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Original Paper

PI3K–AKT Pathway Protects Cardiomyocytes Against Hypoxia-Induced Apoptosis by MitoKATP-Mediated Mitochondrial Translocation of pAKT

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Key Words
Pi3k–AKT • Cardiomyocytes • Hypoxic • Mitochondrial • MitoKATP • Apoptosis

Abstract
Background/Aims: The phosphatidylinositol-3-kinase -AKT (PI3K-AKT) is an important intracellular signal pathway in regulating cell proliferation, differentiation and apoptosis. In previous studies, we've demonstrated that PI3K–AKT pathway protects cardiomyocytes from ischemic and hypoxic apoptosis through mitochondrial function. However, the molecular mechanisms underlying hypoxia-induced cardiomyocyte apoptosis via PI3K-AKT pathway remain ill-defined. Here, we addressed this question. ***Methods:*** Cardiomyocytes were exposed to hypoxia, with/without different inhibitors and then protein levels were assessed by Western blotting. ***Results:*** We found that the PI3K–AKT pathway was activated in cardiomyocytes that were exposed to hypoxia. Moreover, the phospho-AKT (pAKT) translocated from cytosol to mitochondria via mitochondrial adenosine triphosphate-dependent potassium (mitoKATP), leading to an increase in cytochrome c oxidase (CcO) activity to suppress apoptosis. On the other hand, the mitoKATP specific blocker, 5-hydroxydecanote (5-HD), or suppression of CcO using siRNA, inhibited the pAKT mitochondrial translocation to maintain the CcO activity, resulting in mitochondrial dysfunction and cellular apoptosis induced by hypoxia. ***Conclusion:*** These findings suggest that the anti-apoptotic effect of the PI3K-AKT pathway through pAKT translocation to mitochondrial via mitoKATP may be conducted through modification of CcO activity.

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Introduction

Myocardial cell damage is often caused by ischemia and hypoxia of the myocardium in people with severe burns, even after timely resuscitation [1]. Consequently, apoptosis occurrence in cardiomyocytes is considered to contribute to burn-induced cardiac dysfunction. Understanding the mechanism of apoptosis can improve cardiac function in these disorders [2-4]. Mitochondria are essential organelles in cellular metabolic pathways and respiration; at the same time, mitochondrion is also the primary organelle involved in mediating the intrinsic apoptotic pathway [5, 6]. Our previous study showed that activation of PI3K–AKT pathway protects cardiomyocytes from hypoxic apoptosis through mitochondrial function [7]; however, the molecular mechanisms still remain unclear.

In mammalian cells, protective signaling molecules are recruited in response to stress to prevent cell death. The PI3K–AKT signaling pathways play an important role in controlling diverse cellular function, including cell cycle progression, protein synthesis, and cell survival [8]. PI3K-AKT prevents the caspase-9 and p53-dependent apoptosis in HTLV-1-transformed cells by targeting multiple proteins [9]. The activated AKT can then activate or deactivate its myriad of substrates through its kinase activity. Recent studies have shown that the activation of the PI3K–AKT pathway reduces TNF-α production and suppresses inflammation [10, 11]. Activation of the PI3K/AKT pathway limits JNK-mediated apoptosis by phosphorylating and inactivating ASK1 during the Enterovirus 71 (EV71) infection [12]. We have recently established that PI3K–AKT pathway activation contributes to cardiomyocyte hypoxic apoptosis through mitochondrial function [7]. However, the molecular mechanisms have not been clearly explained.

In the present study, we examined the molecular mechanisms of PI3K-AKT pathway involved in anti-apoptotic. We have shown here that the pAKT translocate to the mitochondria from cytoplasm via mitoKATP and this play an anti-apoptotic effect through the modification of CcO activity.

Materials and Methods

Cell culture

The methods of primary culture of neonatal rat cardiomyocytes from 1 to 2-day-old Sprague-Dawley rats were described as the previous study [7]. All of the animal procedures were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University and followed the *Principles of Laboratory Animal Care*. The cardiomyocytes were maintained at a temperature of 37°C in a 5% CO_2 incubator in Dulbe CcO's modified Eagle's medium (DMEM)–F12 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) for hypoxia treatment. The cells were cultured in an air-tight Plexiglas box maintained with 1% O_2, 5% CO_2 and 94% N_2. The oxygen level was measured using an electronic gas analyzer.

Cell treatment

The cells were cultured under normoxic conditions or hypoxic conditions. One hour before the hypoxia, the PI3K-AKT pathway inhibitor, LY294002 (final concentration 50 μmol/L) or activator IGF-1 (final concentration 200 ng/mL; Chiron, Emeryville, CA, USA), and the specific opener, diazoxide (DZ), or blocker, 5-hydroxydecanote (5-HD), of mitoKATP were added to the culture medium and subsequent treatments. The cells were harvested. For measurement of CcO enzyme activity, mitochondrial cytochrome c oxidase subunit IV (CcO IV) was knockdown by siRNA.

Preparation of subcellular fractionation

The subcellular fractionations were isolated according to the method described by Tsuruta, et al. [13]. Briefly, myocardial cells were washed with PBS, scraped into an isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH (pH 7.4), 1 mM dithiothreitol), supplemented with the protease inhibitors described above, and homogenized with a Potter-Elvehjem homogenizer. Nuclei and

unbroken cells were removed by centrifugation at 500 × g for 10 minutes, and the supernatant was further centrifuged at 100, 000 × g for 60 minutes. The resulting supernatant was saved as the cytosolic fraction, and the pellet was washed with the isotonic buffer. It was then resuspended in an extraction buffer which was supplemented with protease inhibitors, and centrifuged at 20, 000 × g for 5 minutes to remove debris. The resulting supernatant was saved as the mitochondrial fraction.

Western blotting

The expression levels of pAKT and total AKT were analyzed through Western blot assay. In brief, the whole-cell protein, or mitochondrial proteins, were extracted from cells and separated using a 10% SDS-PAGE gel and then they were transferred to nitrocellulose membranes. After the blocking procedure, the membranes were immunoblotted with the primary antibody for total AKT or Phospho-AKT (Ser473) (Cell Signaling Technology) and COXIV antibody (CcO IV) (Cell Signaling Technology) at 4°C overnight. We used VDAC and β-actin for normalization for Westerns. The blots were washed, incubated with HRP-conjugated secondary antibodies, and then they were visualized by ECL assay.

Alteration in mitochondrial membrane potential

The cardiomyocytes were cultured on coverslips. After drug treatment under ischemia and hypoxia for 6 hours, the medium was changed to a glucose negative and serum-free DMEM (without Ca^{2+} or Mg^{2+}; Dingguo Bio, Beijing, China) with a tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR, USA), and a fluorescence probe was added (final concentration 10 μmol/L). The cells were cultured at 37°C for 20 minutes and the distribution of fluorescent intracellular TMRE was observed under a confocal laser microscope (Leica TCS NT; Leica, Wetzlar, Germany), with the relative fluorescence intensity being measured and analyzed.

Apoptosis analysis

Cell apoptosis was measured by Flow Cytometry by Annexin V-PE/7-AAD double-staining. The treated cells and the control cells were collected and washed with cold PBS, then the cells were resuspended in 1× binding buffer according to manufacturer's instructions (Annexin V-PE/7-AAD apoptosis detection Kit, BD Biosciences). We added 5 μL Annexin V-PE and 5 μL 7-AAD to 100 μL of cell suspension, then we gently vortexed and incubated this for 15 minutes in the dark, at room temperature. Then a 400 μL 1× binding buffer was added to cells before conducting flow cytometry analysis by flow cytometry. The amounts of early and late apoptosis were determined as the percentage of Annexin V-PE positive /7-AAD negative or Annexin V-PE and 7-AAD positive cells, respectively.

ATP/ADP ratio assay

An ATP/ADP ratio assay was used with an ADP/ATP ratio assay kit (ab65313, Abcam) according to the manufacturer's instructions. The cells were harvested and Nucleotide Releasing Buffer (50 μL Buffer per 10^3 –10^4 cells) was added, and then they were incubated for five minutes, at room temperature, with gentle shaking. We prepared a reaction mix for each reaction, adding 100 μL of the reaction mix to the control, and sampling wells, and reading the background luminescence. We transferred 50 μl of cells (10^3 – 10^4 cells) treated with Nucleotide Releasing Buffer into a luminometer plate. After approximately two minutes, we read the sample with the luminescence capable plate reader. We diluted 10x ADP-Converting enzyme 10-fold with Nucleotide Releasing Buffer. To measure ADP levels in the cells, we read the samples again, and then added 10 μL of 1x ADP Converting Enzyme. The samples were read again after approximately two minutes. The experiments were repeated six times, in triplicate, and then a mean was calculated from the results.

Measurement of ROS accumulation

The cells were initially plated in six well plates at a density of 5 × 10^5 cells/well. At the indicated time points, to monitor intracellular accumulation of ROS, the treated cells were incubated with 5 μM DCFH-DA for 30 minutes at 37 °C and subsequently they were washed twice with D-Hanks and then they were collected. The fluorescence intensity was monitored with excitation wavelength at 488 nm and emission wavelength at 530 nm with a fluorometer (GENios, USA). All of the experiments were performed in triplicate.

Caspase-3 activity measurement

The caspase-3 assay was performed according to the manufacturer's instructions of the Abcam Caspase-3 Kit. Briefly, cells were lysed with lysis buffer and equal amounts of lysates protein were incubated with caspase-3 colorimetric DEVD-pNA substrate at 37 °C for 60 minutes. The results were measured with the Multiskan™ GO microplate spectrophotometer (Thermo) at 405 nm, and caspase-3 activity was calculated as a fold change in comparison to cells without treatment.

Cytochrome oxidase

The activity of the cytochrome oxidase was measured according to previous reports [14]. Homogenate was obtained from cells in 1.0 mL of 1/30 mol/L phosphate buffer, pH 7.4 centrifuged at 10, 000 × g for 30 minutes at 4 °C. Supernatants were used an enzyme source. The enzyme activity at 25 °C was measured in a 10 mm path length curette. A 3.0 mL of reduced cytochrome solution (1.7×10^{-5} mol/L) was taken into the cuvette and 0.2 mL of cell homogenate was added. The reactants were mixed by inverting the cuvette several times and then absorption at 550 nm was monitored for three minutes. A few grains of potassium ferricyanide were added (to oxidase cytochrome c completely) and the extinction was re-determined. The enzyme activity has been expressed as the average enzyme activity/min/mg protein.

Statistical analysis

All data was presented as the mean±SD. The statistical analysis was evaluated with *t*-tests and one-way ANOVA using SPSS 10.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered as significant.

Results

Hypoxia activates the PI3K–AKT pathway in the cardiomyocytes.

Previously, we proved that activation of the PI3K–AKT pathway was found in the myocardium of burn rats. We first looked at the hypoxia activation of the PI3K-AKTpathways in cardiomyocytes, since hypoxia has been reported as being induced by burn wounds. As determined by Western blotting, we found that the expression of pAKT in cardiomyocytes was significantly increased under exposure to hypoxia (Fig. 1). These findings suggested that the hypoxia was associated with activating the PI3K-AKT signaling pathway in cardiomyocytes.

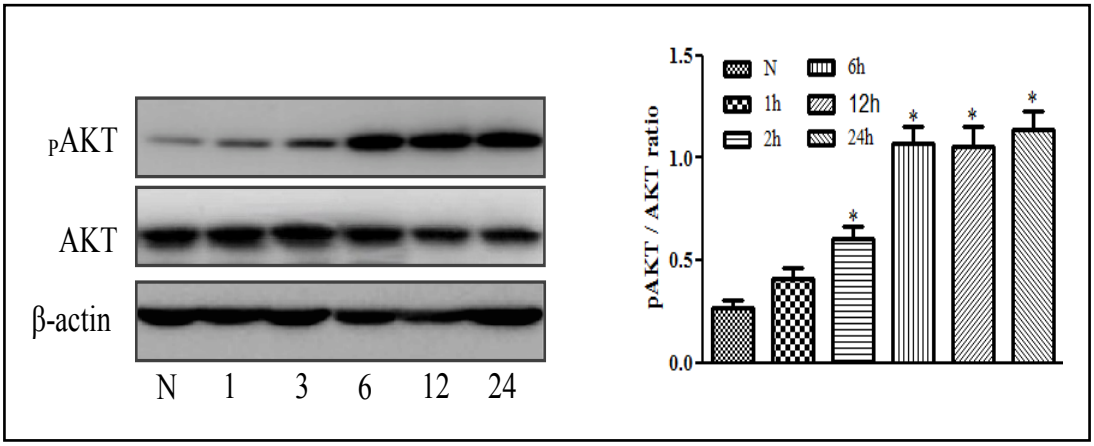

Fig. 1. Activation of the PI3K-AKT pathway induced by hypoxia in cardiomyocytes. The expression of total AKT (T-AKT) and phosphorylated AKT (pAKT) was determined by Western blotting at the following intervals: 0, 1, 3, 6, 12, and 24 hours in hypoxia. The representative bands of pAKT and quantitative analysis results of pAKT / T-AKT ratio.

PI3K-AKT pathway protects cardiomyocytes from hypoxia-induced mitochondrial damage and apoptosis

We further explored whether the PI3K–AKT pathway protects cardiomyocytes from hypoxia-induced mitochondrial damage and apoptosis. Our results showed that mitochondrial membrane potential (MMP) and ATP/ADP ratio were decreased significantly, and reactive oxygen species (ROS), caspase-3 activity, and cell apoptosis were increased significantly compared with the control group. This effect was blocked by the LY294002, a PI3K–AKT pathway inhibitor (Fig. 2 A-E). Pretreatment of cells with IGF-1, an activator of PI3K–AKT, also significantly reversed the effects of hypoxia-induced apoptosis. This showed that the activation of the PI3K-AKT can protect cardiomyocytes from apoptosis induced by hypoxia.

Hypoxia promotes pAKT translocation to the mitochondria via mitoKATP.

To investigate the protection mechanism of the PI3K–AKT pathway in hypoxia, we next studied whether hypoxia promotes pAKT translocation to the mitochondria. Purified mitochondria and cytosol were prepared from cardiomyocytes cultured under normal and hypoxic conditions. The Western blot assay showed that the levels of activated pAKT translocating into the mitochondria were significantly increased in the hypoxic group (Fig. 3A). We continued with additional testing to see if activation of PI3K-AKT has an influence on the pAKT in the mitochondria under hypoxia. The cardiomyocytes were treated with IGF-1 and the purified cytosolic and mitochondrial fractions were detected in pAKT. The IGF-1 treatment resulted in increased pAKT in cytosol and mitochondria and through this the pAKT translocation to mitochondria was inhibited by LY294002. The result of pAKT translocation and accumulation in mitochondria following activation of PI3K-AKT raised the question of how the pAKT are transported to the mitochondria. Mitochondrial ATP-sensitive potassium channel (mitoKATP) was a common end effector of many protective stimuli in myocardial ischemia-reperfusion injuries [15]; however, the specific molecular mechanism has not been expounded on. Next we examined whether pAKT translocation to the mitochondria was dependent of mitoKATP. The specific opener, diazoxide (DZ), or the blocker, 5-hydroxydecanote (5-HD), were given to test whether it can affect the pAKT translocation. We found that DZ treatment caused an increase in the pAKT level in the mitochondria compared to the control group, and the level of pAKT in cytosol was significantly reduced (Fig. 3B). Compared to the control group, mitochondrial pAKT was significantly reduced in the 5-HD treated group, and the pAKT was increased in the cytosol. These results suggest that hypoxia promotes pAKT translocation dependent of the mitoKATP. Our data demonstrated the anti-apoptotic effect of the PI3K–AKT pathway through pAKT translocation to mitochondria which modulates mitochondrial function via mitoKATP.

Mitochondrial translocation of pAKT increases the activity of CcO in hypoxic cardiomyocyte.

CcO is a terminal enzyme complex of the electron transport chain, it plays critical roles in mitochondrial respiration and energy production, and the specific alteration of the CcO facilitates mitochondrial apoptosis [16]. To explore whether the alteration of PI3K-AKT pathway can affect the activity of CcO, we measured the alteration of CcO activity following inhibition or activation of PI3K-AKT pathway. The activity of CcO was first examined in the presence of PI3K-AKT activator IGF1. Remarkably, the activity of CcO was significantly increased in pretreatment with IGF1 group (Fig. 4A). In contrast, the activity of CcO were lower in PI3K-AKT inhibitors LY294002 group (Fig. 4B). These findings identify pAKT as modulator of CcO activity in the hypoxic cardiomyocytes.

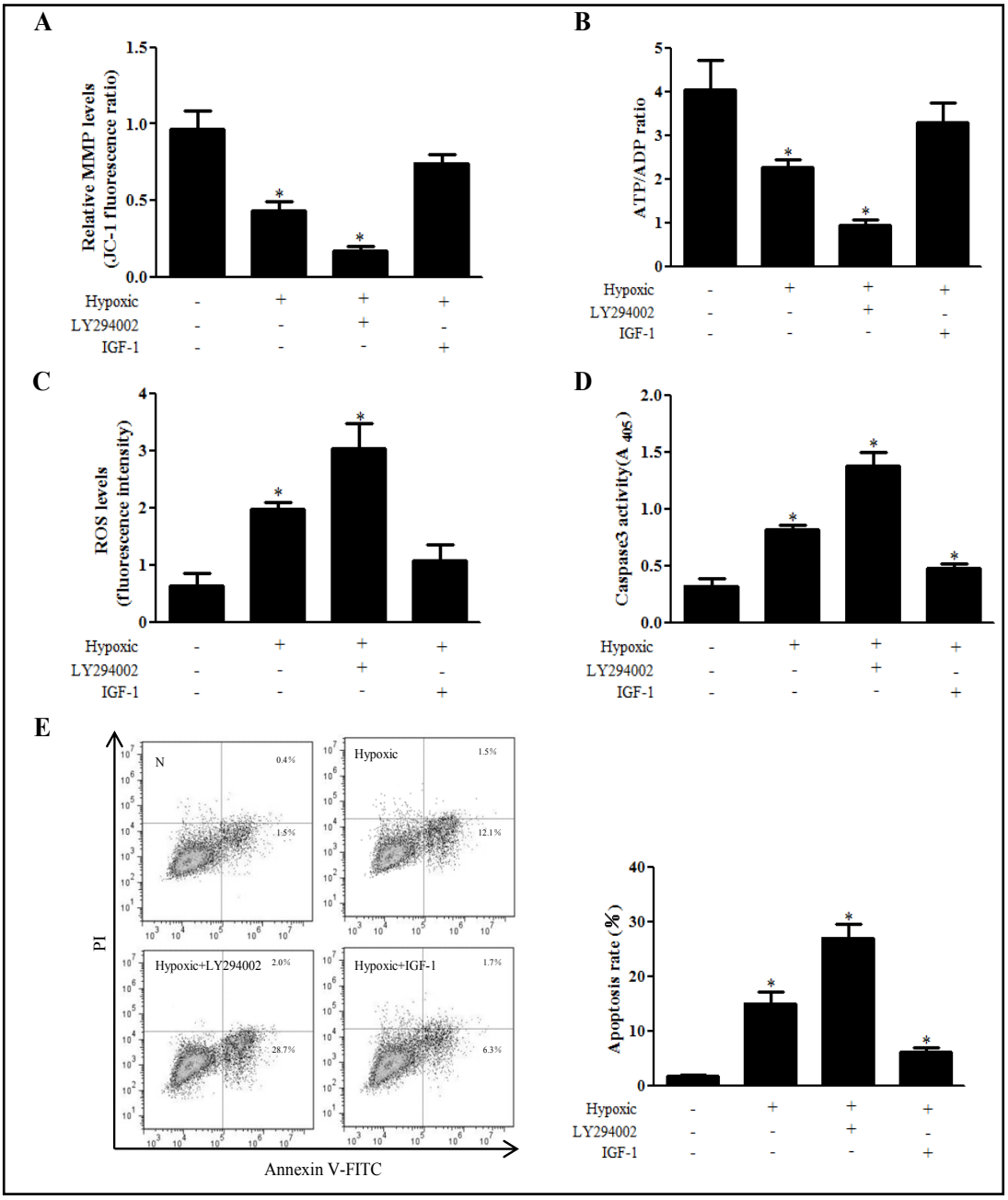

Fig. 2. The effect of the PI3k-AKT on cardiomyocytes under hypoxia. A - Cardiomyocytes in normoxia or hypoxia were treated with LY294002 and IGF-1, mitochondrial membrane potential (MMP) was measured with fluorescent dSye, JC-1, and the fluorescent intensity of the JC-1 was determined by Multimode Reader. B - Cardiomyocytes in normoxia or hypoxia were treated with LY294002 and IGF-1, and the ATP/ADP ratio was measured with a ADP/ATP Ratio Assay Kit by a luminescence capable plate reader. C - Cardiomyocytes in normoxia or hypoxia were treated with LY294002 and IGF-1, and a quantitative analysis of caspase-3 activity calculated as μmol of pNA released per minute per milliliter of cell lysate was measured using the synthetic caspase substrate, DEVD-pNA. D - cardiomyocytes in normoxia or hypoxia were treated with LY294002 and IGF-1, the relative levels of ROS were analyzed using H2DCF-DA by fluorometer. E - Cardiomyocytes in normoxia or hypoxia were treated with LY294002 and IGF-1. The cells were then stained for FITC-Annexin V and Propidium Iodide and then they analyzed quantitatively by flow cytometry. The cell apoptotic rates are reported in the histograms. The mean was ±SEM. ∗P<0.05.

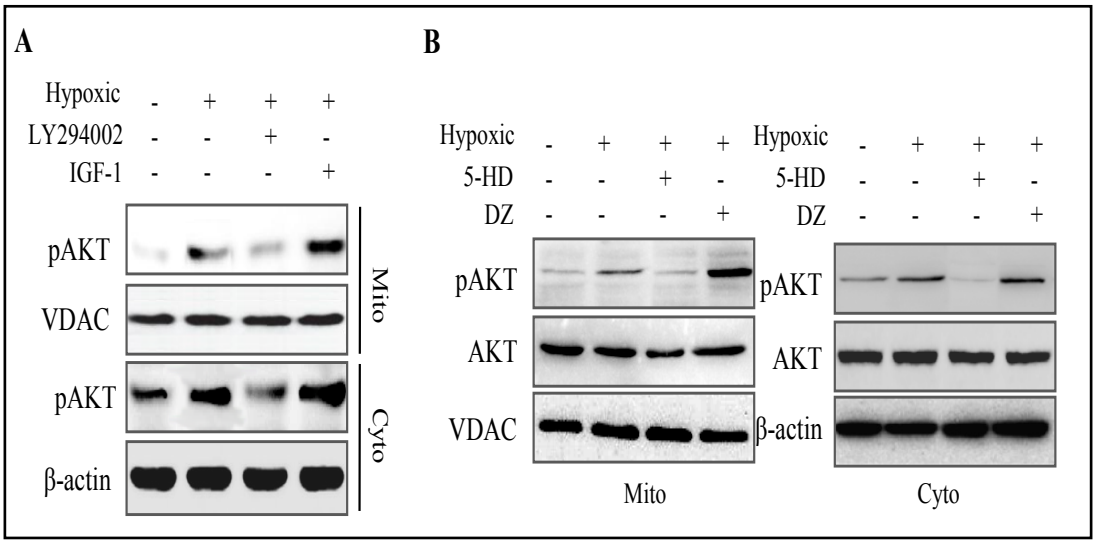

Fig. 3. The mitoKATP promotes pAKT translocation to the mitochondria under hypoxia. A - The PI3K-AKT signaling induces accumulation of AKT in mitochondria in hypoxia. Cardiomyocytes were treated with IGF-1 or LY294002 before being cultured in a hypoxia condition for 6 hours. The cytosolic (Cyto) and mitochondrial (Mito) fractions were prepared, and pAKT was detected by Western blotting. B - The pAKT translocation to the mitochondria via mitoKATP in hypoxia. The cardiomyocytes were treated with 5-DH or DZ before being cultured in hypoxic condition for 6 hours.

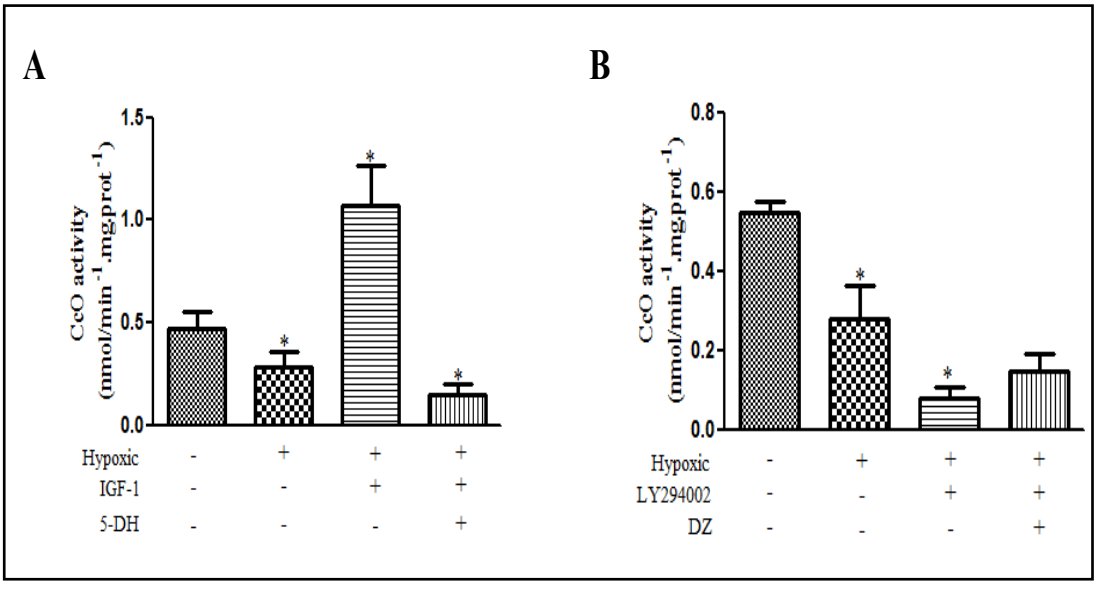

Fig. 4. The activity of CcO in hypoxic cardiomyocyte. A - Cardiomyocytes in normoxia or hypoxia were treated with 5-DH, IGF-1, and the activity of the CcO was analyzed. B, Cardiomyocytes in normoxia or hypoxia were treated with LY294002, DZ, and the activity of the CcO was analyzed. Mean ± SD. *p <0.05.

The protection of cardiomyocytes from hypoxic apoptosis by PI3K-AKT pathway requires activity of CcO

In order to examine whether the PI3K-AKT pathway protects cardiomyocytes from hypoxic apoptosis is dependent on activity of CcO, we analyzed the impairments of mitochondrial function in knockdown CcO by siRNA (Fig. 5A). We found that IGF-1 enhanced cardiomyocytes' mitochondrial membrane potential (MMP), the ATP/ADP ratio (Fig. 4B, C), and the reactive oxygen species (ROS). The caspase-3 activity decreased significantly

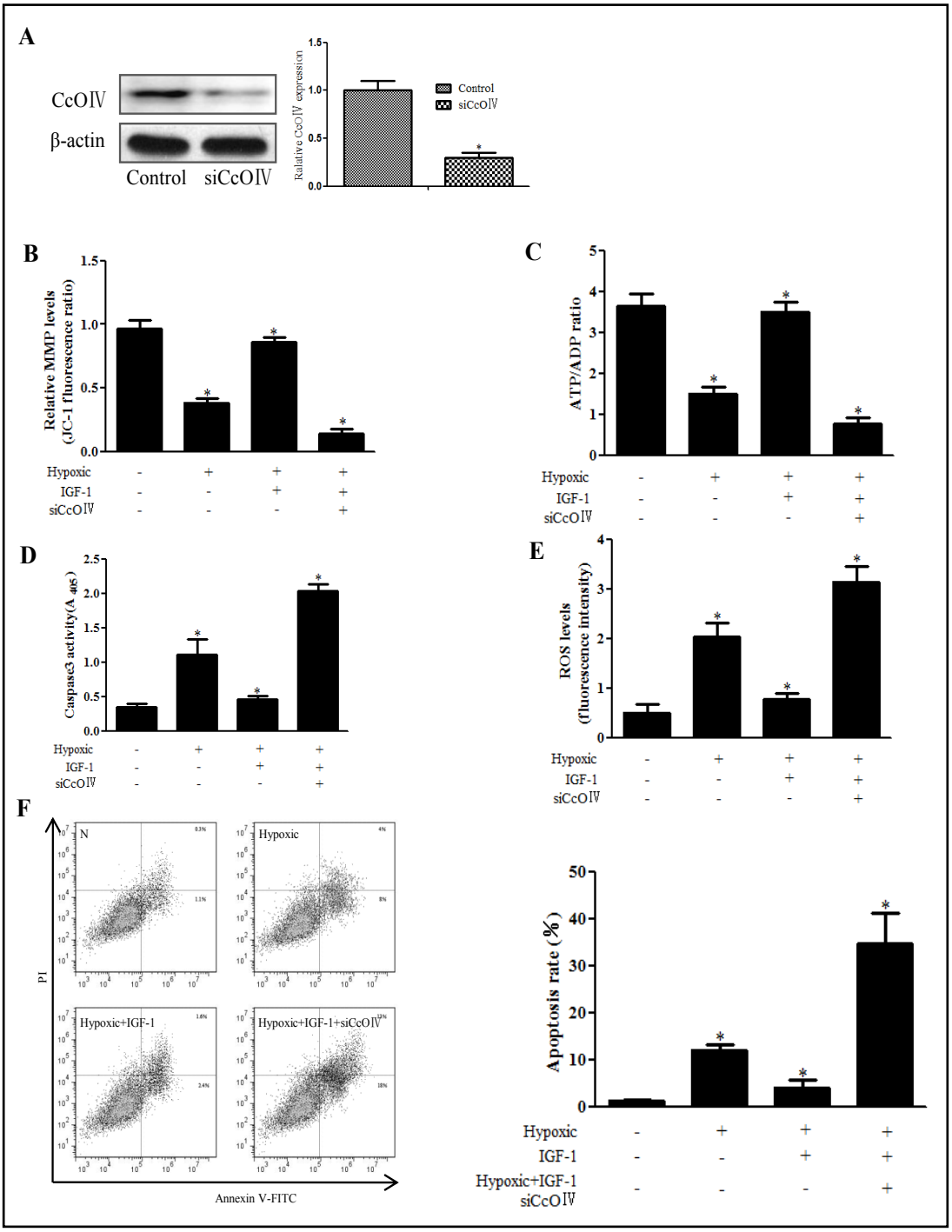

Fig. 5. The effect of PI3k-AKT on cardiomyocytes depends on the activity of CcO in hypoxia. A- Cardiomyocytes were transfected with control siRNA (Control) or siRNA to CcOIV(siCcOIV), and this was analyzed by Western blotting. B - Cardiomyocytes in normoxia or hypoxia were treated with IGF-1 and siCcOIV and the mitochondrial membrane potential (MMP) was measured with the fluorescent dye, JC-1. C - The ATP/ADP ratio was measured with ADP/ATP Ratio Assay Kit by a luminescence capable plate reader. D - The quantitative analysis of caspase-3 activity was measured using a synthetic caspase substrate, DEVD-pNA. E - The relative levels of ROS were analyzed using H2DCF-DA by a fluorometer. F – The cells were stained for FITC-Annexin V and Propidium Iodide and then they were analyzed quantitatively by flow cytometry. The cell apoptotic rates are reported in the histograms. The data's mean was ±SEM. ∗P<0.05.

compared to the hypoxic group (Fig. 5D, E). The cell apoptosis was decreased significantly compared with hypoxic group (Fig. 5F). Furthermore, shRNA silencing of CcO significantly attenuated this protection effect (Fig. 5B-F). These findings suggest that the anti-apoptosis effect of PI3K-AKT on hypoxia-induced apoptosis probably maintains the enzyme activities of CcO.

Discussion

In this study, we have shown that hypoxia activates and promotes the recruitment of pAKT to the mitochondria of cardiomyocytes. The mechanism of how pAKT is translocated to mitochondria in hypoxia was also examined, and we found that hypoxia promotes pAKT translocation to the mitochondria dependent on mitoKATP. In turn, mitochondrial pAKT resulted in increased activity of CcO and prevented hypoxia induced cardiomyocyte apoptosis. We revealed that the mechanism of PI3K-AKT pathway decreases apoptosis and enables cardiomyocytes' survival in hypoxia condition *in vivo*.

In severe burns, ischemia and hypoxia often lead to myocardial damage with apoptosis or necrosis. Two main cell signaling pathways are involved in the process of cardiomyocyte apoptosis: one contributes to apoptosis, such as the p38 kinase pathway. Inhibition of p38 MAP kinase improved survival of cardiac myocytes with hypoxia and in the burn serum challenge [17]. The TNF-α induces apoptosis by stimulation of TNFR1 with subsequent triggering of the caspase cascade and this may be an important contributing factor to cardiomyocyte apoptosis and myocardial dysfunction [18-20]. While the other protective signaling pathways prevent cells from apoptosis, a number of studies found that PI3K-AKT pathway plays an important role in preventing cell apoptosis. Recent studies have revealed that AKT has direct inhibitory effects on apoptotic Bcl-2 family proteins [21]. We also reported that the PI3K–AKT pathway was an activated response to severe burns in rats; however, the function of the activated PI3K–AKT pathway in hypoxic cardiomyocytes simulating burns remains unclear. We found that suppression of the PI3K–AKT pathway led to increased hypoxia induced apoptosis. In contrast, the cardiomyocyte pretreated with IGF-1 resulted in significantly decreased hypoxia induced apoptosis.

We further revealed how the PI3K–AKT pathway inhibits apoptosis. Gautam, et al. has reported that AKT can be recruited to mitochondria under hypoxia. This is the first report to show AKT can be located in mitochondria. Simulation of a cell with IGF-1 or heat shock stress, induced translocation of AKT to the mitochondria within only several minutes after the stimulation, and the mitochondrial AKT was in its phosphorylated and activated form [22]. Young, et al., a study which profiled the mitochondrial phosphoproteome of prostate adenocarcinoma PC3 cells exposed to severe hypoxia found that active AKT accumulates in the mitochondria under hypoxia [23]. When Mitochondrial ATP-sensitive potassium channel (mitoKATP), a common effector of protective stimuli in myocardial ischemia-reperfusion injury (MIRI), opens it plays a physiologic role in triggering cardiomyocytes' energy homeostasis during MIRI [15]. A study by Nauman, et al., also found that the cardioprotective effect of mitoKATP channels is attributed to the translocation of phosphorylated AKT from cytosol to mitochondria [24]. In this study, we found that pAKT translocate to the mitochondria dependent of mitoKATP and inhibit the mitoKATP with 5-HD preventing the accumulation of mitochondrial pAKT in hypoxia.

The findings showed that AKT translocates and accumulates in the mitochondria following its activation. This raised the question that what are the substrates phosphorylated by AKT in mitochondria. Inside mitochondria, phosphorylation of the β-subunit of ATP synthase, GSK3β mediated by AKT was previously reported, and two mitochondrial proteins were identified to be phosphorylated following stimulation of mitochondrial AKT, the beta-subunit of ATP synthase, and GSK3β [22]. To explore the biological function of mitochondrial translocation of pAKT under hypoxic condition, we examined the effect of the pAKT on CcO activity. CcO is a large transmembrane protein complex located on the inner mitochondrial

membrane and it plays a critical role in cellular bioenergetics as component four of the electron transport chain [14]. Previous studies found that the protein kinase C could protect the lens from mitochondria damage under hypoxia through the activation of CcO enzyme activity [25]. In order to further clarify the mechanisms by which the PI3K–AKT pathway inhibits apoptosis, the activity of CcO was evaluated in cultured cardiomyocytes. Our results showed that hypoxia decreased CcO activity and that this was aggravated by IGF-1 pretreatment. Inhibited CcO with siRNA weakened the protective effect of PI3K-AKT. These findings suggest that PI3K–AKT may play an anti-apoptotic role by maintaining CcO activation. We demonstrate, in this paper, that activation of PI3K-AKT and pAKT translocated to mitochondria by hypoxia result in the activation of CcO. This would further result in more efficient mitochondria and may provide a way for the cell to deal with hypoxia.

Conclusion

In conclusion, our results prove that PI3K-AKT pathway inhibits hypoxia induced apoptosis in cardiomyocytes by accumulation of mitochondria in an mitoKATP-dependent manner and anti-apoptosis by activating the oxidase activity of CcO in cardiomyocytes. Additionaly, inhibition of PI3K-AKT pathway, mitoKATP, or CcO expression may also improve mitochondria dysfunction and enhance cells' apoptosis. These findings revealed the molecular mechanisms that PI3K-AKT antiapoptosis effects in cardiomyocytes, and may provide potentially valuable therapeutic targets for the treatment of hypoxia-induced myocardial apoptosis caused by severe burns.

Acknowledgements

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Disclosure Statement

The authors have declared that no competing interests exist.

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