Injury, Int. J. Care Injured 46 (2015) 233–239

Contents lists available at ScienceDirect

Injury

journal homepage: www.elsevier.com/locate/injury

Hypothermia treatment preserves mitochondrial integrity and viability of cardiomyocytes after ischaemic reperfusion injury

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ARTICLE INFO

Article history:
Accepted 22 October 2014

Keywords:
Hypothermia
Cardiomyocyte
Ischaemia reperfusion
Mitochondria

ABSTRACT

Background: Haemorrhagic shock after traumatic injury carries a high mortality. Therapeutic hypothermia has been widely used in critical illness to improve the outcome in haemorrhagic shock by activation of cardiac pro-survival signalling pathways. However, the role played by the mitochondria in the cardioprotective effects of therapeutic hypothermia remains unclear. We investigated the effects of therapeutic hypothermia on mitochondrial function and integrity after haemorrhagic shock using an in vitro ischaemia-reperfusion model.

Methods: H9c2 cardiomyocytes received a simulated ischaemic reperfusion injury under normothermic (37 °C) and hypothermic (31 °C) conditions. The cardiomyocytes were treated with hypoxic condition for 18 h in serum-free, glucose-free culture medium at pH 6.9 and then shifted to re-oxygenation status for 6 h in serum-containing cell culture medium at pH 7.4. Cellular survival, mitochondrial integrity, energy metabolism and calcium homeostasis were studied.

Results: Hypothermia treatment lessened cell death (15.0 ± 12.7 vs. $31.9 \pm 11.8\%$, $P = 0.025$) and preserved mitochondrial number (81.3 ± 17.4 vs. 45.2 ± 6.6, $P = 0.03$) against simulated ischaemic reperfusion injury. Hypothermia treatment ameliorated calcium overload in the intracellular (1.5 ± 0.2 vs. 9.5 ± 2.8, $P < 0.001$) and intra-mitochondrial (1.0 ± 0.3 vs. 1.6 ± 0.3, $P = 0.014$) compartments against the injury. Mitochondrial integrity was more preserved by hypothermia treatment (50.1 ± 26.6 vs. $14.8 \pm 13.0\%$, $P < 0.01$) after the injury. Mitochondrial ATP concentrations were maintained with hypothermia treatment after injury (16.7 ± 9.5 vs. 6.1 ± 5.1 μM, $P < 0.01$).

Conclusions: Hypothermia treatment at 31 °C can ameliorate cardiomyocyte damage caused by simulated ischaemic reperfusion injuries. Mitochondrial calcium homeostasis, energy metabolism, and membrane integrity are preserved and play critical roles during therapeutic hypothermia treatment.

Introduction

Haemorrhagic shock results in death in over two-thirds of cases involving traumatic injury. As perfusion of vital organs is severely compromised in haemorrhagic shock, prevention of haemorrhagic shock is essential in order to maintain vital organ function [1,2]. Metabolic derangement and cardiac dysfunction also lead to worsening perfusion of vital organs [2]. Thus, adequate haemodynamic support with meticulous control of blood pressure can improve the outcome in haemorrhagic shock [3].

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http://dx.doi.org/10.1016/j.injury.2014.10.055
0020–1383/

Therapeutic hypothermia has been successful in treating critical illness including sudden cardiac arrest and neonatal hypoxic encephalopathy [4]. Preclinical studies have shown that survival outcome can also be improved by therapeutic hypothermia intervention in cases of haemorrhagic shock. Cardiac performance and haemodynamic parameters have also shown improvement using hypothermia treatment [5,6]. However, in clinical practice, there exists an association between coagulopathy and spontaneous hypothermia with its potential bleeding complications [7]. It may detrimental if hypothermia is too severe or too long in duration. More studies are needed to explore the mechanism underlying the protective effect of hypothermia with the goal to develop interventions which mimic its beneficial effects while minimizing the side effects associated with hypothermia treatment.

Cardiac function is depressed during haemorrhagic shock because of poor tissue perfusion and ischaemia within cardiomyocytes [8,9]. Hypothermia can decrease oxygen consumption, and functional damage in vital organs [6]. Cardiomyocyte necrosis and apoptosis are ameliorated by employing therapeutic hypothermia after ischaemic reperfusion injury in haemorrhagic shock. Therapeutic hypothermia suppresses expression of cardiac inflammatory cytokines and activates protective Akt pathway in haemorrhagic shock [10,11]. Oxidative stress to the mitochondria is decreased under hypothermia treatment in cardiomyocyte in vitro study [12]. Hypothermia treatment preserves the pro-survival mitochondria protein genes expression after ischaemic reperfusion injuries [13]. However, the mechanism and effect on mitochondria integrity and energy production under hypothermia treatment remains unclear.

Ischaemic reperfusion injuries cause myocardial damage including myocardial stunning, apoptosis and necrosis [14]. In addition to the activation of pro-inflammatory cytokines, intracellular calcium overload induces the opening of mitochondrial permeability transition pores (PTP) and release of pro-apoptotic factors in ischaemia reperfusion injury [15]. Apoptosis is triggered by mitochondria dysfunction and cytochrome C release from the damaged mitochondria membrane. The release of cytochrome C from mitochondria induces the activation of caspase pathways which are critical for progression of apoptosis. The opening of mitochondrial membrane PTPs also collapses the mitochondria membrane potential and causes uncoupling of oxidative–phosphorylation, ATP depletion and cell death [16]. Maintaining the mitochondria integrity by inhibition of opening of mitochondria PTP can ameliorate cell survival against the progression of apoptosis [17]. Whether therapeutic hypothermia targets maintenance of mitochondrial integrity that prevents cell injury and death after ischaemic reperfusion injuries remains unclear. We developed an in vitro cardiomyocyte culture model to mimic ischaemic reperfusion injury after haemorrhagic shock [18]. Our goal was to investigate the effects of therapeutic hypothermia on mitochondrial integrity and function.

Methods

Study design and protocol

The H9c2 embryonic rat heart-derived cell line was obtained from the American Type Culture Collection (CRL1446, Manassas, VA, USA) and was cultured in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Inc., CA, USA) supplemented with 10% heat-inactivated foetal calf serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. H9c2 cells were sub-cultured when they reached 70% confluence at a ratio of 1:4. For each set of experimental conditions, individual experiments (1×10^6 H9c2 cells per experiment) were repeated six times to ensure reproducibility ($n = 6$ per group or experimental condition).

Simulated ischaemic reperfusion injury

To simulate ischaemic reperfusion injury, hypoxia was achieved by modification of the culture environment using an anaerobic jar (AnaeroPack Series, Mitsubishi Gas Chemical Co, Inc.) equipped with an AnaeroPack, disposable O_2-absorbing and CO_2-generating agent, and an indicator to monitor oxygen depletion as described in a previous study [18].

The AnaeroPack jar is capable of depleting the concentration of O_2 down to less than 0.1% in 2 h and of providing a 21% CO_2 atmosphere. By placing flasks containing serum-free, glucose-free medium at a pH of 6.9 in an AnaeroPack jar overnight, the medium was balanced with the hypoxic atmosphere. Cultured cardiac myocytes were subjected to hypoxic conditions by immediate replacement of the medium with the hypoxic medium in the AnaeroPack jar. To maintain hypoxic conditions, all of the procedures were performed in an airtight chamber filled with 95% N_2/5% CO_2.

After incubation under hypoxic conditions, the cells were re-oxygenated by immediate replacement of the hypoxic medium with a normoxic medium to simulate reperfusion. To achieve significant H9c2 cell damage after simulated ischaemic reperfusion in the experiment, the cells were treated with different protocols by adjusting hypoxia and oxygenation durations. More than 50% of the H9c2 cells were damaged after being incubated in hypoxic conditions for 18 h and in re-oxygenated conditions for 6 h as determined by 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake assays.

Experimental hypothermia treatment protocols

The H9c2 cells were incubated at 37 °C before each experiment. The incubator temperature was set and controlled at 37 °C during the entire experiment for the normothermic groups. For the hypothermic groups, the cells were placed in a 31 °C incubator after starting the hypoxic treatment and maintained at that temperature throughout the experiment. In addition, the cell culture mediums were pre-incubated at 37 °C or 31 °C overnight before adding the medium to the cells.

Detection of apoptosis by flow cytometry analysis

Apoptotic cells were detected by both propidium iodide (PI, 50 mg/ml with DNase-free RNase, 200 μg/ml) and annexin-V (2 mg/ml) labelling after treatment with simulated ischaemia reperfusion as described previously [19]. Double labelling was performed and cell staining was analysed with the FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin-V is a protein that binds to phosphatidylserine residues that are exposed on the surface of apoptotic cells, but not on normal cells. A change in the plasma membrane is one of the most important morphological features of apoptosis. When nuclear membranes are disrupted (which is a sign of advanced (late) apoptosis or necrosis, but not early apoptosis) PI enters the nuclei and demonstrates positive staining. The partial loss of membrane integrity or functionality is a useful criterion for distinguishing apoptotic from necrotic and/or living cells.

Measurement of mitochondria mass

The fluorescent dye 1:1 10-N-nonyl acridine orange (NAO, Molecular Probes, Eugene, OR, USA), which binds specifically to cardiolipin on the inner mitochondrial membrane independently