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Role of superoxide ion formation in hypothermia/rewarming induced contractile dysfunction in cardiomyocytes

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ABSTRACT

Rewarming following accidental hypothermia is associated with circulatory collapse due primarily to impaired cardiac contractile (systolic) function. Previously, we found that reduced myofilament Ca^{2+} sensitivity underlies hypothermia/rewarming (H/R)-induced cardiac contractile dysfunction. This reduced Ca^{2+} sensitivity is associated with troponin I (cTnI) phosphorylation. We hypothesize that H/R induces reactive oxygen species (ROS) formation in cardiomyocytes, which leads to cTnI phosphorylation and reduced myofilament Ca^{2+} sensitivity. To test this hypothesis, we exposed isolated rat cardiomyocytes to a 2-h period of severe hypothermia (15 °C) followed by rewarming (35 °C) with and without antioxidant (TEMPOL) treatment. Simultaneous measurements of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyto}$) and contractile (sarcomere shortening) responses indicated that H/R-induced contractile dysfunction and reduced Ca^{2+} sensitivity was prevented in cardiomyocytes treated with TEMPOL. In addition, TEMPOL treatment blunted H/R-induced cTnI phosphorylation. These results support our overall hypothesis and suggest that H/R disrupts excitation-contraction coupling of the myocardium through a cascade of event triggered by excessive ROS formation during hypothermia. Antioxidant treatment may improve successful rescue of accidental hypothermia victims.

1. Introduction

Guidelines for rescue of accidental hypothermia victims have not been standardized due to an incomplete understanding of the key physiological responses to hypothermia and rewarming (H/R) [6]. Clinical evidence indicates high mortality of accidental hypothermia victims related to “rewarming shock”, defined as circulatory failure occurring during rewarming [26]. Experimental models of H/R demonstrate that rewarming shock is associated with insufficient cardiac contractile function during systole attributed by reduced myofilament Ca^{2+} sensitivity rather than altered $[Ca^{2+}]_{cyto}$ levels *per se* [16,36].

In rat papillary muscle [16] and isolated cardiomyocytes [36], Ca^{2+} sensitivity of force generation is reduced following H/R, and is associated with increased phosphorylation of cardiac troponin I (cTnI). In other models of cardiac contractile failure, excessive formation of reactive oxygen species (ROS) has been suggested to play an important role in reducing Ca^{2+} sensitivity [32,40,44]. Although there is *in vitro* evidence of hypothermia-induced ROS formation in a variety of mammalian cells [1,8,33], it is unknown whether increased ROS formation plays a role in the H/R-induced reduction in Ca^{2+} sensitivity and contractile dysfunction in cardiomyocytes.

We hypothesize that H/R induces excessive ROS formation in cardiomyocytes, which underlies cTnI phosphorylation and reduced Ca^{2+} sensitivity. To test this hypothesis, we measured ROS formation in isolated ventricular cardiomyocytes during H/R. We also determined whether treatment of cardiomyocytes with an antioxidant, TEMPOL, mitigates H/R-induced changes in cardiomyocyte contraction, cTnI phosphorylation and Ca^{2+} sensitivity.

2. Materials and methods

2.1. Animals

The use and handling of rats for this study was in accordance with the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). A total of 24 male Sprague-Dawley rats (250–350 g) were used in this study. These rats were equally assigned to 4 experimental conditions: 1) control (CTL), 2) CTL + TEMPOL, 3) hypothermia/rewarming (H/R),

Abbreviations: $[Ca^{2+}]_{cyto}$, cytosolic Ca^{2+} concentration; CTL, time-matched controls; cTnC, cardiac troponin C; cTnI, cardiac troponin I; DHE, dihydroethidium; ETC, mitochondrial electron transport chain; H/R, hypothermia/rewarming; H/R + TEMPOL, hypothermia/rewarming with TEMPOL treatment; HPLC, high-performance liquid chromatography; PDE3, phosphodiesterase 3; ROS, reactive oxidant species; RyR, ryanodine receptors; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; UCP, mitochondrial uncoupling protein

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and 4) H/R + TEMPOL. The animals were anesthetized by intramuscular injection of 90 mg/kg ketamine with 10 mg/kg xylazine, and the hearts were excised.

2.2. Cardiomyocyte isolation

The method for isolating cardiomyocytes has been described previously [36]. Briefly, the freshly-excised heart was cannulated via the aorta, connected to a modified Langendorff apparatus and perfused with warmed (37 °C) enzyme solution containing collagenase type II (0.6 mg/ml, Worthington) to isolate cardiomyocytes from the myocardium. Isolated cardiomyocytes were washed by resuspending cells following centrifugation. Finally, cardiomyocytes were resuspended in a M199 culture media containing 5% fetal bovine serum (FBS) and incubated at 35 °C for 30 min before evoking contractions.

With this isolation technique, most of freshly-isolated cardiomyocytes maintained a straight, rod-shaped morphology with clear sarcomere patterns throughout the cell. Only rod-shaped cells with a clear contractile response to stimulation (see below) were included in this study. Using this technique, robust cardiomyocyte contractile responses can be maintained for up to 5 h at 100% survival during continuous pacing at 0.5 Hz.

2.3. Hypothermia/rewarming protocol and contractile and $[Ca^{2+}]_{cyto}$ measurements

After cardiomyocyte isolation was complete, cells were placed in a glass coverslip based cell chamber. The cell chamber allowed for continuous perfusion of cells with oxygenated Tyrode solution (95% O_2 and 5% CO_2). A pair of platinum electrodes were attached to the cell chamber and stimulated at 0.5 Hz using the MyoPacer (IonOptix) to evoke contractile responses over time. Electrical pacing during continuous solution perfusion was done for 30 min before measurement to allow sufficient time for stabilization of contractile responses.

The cell chamber and perfusing solutions were surrounded by water flowing through a precision digital circulating water bath. Using a feedback circuit, the temperature of the perfusion solution was either maintained at 35 °C (time-matched control) or in the H/R group changed as follows (illustrated in Fig. 1): cooled from 35 °C to 15 °C over a 30-min period, then maintained at 15 °C for 2 h, followed by rewarming to 35 °C over a 30-min period (overall experiment completed in 3 h).

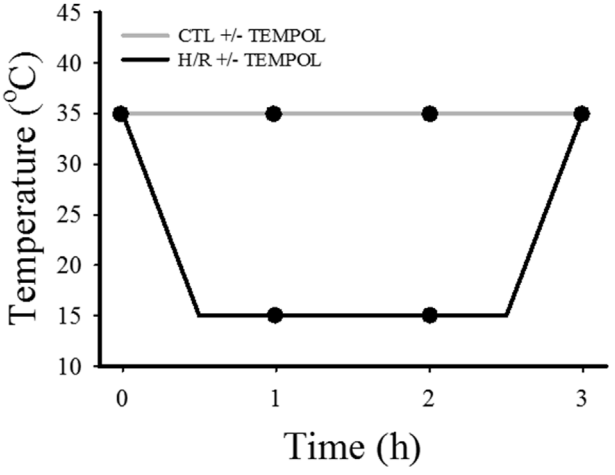

Fig. 1. Temperature over time of H/R and CTL groups. As shown, After an initial measurement (time 0), the CTL group was maintained at a constant temperature of 35 °C (± TEMPOL). On the other hand, after an initial measurement, the H/R group was cooled from 35 °C to 15 °C within 0.5 h, maintained at 15 °C for 2 h, and finally rewarmed from 15 °C to 35 °C within 0.5 h (± TEMPOL). Evoked $[Ca^{2+}]_{cyto}$ and contractile responses in cardiomyocytes in both CTL and H/R groups were measured at 0, 1, 2, and 3 h (solid circles).

In the TEMPOL-treated group, 200 μM of TEMPOL (4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) was added to the perfusion solution at the onset of hypothermia and treatment (or time CTL) and continued throughout the remaining protocol. TEMPOL is a widely used superoxide dismutase (SOD) mimetic and a pleiotropic intracellular antioxidant. Importantly, TEMPOL is cell membrane permeable. We tested the effects of TEMPOL in time-matched control myocytes, and if anything, TEMPOL stabilized contractility over the 3-h time period of the experiment. This is likely due to the production of ROS over time, which may account for some rundown of the preparation.

In isolated cardiomyocytes, evoked $[Ca^{2+}]_{cyto}$ and contractile responses were measured simultaneously using an IonOptix system, as previously described [16,36]. Briefly, cardiomyocytes were incubated with 0.5 μM Fura-2 AM for 10 min at 35 °C. Fura-2 fluorescence was excited using alternate wavelengths of 340 and 380 nm and fluorescence emission was measured at 510 nm. Contractility was assessed by measuring sarcomere length based on fast Fourier analysis of sarcomeric striation pattern (IonOptix). Cardiomyocytes were stimulated using electrical field stimulation (5 ms pulse width, 0.5 Hz), Only cardiomyocytes with robust $[Ca^{2+}]_{cyto}$ (peak > 500 nM) and contractile responses (> 5% shortening from resting sarcomere length) were included for further analysis. Approximately 75% of isolated cardiomyocytes met these inclusion criteria.

2.4. Measurements of intracellular superoxide anions

Two complementary techniques were used to determine ROS generation in cardiomyocytes following H/R exposure. HPLC-based measurement of oxy-ethidium was performed as described in Refs. [10,15] with minor modification for use in isolated cardiomyocytes. Briefly, the accumulation of oxy-ethidium is used to indicate the conversion of dihydroethidium (DHE) by superoxide anions. Using fluorescence detection, oxy-ethidium is quantified as the area under the curve at a unique retention time distinct from other fluorescent products such as ethidium [51]. Cells were incubated in phosphate based saline (PBS) solution containing 10 μM of dihydroethidium (Molecular Probes) at 37 °C for 15 min. The cells were washed to remove free dihydroethidium in Krebs-HEPES buffer for 1 h at 37 °C. After the wash, the cells were centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was eluted and analyzed to quantify oxy-ethidium from the reaction of DHE and superoxide anions using high-performance liquid chromatography (HPLC)/fluorescence assay [10]. The pellets in 2% SDS were saved for protein assay (Bio-Rad DC protein assay). Intracellular superoxide anion levels were determined by normalizing the quantity of oxy-ethidium for protein concentration.

Changes in fluorescence of MitoSOX Red (Invitrogen) was used as a second technique for ROS detection in individual cardiomyocytes. Using this technique, cardiomyocytes were incubated with 5 μM MitoSOX for 10 min at 35 °C, and subsequently imaged using a Nikon A1R confocal system equipped with a Plan-Apo 60×/1.4 numerical aperture oil objective. MitoSox Red was excited at 488 nm and resulting emission was measured at 590 nm. MitoSox Red is targeted to the mitochondria and fluoresces after reaction with superoxide anions.

2.5. Western blot

Cardiomyocytes samples were lysed and protein extracted in radioimmunoprecipitation assay (RIPA) buffer supplemented with 2% sodium dodecyl sulfate (SDS) and phenylmethane sulfonyl fluoride (PMSF). Protein lysates were collected by centrifuging at 10,000 g for 15 min at 4 °C. To prepare the sample for Western blot, total protein content was measured using a Lowry assay (Bio-Rad DC protein assay) in order to achieve equal loading of protein sample during SDS-PAGE. Samples were denatured by boiling at 100 °C for 3 min. Using SDS-PAGE, protein samples were fractionated over a gel and then