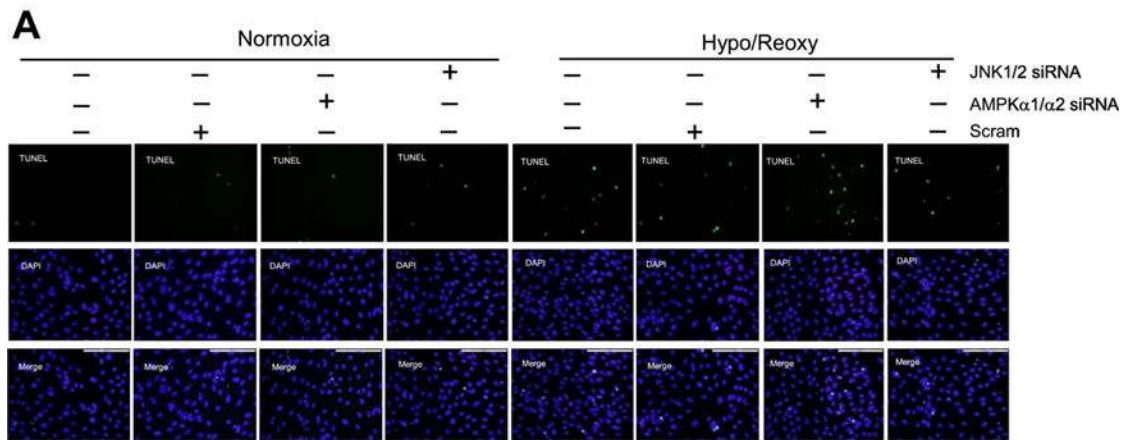
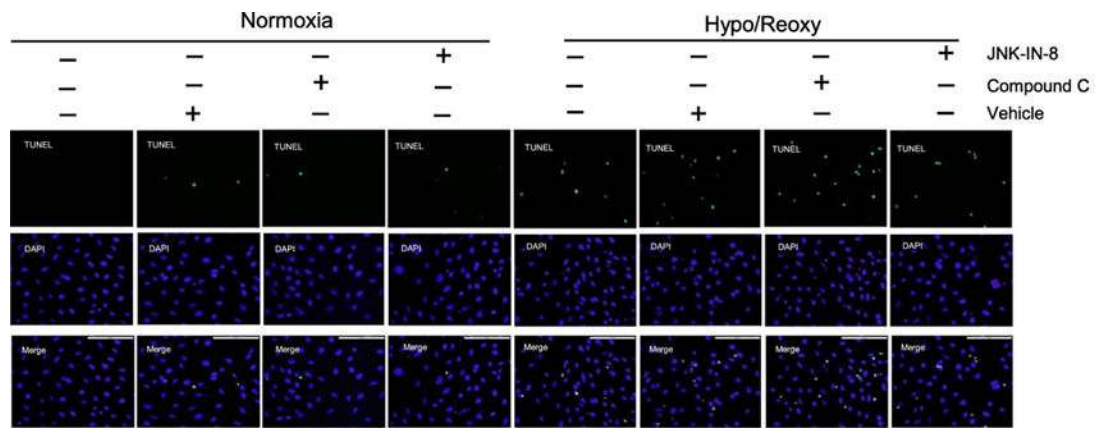
secreted TNF- $\alpha$  and IL-6 in the culture medium were measured with ELISA kit. Values are mean  $\pm$  SEM. N=5, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypo/reoxy vehicle. (D) Real-time RT-PCR measured the mRNA levels of TNF $\alpha$  and IL-6 in H9c2 cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. The secreted TNF- $\alpha$  and IL-6 in the culture medium were measured with ELISA kit. Values are mean  $\pm$  SEM. N=5, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypo/reoxy scram.

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**Figure 6. AMPK inhibits while JNK promotes apoptosis of H9c2 cells during hypoxia and reoxygenation**

(A) Apoptotic cells were detected by staining with TUNEL and DAPI, as described in Methods. Apoptosis in H9c2 myoblast cells treated with compound C (10  $\mu$ M) or JNK-IN-8 (1  $\mu$ M) under normoxia and hypoxia/reoxygenation conditions. (B) Apoptosis in H9c2 myoblast cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. (C) Quantitative analysis of apoptosis in H9c2 cells treated with compound C or JNK-IN-8 under normoxia and hypoxia/reoxygenation conditions. Value are mean  $\pm$  SEM. N=3, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs.

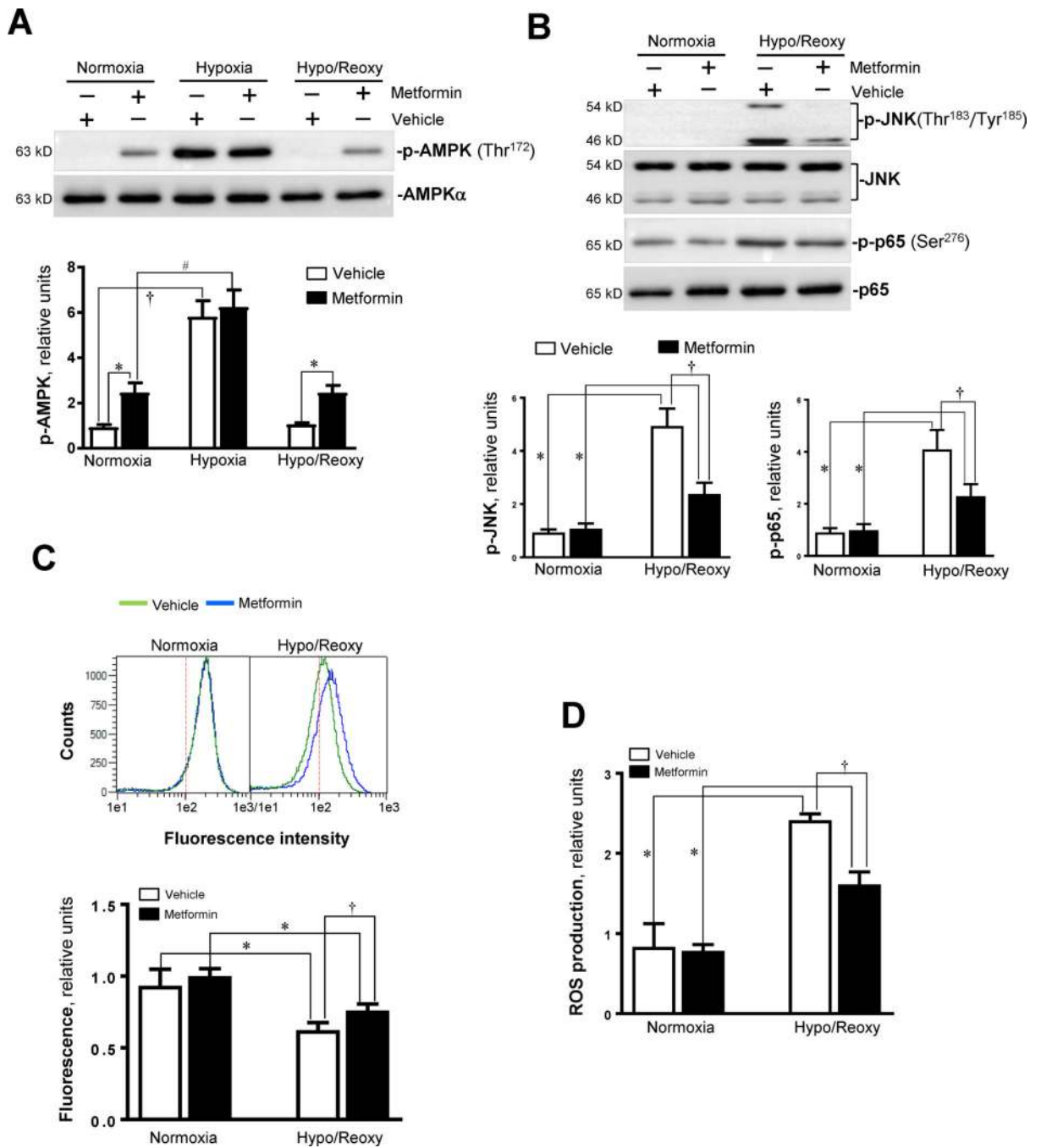
hypo/reoxy vehicle. (D) Quantitative analysis of apoptosis in H9c2 cells treated with AMPK $\alpha$ 1/ $\alpha$ 2 siRNA or JNK1/2 siRNA under normoxia and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \*p<0.05 vs. normoxia, respectively; †p<0.05 vs. hypo/reoxy scram.

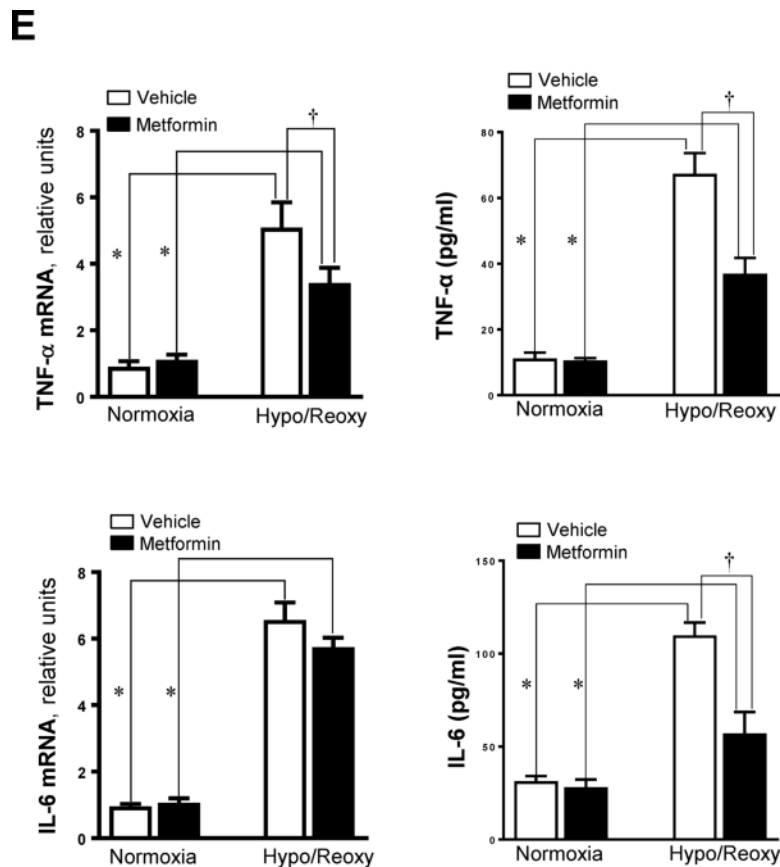
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**Figure 7. AMPK activator inhibits JNK activation by hypoxia and reoxygenation and modulates inflammatory response during hypoxia and reoxygenation**

(A) Immunoblotting showed the phosphorylation of AMPK  $\alpha$  catalytic subunit at Thr<sup>172</sup> site in H9c2 cells treated with AMPK activator metformin (5 mM) under normoxia, hypoxia, and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \* $p$ <0.05 vs. vehicle, respectively; † $p$ <0.05 vs. normoxia vehicle. # $p$ <0.05 vs. normoxia metformin. (B) Immunoblotting showed the phosphorylation of JNK and p65 in H9c2 cells treated with AMPK activator metformin (5 mM) under normoxia, hypoxia, and hypoxia/reoxygenation conditions. Values are mean  $\pm$  SEM. N=3, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs. hypo/reoxy vehicle. (C) The mPTP opening in H9c2 myoblast cells treated with or without metformin. Values are mean  $\pm$  SEM. N=5, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs. hypo/reoxy vehicle. (D) The ROS production in H9c2 myoblast cells treated with or without metformin. Values are mean  $\pm$  SEM. N=6, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs. hypo/reoxy vehicle. (E) The total RNA was isolated and subjected to real time RT-PCR to analyze the products of pro-inflammatory cytokines, TNF- $\alpha$  and IL-6 in H9c2 myoblast cells treated with or without metformin. 18S rRNA was used as an internal control. The secreted TNF- $\alpha$  and IL-6 in the culture medium were measured with ELISA kit. Values are mean  $\pm$  SEM. N=5, \* $p$ <0.05 vs. normoxia, respectively; † $p$ <0.05 vs. hypo/reoxy vehicle.