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Reactive oxygen species inhibit hyposmotic stress-dependent volume regulation in cultured rat cardiomyocytes

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Abstract

Cells have developed compensatory mechanisms to restore cell volume, and the ability to resist osmotic swelling or shrinkage parallels their resistance to necrosis or apoptosis. There are several mechanisms by which cells adapt to hyposmotic stress including that of regulatory volume decrease. In ischemia and reperfusion, cardiomyocytes are exposed to hyposmotic stress, but little is known as to how their volume is controlled. Exposure of cultured neonatal rat cardiomyocytes to hyposmotic media induced a rapid swelling without any compensatory regulatory volume decrease. The hyposmotic stress increased the production of reactive oxygen species, mainly through NADPH oxidase. Adenoviral overexpression of catalase inhibited the hyposmosis-dependent $OH^{\cdot}$ production, induced the regulatory volume decrease mechanism, and prevented cell death. These results suggest that hyposmotic stress of cardiomyocytes stimulates production of reactive oxygen species which are closely linked to volume regulation and cell death.

Keywords: Hyposmotic stress; Reactive oxygen species; Volume regulation; Cardiomyocytes; Heart

Cell membranes are, with few exceptions, highly permeable to water so that any imbalance between extracellular and intracellular osmolarity induces a movement of water which modifies the cell volume [1]. Cells have developed compensatory homeostatic mechanisms including ion transport across the membrane and changes in metabolism [1]. The ability of cells to resist osmotic swelling or shrinkage by cell volume regulation, parallels their resistance to necrosis or apoptosis after osmotic shock [1,2]. Cells adapt to hyposmotic stress by a variety of mechanisms that recover cell volume by restoring intracellular salt and osmolyte concentrations [3]. Restitution of cell volume after cell swelling in mammalian cells is achieved by the loss of solutes (K^+, Cl^-, and organic osmolytes) and the subsequent osmotically driven efflux of water. This process is generally known as regulatory volume decrease (RVD) [1,4,5].

Isolated cardiac cells have lower water permeability than do renal or blood cells and are not normally exposed to changes in extracellular osmolarity [1,5]. In ischemia and reperfusion, however, cardiomyocytes are exposed to hyposmotic as well as oxidative stress [5,6]. In HTC and HeLa cells, hyposmolarity activates an NADPH oxidase which generates reactive oxygen species (ROS) [7]. Although this enzyme occurs in cardiomyocytes, its potential role in hyposmotic stress remains unknown [8].

Here we describe the effects of hyposmotic stress on volume regulation and ROS production in cultured rat cardiomyocytes. We have shown that cardiomyocytes exposed to hyposmotic stress did not spontaneously exhibit RVD. Hyposmotic stress induced ROS production, particularly

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$OH^{\cdot}$, most likely through NADPH oxidase. The production of $OH^{\cdot}$ was inhibited by over-expression of both catalase and cytoplasmic superoxide dismutase. Overexpression of catalase also inhibited hyposmotic cell death by inducing RVD.

Materials and methods

Cell culture. Neonatal rat cardiac myocytes and HeLa cells were cultured as described previously [9,10]. Hyposmotic culture media (248 ± 5 and 202 ± 5 mosmol (kg water)$^{-1}$) were made by diluting culture media with distilled water (15 and 30% dilution, respectively). When cell volume was measured, experiments were also performed using an isosmotic solution containing 95 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM Hepes, pH 7.4, and sucrose was added to yield a final osmolarity of 310 mosmol (kg water)$^{-1}$. The hyposmotic NaCl solution had the same composition as the isosmotic solution but without sucrose (210 mosmol (kg water)$^{-1}$). In the hyposmotic NaCl-free solution, NaCl was replaced by NMDG-Cl.

Measurements of cell volume. Changes in cell water volume were assessed in single cardiomyocytes and HeLa cells by measuring changes in concentration of an intracellularly trapped fluorescent dye (calcein) as described previously [11,12].

Measurement of ROS production. Cardiomyocytes exposed to hyposmotic solutions were treated 10 min before cell lysis with dichlorofluorescein diacetate acetyl ester (DCF-DA, 10 μM). Cells were lysed with 100 μL NaOH (100 mM) and fluorescence was determined in cell extracts (excitation: 490, emission: 525 nm). Arbitrary units of fluorescence were corrected for protein content. Additionally, cardiomyocytes were exposed to hyposmotic solutions in the presence of 5,5-dimethylpyrroline 1-oxide (DMPO, 200 mM). Cells were incubated for 30 min, lysed with 0.5 mL Triton X-100 (0.8% v/v), DMPO 200 mM in DME/M199 4:1, and incubated for 10 min at 37 °C. ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. Total intracellular glutathione levels were determined as described in [11].

Cell viability and caspase activation. Cell viability was determined by trypan blue exclusion [11]. Activation of caspase-9 and caspase-3 was assessed by Western blotting using anti-caspase-9 (Cell Signaling) or anti-caspase-3 (Cell Signaling) antibodies.

Adenovirus transduction. Adenovirus catalase (AdCAT) [13], cytosolic superoxide dismutase (AdSOD1) [14], and mitochondrial superoxide dismutase 2 (AdSOD2) [15] were transduced at a multiplicity of infection (MOI) of 300. As control, an adenovirus β-galactosidase (AdLacZ) construct was used. Cells were used after incubation for 24 h.

Statistical analysis. Values are presented as means ± SEM. Statistical analysis of the data was performed by ANOVA, comparisons were performed using a protected Tukey's test and considered significant at $p < 0.05$.

Results and discussion

Cell volume increase in cardiomyocytes exposed to hyposmotic stress

Hyposmotic stress causes a rapid influx of water into cardiomyocytes. In eight independent experiments, exposure of rat cardiomyocytes to culture media containing either 248 or 202 mosmol (kg water)$^{-1}$ resulted in a sudden and osmodependent increase in volume (to 1.40 ± 0.05 or 1.60 ± 0.15-fold, respectively). No measurable regulatory volume decrease (RVD) response was observed after 6 min (Fig. 1A). To confirm the absence of RVD,

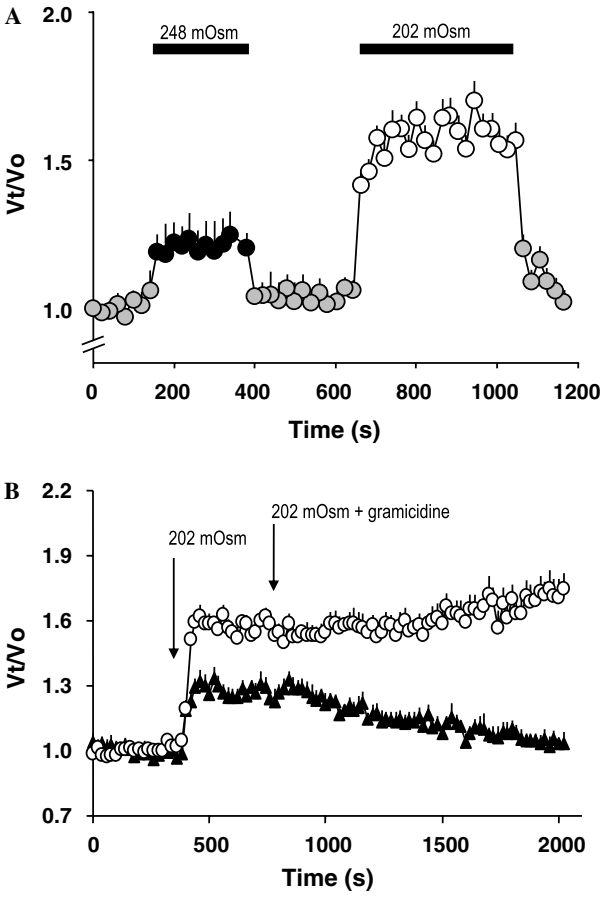

Fig. 1. Hyposmotic stress induces osmotic swelling in cardiac myocytes. Cultured rat cardiomyocytes were ester-loaded with calcein (5 μM) for 10 min and washed with isosmotic medium (290 mosmol (kg water)$^{-1}$. (A) Cardiomyocytes were maintained in isosmotic medium (grey circles) and incubated for 4 or 6 min with media containing 248 or 202 mosmol (kg water)$^{-1}$ (black and white circles). (B) Cardiomyocytes (white circles) and HeLa cells (black triangles) were maintained in isosmotic solution, incubated for 5 min with a medium containing 202 mosmol (kg water)$^{-1}$, and then treated for 30 min with a similar medium containing gramicidine (10 μM). Relative cell volume was estimated as described in Materials and methods. Changes in intracellular calcein concentration, as an indicator of relative cell volume, were determined using confocal microscopy with Vt/Vo being calculated for each point. Data are means ± SEM ($n = 3$ independent experiments).

cardiomyocytes were exposed to 210 mosmol (kg water)$^{-1}$ and incubated in presence of gramicidin, a monovalent-cation- selective channel-forming decapeptide [16]. Gramicidin did not induce measurable RVD in cardiomyocytes, while, in contrast, HeLa cells recovered almost completely (>90% of their initial volume) after incubation for 25 min (Fig. 1B). When exposed to 210 mosmol (kg water)$^{-1}$ in a NaCl-containing solution, cardiomyocytes increased cell volume more slowly than that observed in water-diluted culture media and the addition of gramicidin in a hyposmotic NaCl-free solution also did not induce measurable RVD (data not shown).

Although there is solid evidence that heart cells from fish and marine invertebrates are capable of regulating their volume when exposed to dilute media [17], data for higher