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Extracellular Cl^--free-induced cardioprotection against hypoxia/reoxygenation is associated with attenuation of mitochondrial permeability transition pore

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ABSTRACT

The isotonic substitution of extracellular chloride by gluconate (extracellular Cl^--free) has been demonstrated to elicit cardioprotection by attenuating ischaemia/reperfusion-induced elevation of intracellular chloride ion concentration ($[Cl^-]_i$). However, the downstream mechanism underlying the cardioprotective effect of extracellular Cl^--free is not fully established. Here, it was investigated whether extracellular Cl^--free attenuates mitochondrial dysfunction after hypoxia/reoxygenation (H/R) and whether mitochondrial permeability transition pore (mPTP) plays a key role in the extracellular Cl^--free cardioprotection. H9c2 cells were incubated with or without Cl^--free solution, in which Cl^- was replaced with equimolar gluconate, during H/R. The involvement of mPTP was determined with atractyloside (Atr), a specific mPTP opener. The results showed that extracellular Cl^--free attenuated H/R-induced the elevation of $[Cl^-]_i$, accompanied by increase of cell viability and reduction of lactate dehydrogenase release. Moreover, extracellular Cl^--free inhibited mPTP opening, and improved mitochondria function, as indicated by preserved mitochondrial membrane potential and respiratory chain complex activities, decreased mitochondrial reactive oxygen species generation, and increased ATP content. Intriguingly, pharmacologically opening of the mPTP with Atr attenuated all the protective effects caused by extracellular Cl^--free, including suppression of mPTP opening, maintenance of mitochondrial membrane potential, and subsequent improvement of mitochondrial function. These results indicated that extracellular Cl^--free protects mitochondria from H/R injury in H9c2 cells and inhibition of mPTP opening is a crucial step in mediating the cardioprotection of extracellular Cl^--free.

1. Introduction

Chloride ion (Cl^-) is the primary intracellular anion and partakes in a great diversity of cell and intracellular organelle functions, including regulation of intracellular pH, electrical activity, cell volume, apoptosis, and Ca^{2+} homeostasis, etc. [1,2]. It is now becoming clear that an increase of intracellular Cl^- concentration ($[Cl^-]_i$) serves a crucial role in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury [3–8]. Many studies showed that the increased $[Cl^-]_i$ activates the Cl^-–OH^- exchanger to increase intracellular concentration of OH^-, an important member of the reactive oxygen species (ROS) family, to induce oxidative stress [3,9]; Moreover, the increased $[Cl^-]_i$ also promotes the release of intracellular Ca^{2+} to elicit Ca^{2+} overload through the Cl^--increase induced Ca^{2+} release from intracellular stores [3,9,10]. Therefore, the application of Cl^--substitution and Cl^--blockers to prevent I/R injury may be a promising strategic devisal. Indeed, it was reported that the substitution of extracellular Cl^- with equimolar NO_3^- prevented ischemia and reperfusion-induced ventricular fibrillation and promoted recovery of the contractive force in rat Langendorff hearts [11–13]. Additionally, some studies also showed that Cl^--free solution, in which Cl^- was replaced with equimolar gluconate, inhibited the hypoxia/reoxygenation (H/R)-induced $[Ca^{2+}]_i$-increase and elicited cardioprotection by suppressing the elevation of $[Cl^-]_i$ in adult rat ventricular myocytes [6].

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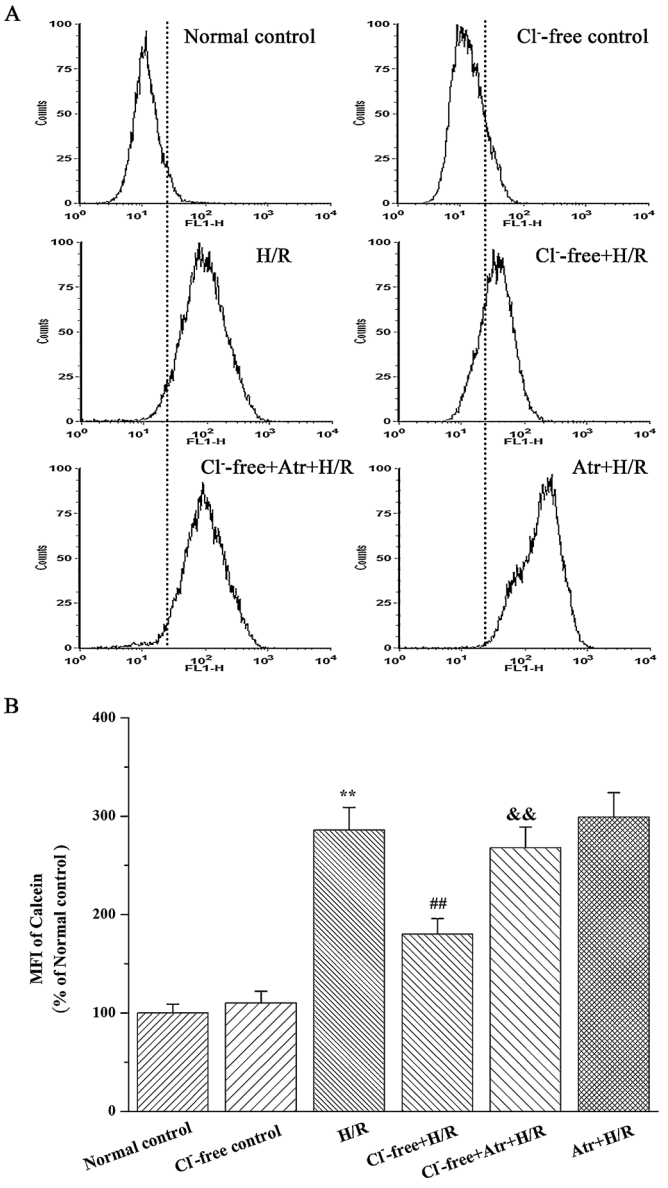

Fig. 1. Effect of extracellular Cl^--free on mPTP opening in H9c2 cells subjected to hypoxia/reoxygenation (H/R). The mPTP opening was measured directly by a combined use of Calcein-AM and $CoCl_2$, as described in Materials and methods section. (A) Representative flow cytometric histograms of Calcein fluorescence in different treatment cells. (B) Column bar graph of mean fluorescence intensity (MFI) for Calcein evaluated for different treatment cells. Values obtained from four independent experiments are expressed as mean ± SEM. $^{**}P < 0.01$ vs. Normal control group; $^{\#\#}P < 0.01$ vs. H/R group; $^{\&\&}P < 0.01$ vs. Cl^--free + H/R group.

However, the downstream mechanism underlying the cardioprotection effect of extracellular Cl^--free condition remains to be determined.

Substantial evidence revealed that the critical mechanisms of cardioprotection converge on the mitochondria [14] and the mitochondrial permeability transition pore (mPTP) seems to be an end-effector of cardioprotection against I/R injury [15–17]. The mPTP is a non-selective pore that penetrates the inner and outer mitochondrial membranes and closely associated with mitochondrial function. Under physiological conditions the mPTP is either not present or mostly closed, but opens under conditions associated with postischaemic reperfusion, such as ROS accumulation, increases in $[Ca^{2+}]_i$, and a reduction of the inner membrane potential [18–20]. The massive opening of mPTP results in mitochondrial swelling, collapse of mitochondrial membrane potential, and resultant uncoupling of mitochondrial oxidative phosphorylation, which ultimately leads to ATP depletion and cell apoptosis and necrosis [18,19]. It is demonstrated that the direct or indirect inhibition of mPTP opening at this time not only provides a protective strategy against reperfusion injury but also is a key convergence point of cardioprotective mechanisms [15,21,22].

The regulation of mPTP opening is a complex process wherein several critical factors are involved, including ROS, Ca^{2+}, inorganic phosphate, and ADP, etc. [20]. Interestingly, Huang's group recently reported that the increased $[Cl^-]_i$ can cause the mPTP opening, which results in ROS burst and subsequent oxidative stress in human umbilical vein endothelial cells [23]. It implies that the increased $[Cl^-]_i$ could also be a prominent factor on mPTP opening and suppressing the elevation of $[Cl^-]_i$ could display the inhibitory effect on mPTP opening.

Therefore, considering that extracellular Cl^--free condition inhibits H/R-induced elevation of $[Cl^-]_i$, and that inhibition of the increased $[Cl^-]_i$ could block the mPTP opening, it was accordingly speculated that extracellular Cl^--free induces cardioprotection probably by preventing mPTP opening via inhibition of H/R-induced elevation of $[Cl^-]_i$ and subsequent improving mitochondrial function. The present investigation was undertaken to elucidate the possibility in H9c2 cells under both control and H/R conditions.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibcol (Grand Island, NY, USA). Lactate dehydrogenase (LDH) assay kit and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Atractyloside (Atr) and Rhodamine 123 (Rh123) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MitoSOX™ Red Mitochondrial Superoxide Indicator and *N*-ethoxycarbonyl-methyl-6-methoxyquinolinium bromide (MQAE) were from Invitrogen (Carlsbad, CA, USA). Other chemicals were all from Sigma Chemical Co., unless otherwise stated.

2.2. Cell culture

The rat embryonic heart-derived H9c2 cell line (Catalog No. CRL-1446) was purchased from American Type Culture Collection and cultured in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4.5 mM L-glutamine. The H9c2 cells were incubated in a humidified CO_2 incubator (Sanyo, Japan) at 37 °C with 5% CO_2. The medium was replaced every other day. H9c2 cells were allowed to grow to 60–80% confluence within 24 h prior to experiment.

2.3. H/R injury model

The model of H/R injury used in the present study performed as previously described by Xu et al. [24]. Briefly, H9c2 cells were initially incubated for 30 min with normal Tyrode solution (pH 7.4 at 37 °C), which contained (in mM) 125 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 25 HEPES, and 25 glucose. Subsequently, the cells were incubated in glucose-free Tyrode solution (pH 6.8, equilibrated with a gas mixture of 95% N_2 and 5% CO_2 for 15 min) and placed into a hypoxia chamber (Stemcell Technologies, Vancouver, Canada). To induce complete anoxia, the chamber