Lidocaine protects H9c2 cells from hypoxia-induced injury through regulation of the MAPK/ERK/NF-κB signaling pathway

HAIBIN JIN[1] and JIN YU[2]

[1]Department of Cardiology, Tianjin Hospital, Tianjin 300211; [2]Department of Anesthesiology, People's Liberation Army 951 Hospital, Korla, Xinjiang 841000, P.R. China

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Abstract. The aim of the present study was to investigate the effect of Lidocaine on hypoxia-induced injury in cardiomyoblasts whilst exploring the associated molecular mechanism. In the present study, hypoxia was induced in H9c2 cells to establish an *in vitro* model of myocardial infarction. The cells were treated with lidocaine (0.5, 1, 5, 10 mM) for 48 h under hypoxic conditions. Cell viability and apoptosis levels were determined by MTT assay and flow cytometry, and ELISA was used to measure the levels of inflammatory cytokines released. A creatine kinase isoenzyme/cardiac troponin I detection kit was used to show that lidocaine significantly reduced hypoxia-induced cardiac troponin 1 and creatine kinase-muscle/brain release in a dose-dependent manner. Mitochondrial viability staining suggested that lidocaine significantly enhanced mitochondrial viability under hypoxic conditions. Lidocaine also significantly reduced hypoxia-induced apoptosis and increased H9c2 viability in a dose-dependent manner. Additionally, under hypoxic conditions, lidocaine dose-dependently promoted Bcl-2 expression, while decreasing Bax and caspase-3 expression in H9c2 cells. ELISA and reverse transcription quantitative PCR were used to detect the levels of tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6 released by H9c2 cells. Results showed that lidocaine markedly reduced the hypoxia-induced expression levels of IL-1β, TNF-α and IL-6 in a dose-dependent manner. In addition, protein levels of phosphorylated (p)-ERK1/2 and NF-κB p-p65 were analyzed by western blotting, and results indicated that lidocaine significantly increased the protein levels of p-ERK1/2 and decreased the protein level of NF-κB p-p65 in a dose-dependent manner under hypoxic conditions. These data suggested that lidocaine might protect cardiomyoblasts from hypoxia-induced injury via activation of the mitogen activated protein kinase/ERK/NF-κB signaling pathway.

Correspondence to: Dr Jin Yu, Department of Anesthesiology, People's Liberation Army 951 Hospital, 7 Tiemenguan Road, Korla, Xinjiang 841000, P.R. China
E-mail: yujin190225@163.com

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Introduction

Myocardial infarction (MI) has a high rate of mortality and morbidity worldwide and due to the growth of an aging population the healthcare burden of MI is increasing (1). MI is characterized by inflammation and cardiomyocyte apoptosis, and can lead to left ventricular enlargement, thoracalgia, breathing difficulty, asthenia, fatigue, diaphoresis, cardiopalmus, heart failure and coma (2,3). Coronary artery occlusion is one of the main causes of MI. With the discovery of an increasing number of therapeutic approaches, long-term survival after MI is increasing worldwide (4). Experimental and clinical studies have shown that cardiomyocyte apoptosis following MI is caused by hypoxic injury, ischemia-reperfusion injury and oxidative stress. Mitigation of these processes is a feasible way to treat MI (5).

Cardiomyocyte dysfunction is regarded as a leading cause of cardiac abnormality and may be responsible for the high mortality of patients with heart failure (6). Evidence has shown that myocardial ischaemia-induced cardiomyocyte dysfunction may be a contributor to cardiac cell death (7). MI is the main cause of death in patients with cardiovascular disease (8). Myocardial damage in hypoxia leads to various chemical and physical changes, including scarring, inflammation, necrosis, cell apoptosis and cardiac remodeling (9). An increasing number of studies have highlighted the significance of cardiomyocyte apoptosis and inflammatory response in MI development, due to their role in regulating heart and cardiac muscle function (9,10). A previous study has shown that increased levels of inflammatory mediators in cardiac dysfunction, especially increased levels of tumor necrosis factor-α (TNF-α) in the locally infarcted myocardium, directly lead to cardiomyocyte apoptosis and myocardial dysfunction (11). Though signaling pathways, including inflammatory and calcium signaling, and reactive oxygen species (ROS) have been shown to play important roles in hypoxia-induced cardiomyocyte apoptosis, the precise molecular mechanisms underlying MI-induced cardiac damage remain unclear (12).

Lidocaine, also known as N-diethylaminoacetyl-2, 6-dimethylaniline, is one of the most commonly used local

anesthetics (13) and an anti-arrhythmic drug in a number of heart conditions (14,15). Lidocaine has a variety of pharmacological effects, including immunomodulation, anti-inflammatory, anti-oxidative effects and anti-tumor effects (16-21). One study assessing lidocaine toxicity showed that it can inhibit major cell signaling pathways, including AKT and ERK (22). Studies have indicated that lidocaine has a protective effect against cardiomyocyte injury (23,24). Lidocaine-induced blockade of voltage-gated sodium channels has been shown to rescue the function of ischemic myocardium *in vivo* (23) and has been confirmed to protect mice from myocardial damage due to ischemia-reperfusion injury (24). In particular, Okamoto *et al* (25) previously reported that hypoxia inducible factor 1α overexpression reduces lidocaine-induced renal cell-derived RCC4 cell and neuronal SH-SY5Y cell death. However, lidocaine has not been previously investigated as a potential treatment for hypoxia-induced cardiomyocyte cell death, and the protective mechanisms of lidocaine in this process remain unclear. Based on existing research data (23-25), it was hypothesized that lidocaine may have a protective effect against hypoxia-induced apoptosis and cardiac damage through the activation of the mitogen activated protein kinase (MAPK) /ERK/NF-κB signaling pathway. The purpose of this study was to explore this hypothesis and the underlying mechanisms of lidocaine action to provide the basis for developing new treatments for MI.

Materials and methods

Cell culture and hypoxia treatment. The rat cardiac myoblast cell line H9c2 was obtained from the American Type Culture Collection and they are used here as model for cardiac myocytes. H9c2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml of streptomycin (Sigma-Aldrich; Merck KGaA). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2. Culture medium was changed every 2 days.

To induce hypoxia, H9c2 cells ($5x10^4$/well) at 80% confluence were placed in a hypoxic chamber (Thermo Fisher Scientific, Inc.) containing 1% O_2, 5% CO_2 and 94% N_2 for 48 h. Cells were cultured with a range of lidocaine (Sigma-Aldrich, Merck KGaA; dissolved in sterile water) concentrations (0.5, 1, 5, 10 mM) for 48 h from the onset of hypoxia. The concentrations of lidocaine used were based on a previous study (26). Cells in the control group were incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Cellular injury assessment. H9c2 cells were treated with lidocaine (0.5, 1, 5, 10 mM) for 48 h under hypoxic conditions before a rat cardiac troponin (cTnI) ELISA kit (cat. no. CSB-E08594r; Cusabio Technology LLC) and a rat creatine kinase-muscle/brain (CK-MB) ELISA kit (cat. no. CSB-E14403r; Cusabio Technology LLC) were used to evaluate cTnI and CK-MB release into the cell culture medium. Data were presented as the fold change of the control group (6). During these experiments, the activity of mitochondria in the different treatment groups was measured using Mitochondrial Viability Stain (cat. no. ab129732; Abcam) according to manufacturer's protocol (6).

Cell viability assay. Cell viability was determined using the MTT assay (Beyotime Institute of Biotechnology). H9c2 cells were cultured in 96-well plates, and ~5,000 cells per well adhered to the culture dish wall. After incubation of cells with 0.5, 1, 5 and 10 mM lidocaine for 48 h under hypoxic conditions, MTT (5 mg/ml) was added to each well. The cells were cultured for a further 4 h before 100 µl of dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added per well. Finally, absorbance was measured at 490 nm using an automated micro-plate reader (BioTek Instruments, Inc.).

Quantitative analysis of apoptosis. Cell apoptosis was quantified using the FITC-Annexin V/PI detection kit (Beijing Biosea Biotechnology Co. Ltd.) according to manufacturer's protocols. Briefly, cells were treated with lidocaine (0.5, 1, 5, 10 mM) for 48 h under hypoxic conditions. A total of $1x10^5$ cells were collected from each sample and resuspended in 200 µl of binding buffer containing 10 µl of FITC-Annexin V. The samples were then incubated at room temperature for 30 min, and 300 µl of PBS and 5 µl of propidium idodide (PI) were added. The samples were immediately analyzed using a flow cytometer (Beckman Coulter, Inc.). FlowJo software (version 7.2.4; FlowJo LLC) was used to analyze the data and calculate levels of early and late stage apoptosis.

Western blot analysis. Total protein from H9c2 cells was isolated using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein concentration in whole cell extracts was quantified using a BCATM Protein Assay kit (Pierce, Thermo Fisher Scientific Inc.). Protein (0.1 mg) from each sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature and then probed with primary antibodies: Bcl-2 (cat no. ab196495; 1:1,000; Abcam), Bax (cat no. 14796; 1:1,000; Cell Signaling Technology, Inc.), caspase-3 (cat no. 14220; 1:1,000; Cell Signaling Technology, Inc.), NF-κB p65 (cat no. 8242; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p)-p65 (cat no. 3033; 1:1,000; Cell Signaling Technology, Inc.), ERK1/2 (cat no. 4695; 1:1,000; Cell Signaling Technology, Inc.), p-ERK1/2 (cat no. 4376; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat no. 4970; 1:1,000; Cell Signaling Technology, Inc.), at 4˚C overnight. The membranes were then incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (cat no. 7074; 1:2,000; Cell Signaling Technology, Inc.). Positive bands from each group of samples were visualized using enhanced chemiluminescent reagent (ECL Advance Western Blotting Detection Kit; GE Healthcare Life Sciences) (27). The intensity of each band was quantified using Image Lab™ Software (version 5.2.1; Bio-Rad Laboratories Inc.).

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was collected from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was generated using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) according to the manufacturer's protocol. The temperature protocol for the reverse transcription reaction consisted of primer annealing

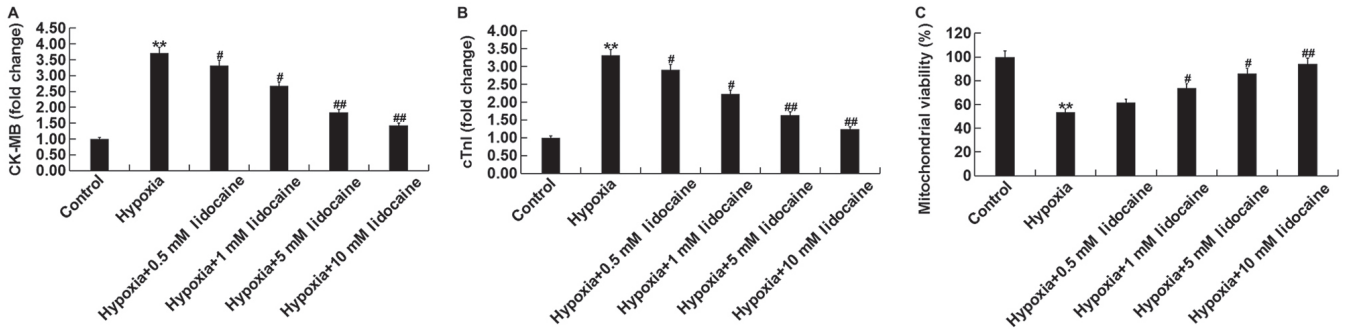

Figure 1. Effect of lidocaine treatment on H9c2 cell damage induced by hypoxia. The release of (A) CK-MB and (B) cTnI and (C) the mitochondrial survival rate were measured to determine the therapeutic effect of lidocaine on H9c2 cells under hypoxic conditions. The experimental data are presented as the mean ± SD of fold change compared to control and the data are from three independent experiments. **P<0.01 vs. Control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. Hypoxia. cTnI, cardiac troponin I; CK-MB, creatine kinase-muscle/brain.

at 25˚C for 5 min, cDNA synthesis at 42˚C for 60 min and termination at 80˚C for 2 min. FastStart Universal SYBR Green Master (Roche Diagnostics) was used to analyze cDNA levels. The thermocycling conditions were conducted as follows: Initial denaturation 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. The primer sequences used for qPCR were as follows: GAPDH forward, 5'-CTTTGGTATCGTGGAAGG ACTC-3' and reverse, 5'-GTAGAGGCAGGGATGATGTTC T-3'; IL-1β forward, 5'-TGTGAAATGCCACCTTTTGA-3' and reverse, 5'-TGAGTGATACTGCCTGCCTG-3'; TNF-α forward, 5'-GAACTGGCAGAAGAGGCACT-3' and reverse, 5'-GGTCTGGGCCATAGAACTGA-3' and IL-6 forward, 5'-CCGGAGAGGAGACTTCACAG-3' and reverse, 5'-CAG AATTGCCATTGCACA-3'. The target gene expression level was normalized to GAPDH. The $2^{-\Delta\Delta Cq}$ method (28) was used to determine relative gene expression.

ELISA for inflammatory cytokines. Cells were treated with lidocaine (0.5, 1, 5, 10 mM) for 48 h under hypoxic conditions and the supernatants collected by centrifugation (500 x g; 5 min; 4˚C) for the determination of levels of the inflammatory cytokines TNF-α (Rat TNF-α ELISA kit; cat. no. PT516), interleukin (IL)-6 (Rat IL-6 ELISA kit; cat. no. PI328) and IL-1β (Rat IL-1β ELISA kit; cat. no. PI303). All kits were obtained from Beyotime Institute of Biotechnology. ELISAs were performed according to the manufacturer's protocols and repeated three times.

Statistical analysis. The experimental results were expressed as the mean ± SD from three independent experiments. Experimental data were analyzed using SPSS 16.0 software (SPSS, Inc.). Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of lidocaine on hypoxia-induced cell damage. Rat cardiac myoblast cell line H9c2 was used in this study and they were used in the present study as a model for cardiac myocytes. The release of two biomarkers of cardiomyoblast injury, CK-MB and cTnI, was measured to assess the effect of lidocaine on hypoxia-induced cell injury. Fig. 1A and B shows that the expression of both biomarkers, CK-MB and cTnI, were significantly increased relative to the control group due to hypoxia. Lidocaine significantly reduced the hypoxia-induced expression levels of CK-MB and cTnI in a dose-dependent manner. In addition, the results showed that hypoxia treatment of H9c2 cells significantly reduced mitochondrial viability, and lidocaine treatment significantly increased mitochondrial viability under hypoxic conditions in a dose-dependent manner relative to the control group (Fig. 1C).

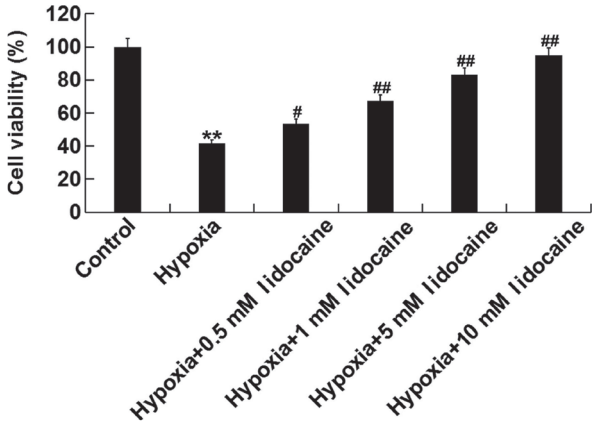

Figure 2. Effect of lidocaine on the viability of H9c2 cells under hypoxic conditions. H9c2 cell viability was examined by MTT assay. The experimental data are presented as the mean ± SD and are from three independent experiments. **P<0.01 vs. Control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. Hypoxia.

Effect of lidocaine on cell viability of hypoxic cardiomyoblasts. MTT assay was used to determine the effect of lidocaine on H9c2 cell viability under hypoxia. As shown in Fig. 2, H9c2 cell viability under hypoxia was significantly reduced relative to the control group, but lidocaine could significantly improve H9c2 cell viability under hypoxia in a dose-dependent manner.

Effect of lidocaine on hypoxia-induced apoptosis of cardiomyoblasts. A FITC-Annexin V/PI detection kit was used to study the effects of lidocaine on hypoxia-induced apoptosis. As shown in Fig. 3A and B, compared with the control group,

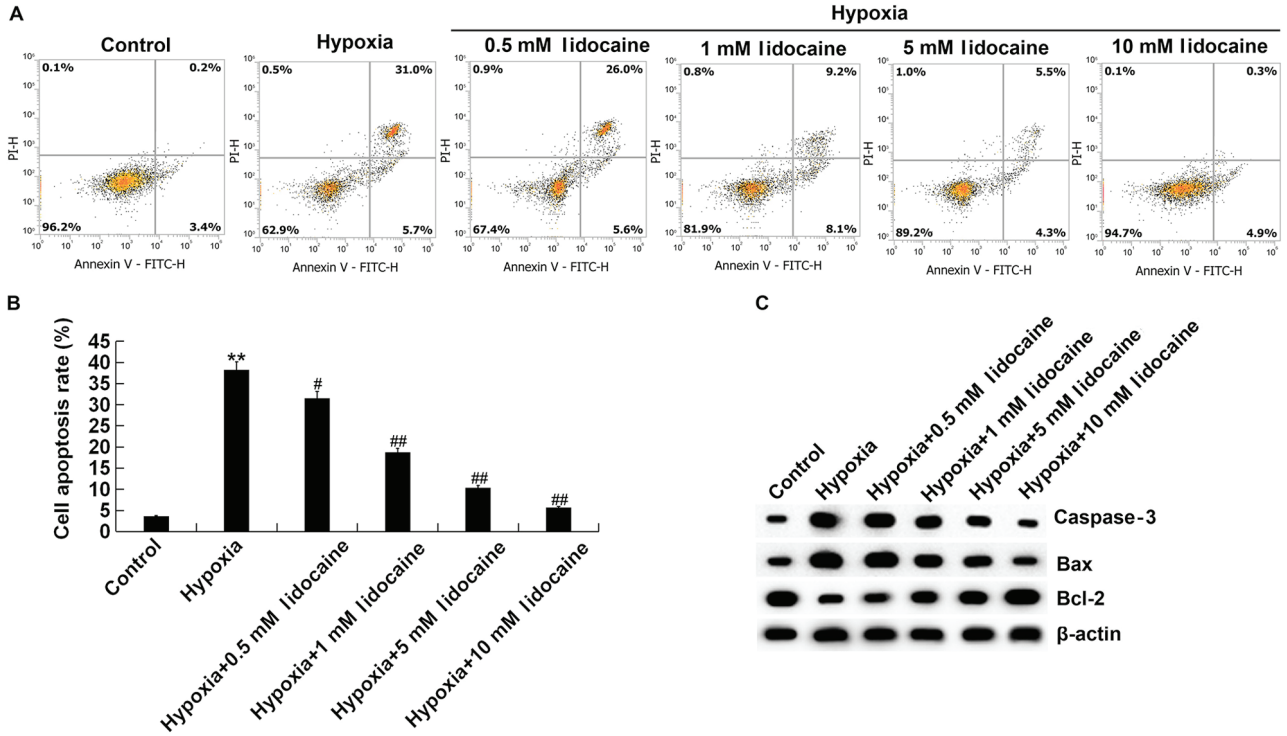

Figure 3. Effect of lidocaine on hypoxia-induced apoptosis of H9c2 cells. Cell apoptosis was measured using a FITC-Annexin V/PI detection kit. (A) Representative images of flow cytometry data and (B) apoptosis rates. (C) Representative images of western blotting analysis of Bax, Bcl-2 and caspase-3. **P<0.01 vs. Control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. Hypoxia.

hypoxia significantly induced H9c2 cell apoptosis, and lidocaine significantly decreased hypoxia-induced H9c2 cell apoptosis in a dose-dependent manner. To further determine the protective effect of lidocaine against hypoxia-induced H9c2 cell apoptosis, the expression levels of apoptosis-related proteins were determined experimentally. Western blotting showed that hypoxia-induction markedly increased Bax expression and decreased Bcl-2 expression relative to the control group, but lidocaine dose-dependently increased Bcl-2 and decreased Bax expression in the hypoxic cells. Western blotting was also used to detect the protein level of caspase-3 in H9c2 cells. Levels of caspase-3 were markedly increased in hypoxic cells, but were reduced in these cells after lidocaine treatment in a dose-dependent manner (Fig. 3C). In summary, the results showed that lidocaine inhibited hypoxia-induced cardiomyoblast apoptosis, indicating that the drug had anti-apoptotic effects.

Lidocaine treatment inhibits the hypoxia-induced inflammatory response in cardiomyoblasts. The therapeutic effect of lidocaine on hypoxic cardiac myocytes was determined by measuring the levels of inflammatory cytokines IL-1β, TNF-α and IL-6. The results of ELISA assay showed that the release of inflammatory cytokines increased significantly in H9c2 cells under hypoxic conditions when compared with control. Lidocaine treatment significantly decreased the level of inflammatory cytokines released by hypoxic H9c2 cells in a dose-dependent manner (Fig. 4A-C). Similar results were obtained from the RT-qPCR analysis of cytokine expression at the mRNA level (Fig. 4D-F). Together, these findings indicated that treatment with lidocaine inhibited the hypoxia-induced inflammatory response in cardiomyoblasts.

Effect of lidocaine on the MAPK/ERK/NF-κB signaling pathway in hypoxic cardiomyoblasts. The MAPK/ERK/NF-κB signaling pathway was investigated in order to demonstrate the potential protective mechanism of lidocaine against hypoxia-induced cardiomyoblast injury. Western blot analysis showed that hypoxia inhibited MAPK/ERK/NF-κB signaling in H9c2 cells, as the cellular p-ERK1/2/ERK1/2 ratio was downregulated, whilst the p-p65/p65 ratio was upregulated compared with the control. Hypoxia-induced changes in expression of these phosphorylated proteins were alleviated by lidocaine treatment (Fig. 5).

Discussion

MI is pathologically defined as myocardial cell death caused by prolonged ischemia (29). The two most prominent features that can enhance each other during MI-induced cardiac injury are the physiological defects of the ischemic tissue and the sustained inflammatory response, which ultimately lead to heart failure. One of the major complications of MI treatment is hypoxia-induced cardiomyocyte death (30). Previous studies have shown that protection of cardiomyocytes from hypoxia-induced injury could lead to a potential treatment for MI (31,32). The findings of the present study are consistent with previous studies (31,32). After 48 h of hypoxia H9c2 cell viability was significantly impaired and cell apoptosis was induced, indicating that hypoxia does indeed cause cell damage *in vitro*.

cTnI and CK-MB are two well-known bio-markers of myocardial cell damage (6,33). The present study found that after treatment with lidocaine, the levels of cTnI and

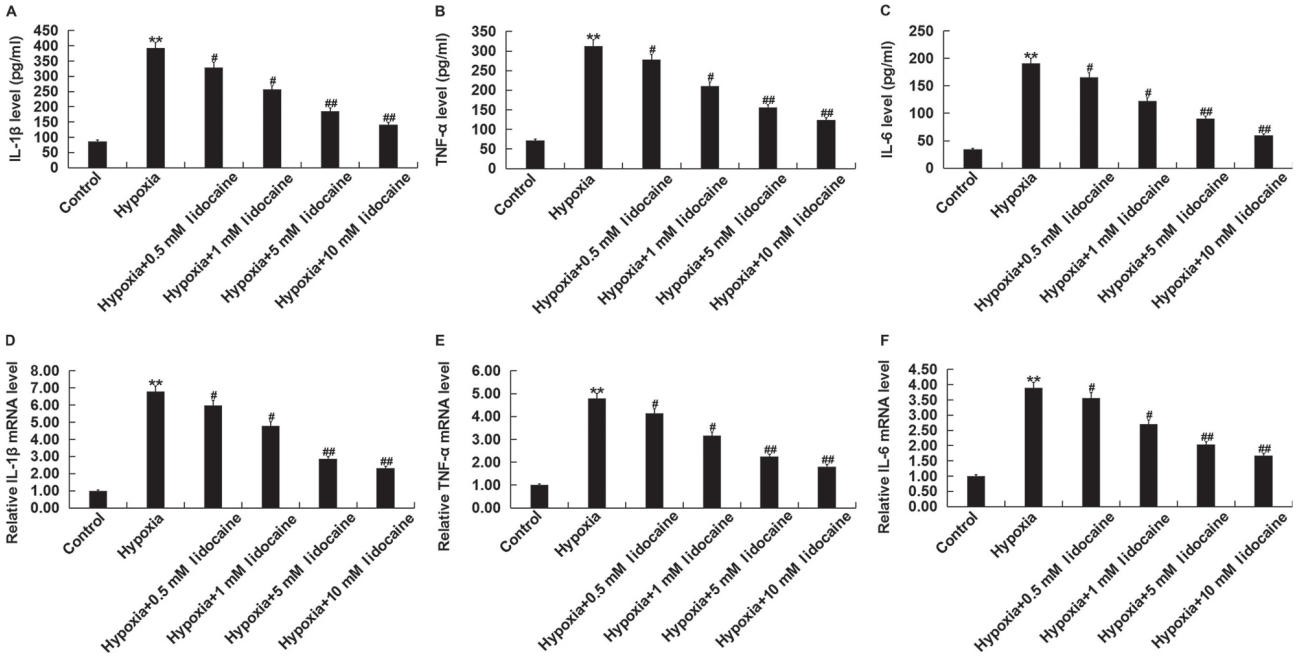

Figure 4. Effect of lidocaine on the hypoxia-induced inflammatory response of H9c2 cells. The concentrations of (A) IL-1β, (B) TNF-α and (C) IL-6 were measured using ELISA kits. The mRNA levels of (D) IL-1β, (E) TNF-α and (F) IL-6 were measured by reverse transcription-quantitative PCR. Data are presented as the mean ± SD. **P<0.01 vs. Control; $^{#}$P<0.05 and $^{##}$P<0.01 vs. Hypoxia. IL, interleukin; mRNA, microRNA; TNF-α, tumor necrosis factor-α.

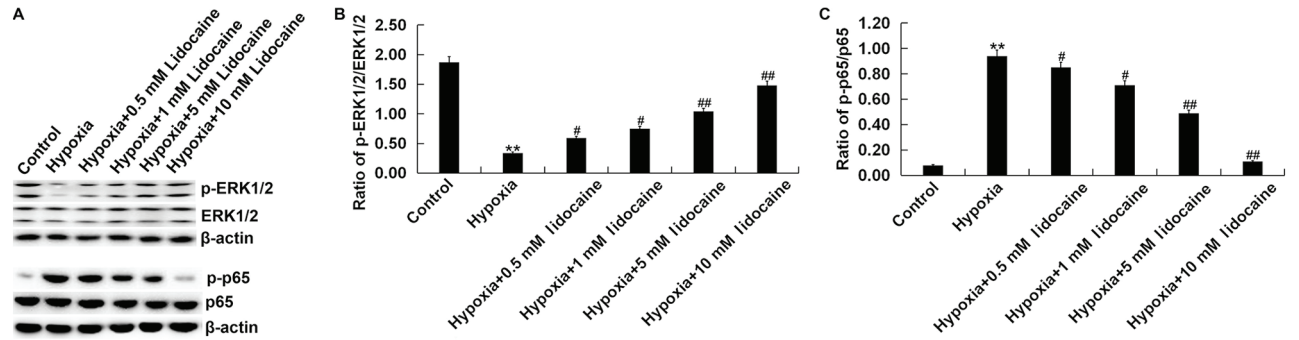

Figure 5. Effect of lidocaine on the MAPK/ERK/NF-κB signaling pathway in H9c2 cells under hypoxic conditions. (A) Protein levels of ERK1/2, p-ERK1/2, NF-κB p65 and NF-κB p-p65 were detected by western blotting after treatment with lidocaine for 48 h under hypoxic conditions. (B) Calculated and quantified presented the ratios of p-ERK1/2/ERK1/2 and (C) p-p65/p65. Data are presented as the mean ± SD. **P<0.01 vs. Control; $^{#}$P<0.05 and $^{##}$P<0.01 vs. Hypoxia. MAPK, mitogen activated protein kinase; p, phosphorylated.

CK-MB in hypoxia-treated cells were significantly reduced when compared with controls, indicating that treatment with lidocaine reduced myocardial cell damage. In addition, it was found in the present study that lidocaine promoted cell viability and inhibited apoptosis in a dose-dependent manner to protect H9c2 cells from damage.

Lidocaine is used in the treatment of acute myocardial infarction and other heart diseases complicated by rapid ventricular arrhythmia (34). To evaluate the molecular mechanisms underlying the potential benefits of lidocaine in MI, hypoxic H9c2 cells were used as a cellular model of MI. The findings of the present study indicated that a hypoxic environment significantly inhibited myocardial cell viability and induced apoptosis, which were reversed by lidocaine treatment. To further confirm the anti-apoptotic activity of lidocaine on hypoxia induced H9c2 cells, proteins associated with apoptosis, including Caspase 3, Bax and Bcl-2, were detected in the present study. The Bcl-2 family consists of a group of proteins which regulate apoptosis (35). In particular, increases in the Bax/Bcl-2 expression ratio has been reported to activate caspase-3 protease activity in cardiomyocytes, leading to programmed cell death (36). Consistent with the findings of Zhang *et al* (37) and Li *et al* (38), the present study demonstrated that hypoxia-induction markedly increased Bax and Caspase-3 expression whilst decreasing Bcl-2 expression compared with the control group. However, lidocaine dose-dependently increased Bcl-2 and decreased Bax and Caspase-3 expression in hypoxic H9c2 cells.

Accumulating evidence demonstrates that the inflammatory response serves critical roles in MI pathogenesis (5,39). Increased expression of a multiple endogenous inflammatory cytokines can lead to myocardial dysfunction. The findings of the present study were consistent with a previous study (9),

which indicated that hypoxia promotes an inflammatory response in cardiomyocytes due to increased secretion of the cytokines IL-6, IL-1β and TNF-α. The results of the present study suggested that lidocaine treatment might effectively reduce hypoxia-induced inflammation.

Myocardial ischemia and hypoxia activate several protein kinase pathways (40). Increased activation of the p38-MAPK, ERK1/2 and JNK pathways occurs during ischemia-reperfusion (41). Research has shown that during myocardial damage, pro-inflammatory cytokine levels are elevated in a manner that is dependent on NF-κB activation (42). It has been shown that, in treatment of the ischemic myocardium, activation of the MAPK/ERK/NF-κB pathway is essential in preventing cardiomyocyte death, which can be achieved by ischemic post-conditioning or administration of certain pharmacological agents (6,41,43). The results of the present study suggested that lidocaine activated the MAPK/ERK/NF-κB signaling pathway through a significant upregulation in ERK1/2 phosphorylation and a downregulation of p-p65 levels, and may protect cells against hypoxia-induced damage.

In summary, the present study highlighted the protective effect of lidocaine against hypoxia-induced damage to cardiomyoblasts and suggested a role for lidocaine treatment in MI. However, this study was only preliminary and further *in vitro* and *in vivo* study with a greater range of lidocaine concentrations is necessary to confirm these results.

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Availability of data and materials

All datasets used and/or generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HBJ contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. JY contributed to data collection and statistical analysis.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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