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Trimetazidine protects cardiomyocytes against hypoxia-induced injury through ameliorates calcium homeostasis

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ABSTRACT

Intracellular calcium (Ca_i^{2+}) overload induced by chronic hypoxia alters Ca_i^{2+} homeostasis, which plays an important role on mediating myocardial injury. We tested the hypothesis that treatment with trimetazidine (TMZ) would improve Ca_i^{2+} handling in hypoxic myocardial injury. Cardiomyocytes isolated from neonatal Sprague–Dawley rats were exposed to chronic hypoxia (1% O_2, 5% CO_2, 37 °C). Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with Fura-2/AM. Perfusion of cardiomyocytes with a high concentration of caffeine (10 mM) was carried out to verify the function of the cardiac Na^+/Ca^{2+} exchanger (NCX) and the activity of sarco(endo)-plasmic reticulum Ca^{2+}-ATPase (SERCA2a). For TMZ-treated cardiomyocytes exposured in hypoxia, we observed a decrease in mRNA expression of proapoptotic Bax, caspase-3 activation and enhanced expression of anti-apoptotic Bcl-2. The cardiomyocyte hypertrophy were also alleviated in hypoxic cardiomyocyte treated with TMZ. Moreover, we found that TMZ treatment cardiomyocytes enhanced "metabolic shift" from lipid oxidation to glucose oxidation. Compared with hypoxic cardiomyocyte, the diastolic $[Ca^{2+}]_i$ was decreased, the amplitude of Ca_i^{2+} oscillations and sarcoplasmic reticulum Ca^{2+} load were recovered, the activities of ryanodine receptor 2 (RyR2), NCX and SERCA2a were increased in cardiomyocytes treated with TMZ. TMZ attenuated abnormal changes of RyR2 and SERCA2a genes in hypoxic cardiomyocytes. In addition, cholinergic signaling are involved in hypoxic stress and the cardioprotective effects of TMZ. These results suggest that TMZ ameliorates Ca_i^{2+} homeostasis through switch of lipid to glucose metabolism, thereby producing the cardioprotective effect and reduction in hypoxic cardiomyocytes damage.

1. Introduction

Hypoxia is the natural consequence of some environments (e.g., high altitude, diving), it is also a common feature of many clinical diseases (e.g., sleep apnea syndrome, chronic obstructive pulmonary disease, heart failure, vascular diseases, stroke, sepsis, metabolic myopathies). Hypoxia is generally associated with cardiovascular diseases, and it elicits a variety of functional responses in cardiomyocytes, including cell proliferation [1], cell hypertrophy [2,3] and cell death [4]. Many cellular responses to hypoxia are mediated by Ca^{2+} signals [5,6]. Intracellular calcium (Ca_i^{2+}) plays a central role in regulating contractility, gene transcription, energy balance, hypertrophic growth, and apoptosis in the heart [7–10]. In many instances, one of the constant responses to hypoxia is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) via the activation of various plasma membrane Ca^{2+} conductances, such as voltage-gated calcium channels, ligand-operated Ca^{2+} channels, and non-specific cation channels [11]. Ca^{2+} homeostasis is very critical to cell survival and hypoxia can induce cell death by increasing Ca_i^{2+}. Cardiomyocyte Ca^{2+} loading is recognized as a major factor in acute hypoxia pathology, promoting cell death, contractile dysfunction and arrhythmogenic activity [5]. Dysregulation of Ca_i^{2+} homeostasis can lead not only to loss of normal physiological control mechanisms but also to pathological changes in cell growth. Thus, to clarify mechanism about how hypoxia affects on calcium homeostasis in cardiomyocytes is required.

Trimetazidine [TMZ; 1-(2, 3, 4-trimethhoxibenzyl)-piperazine dihydrochloride] is a well-known antianginal, has shown beneficial effects on ischemic injury, termed as a cellular anti-ischaemic agent [12]. Numerous experimental studies on myocardial ischemia demonstrated that TMZ inhibits the production of free radicals, protects against oxygen-free radical-induced toxicity, counteracts calcium overload, protects myocyte structure and function, reduces the area of necrosis, and increases cell resistance to hypoxic stress [13–15]. In addition, TMZ selectively inhibits the

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fatty acid beta-oxidation of the final enzyme: long chain 3-ketoacyl-coenzyme A thiolase (3-KAT), resulting in a reduction in fatty acid oxidation and an increase in glucose oxidation, directly improving myocardial energy metabolism in the heart [16]. Consequently, TMZ reduces intracellular acidosis and electrolyte abnormalities by optimizing the oxygen demand of mitochondria and by preventing the decrease in intracellular ATP levels [13]. It has also been demonstrated that TMZ exerts its protective effects partially through this mechanism [17]. Moreover, previous evidence showed that TMZ modulates free cytosolic Ca^{2+} concentration during hypoxia, and reduces basal cytosolic Ca^{2+} concentration during hypoxia in single skeletal myocytes and corrects disturbances of transmembrane ion exchange leading to Ca^{2+} overload in ischaemic cardiomyopathy [18]. It suggests that TMZ might have an effect on intracellular Ca^{2+} regulation under pathological conditions.

However, the effects of TMZ on Ca_i^{2+} handling and mechanisms involved in its cytoprotective effects during myocardial injury induced by hypoxia remain largely unknown. The present study examined the protective effect of TMZ and the mechanism underlying this protection against hypoxia-induced injury in neonatal rat cardiomyocytes.

2. Materials and methods

2.1. Animals

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Animal Administrative Committee of Xi'an Jiaotong University (Xi'an, Shaanxi, China). Animals were supplied by the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China).

2.2. Cell culture and hypoxia exposure

Neonatal ventricular myocytes were isolated from 1-day-old Sprague–Dawley rats as described previously [19]. In brief, neonatal rats were anesthetized with isoflurane (1.5–2.0% vol/vol in air) and disinfected with 75% ethanol and then decapitated under aseptic conditions. The heart was quickly removed and placed in ice-cold phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The ventricles were minced into approximately 1 mm^3 fragments and dissociated with 0.1% collagenase II (GIBCO, Grand Island, NY, USA) for 30 min at 37 °C. After the cells were precipitated by centrifugation (at room temperature, 50×*g* for 5 min) and the collagenase solution was removed, the pellet of cells was resuspended in Kraft-Brühe (KB) solution (composed of (in mM): KCl 85, K_2HPO_4 30, $MgSO_4$ 5, EGTA 1, Na_2ATP 5, pyruvate 5, creatine 5, taurine 20, and glucose 20; titrated to pH = 7.3 with KOH) at room temperature for 15 min. Cardiomyocytes were then plated on a 22 × 22 mm^2 glass coverslip (Fisher Scientific, Pittsburgh, PA, USA) and incubated in Dulbecco's modified Eagle medium (DMEM; Invitrogen Corporation, Grand Island, NY, USA) supplemented with 15% fetal calf serum (FCS; Hyclone, Logan, UT, USA) in 5% CO_2 at 37 °C for 24 h. The medium was replaced with a serum-free medium before the cells were exposured to hypoxia.

During hypoxia culture, cells were placed in a hypoxic (1% O_2, 5% CO_2, 37 °C) incubator (Galaxy oxygen control incubator, RS Biotech, Irvine, UK) for 12 h. Control (Normoxia group) cells were incubated for equivalent periods under normoxic conditions (21% O_2, 5%CO_2, 37 °C). TMZ treated cells (TMZ + Hypoxia group) were incubated with 100 μM TMZ for 1 h before hypoxic conditions.

2.3. Cardiomyocyte morphological analysis

Cardiomyocyte images were captured with a CCD (600ES-CU; Pixera, Los Gatos, CA, USA) camera fixed to an inverted microscope (Leica DMIRB, Leica Microsystems, Wetzlar, Germany). From each coverslip, 30 regular myocytes were selected at random and viewed at 200× magnification. Cardiomyocytes were outlined and the cell surface area (CSA) was measured with a Simple PCI software (High Performance Imaging Software, Compix, Cranberry, PA, USA).

2.4. Protein content

Cultured cardiomyocytes were treated with the three conditions for 12 h. The cells were washed with PBS and then treated with 10% trichloroacetic acid (Sigma, St. Louis, MO, USA) at 4 °C for 1 h to precipitate the protein. The precipitates were dissolved in 0.15 N NaOH. Thereafter, the protein content was determined using a BCA protein assay kit (Beyotime, Haimen, Jiangsu, China).

2.5. Cytotoxicity assay

Cell death was assessed by the trypan blue exclusion assay performed in each treated group. Trypan blue stain (Sigma, St. Louis, MO, USA) was prepared fresh as a 0.4% solution in 0.9% sodium chloride. The cells were washed in PBS twice, and then suspended in 0.25% trypsin (Sigma) for 5 min and centrifuged at 50×*g* for 5 min. Supernatants were removed and pellets were resuspended in 100 μL 0.4% trypan blue solution and incubated for 5 min at room temperature. Cells were microscopically counted in a hemocytometer, and the cell death rate was expressed as a percentage of the trypan blue-positive cells.

Cell viability was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) performed in each treated group. Briefly, purified neonatal rat cardiomyocytes were seeded in a 96-well cell culture plates at 10^4 cells per well and were exposed to TMZ and/or hypoxia. After treatment, 20 μL of the CellTiter 96® AQueous One Solution was added to each well containing 100 μL medium, and the cells were incubated at 37 °C for 3 h. The plates were then agitated on a plate shaker. The absorbance was measured at 490 nm using a microplate reader (Multiskan MK3, Thermo, Waltham, MA, USA). The relative number of viable cells was determined by comparison to untreated cells, in which viability was assumed to be 100%.

Cell proliferation was quantified using a DNA bromodeoxyuridine (BrdU) incorporation assay (Roche Applied Science, Mannheim, Germany). The amount of incorporated BrdU is a measure of the rate of DNA synthesis of the cells and thus indirectly of cell proliferation. Briefly, purified neonatal rat cardiomyocytes were seeded in a 96-well cell culture plates at 10^4 cells per well and were exposed to TMZ and/or hypoxia. After treatment, BrdU labeling solution was added to each well at a final concentration of 10 μM. At the end of the stimulation period, the cells were fixed (60 min.) and then incubated for 90 min. At room temperature, with 1/100 dilution of peroxidase-labeled anti-BrdU antibody. The wells were then washed three times and incubated for 5 min. At room temperature with substrate solution, and the luminescence was measured using a microplate reader (Multiskan MK3, Thermo, Waltham, MA, USA). Proliferating cells were