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,

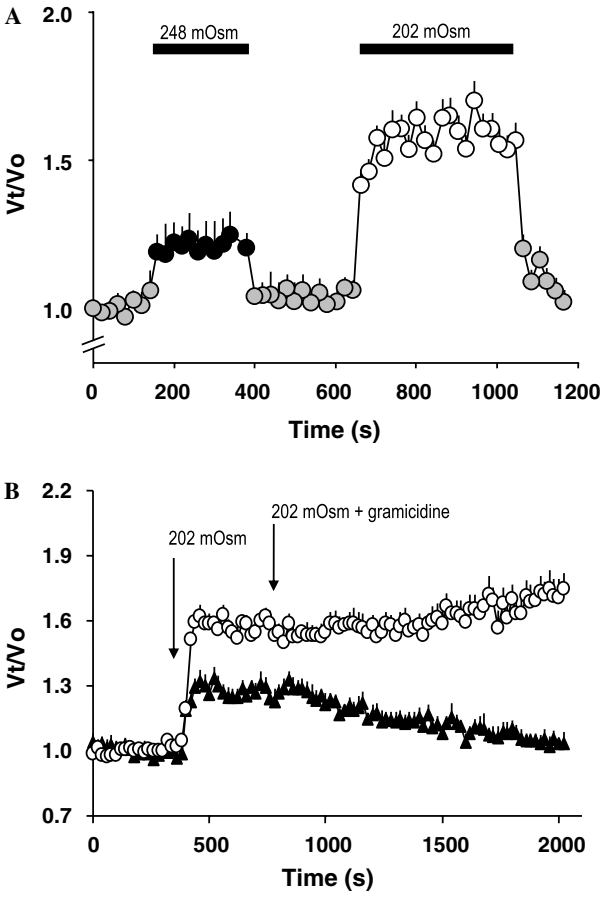

Fig. 1. Hyposmotic stress induces osmotic swelling in cardiac myocytes. Cultured rat cardiomyocytes were ester-loaded with calcein (5 μM) for 10 min and washed with isosmotic medium (290 mosmol (kg water)$^{-1}$. (A) Cardiomyocytes were maintained in isosmotic medium (grey circles) and incubated for 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

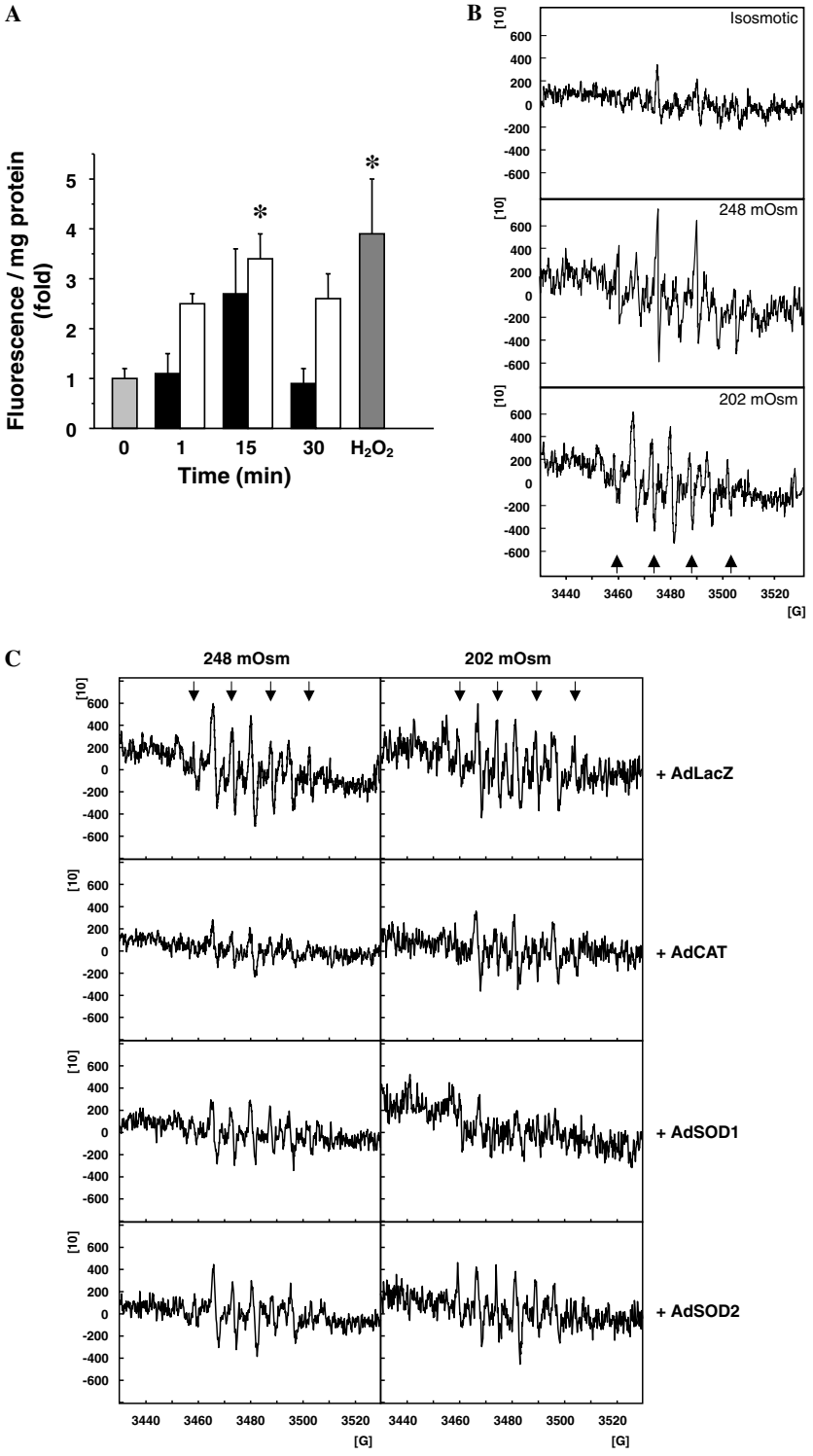

Fig. 2. Hyposmotic stress induces ROS production in cardiomyocytes. (A) Cultured cardiomyocytes were preincubated with DCF-DA (10 μM) and afterwards treated with isosmotic medium (290 mosmol (kg water)$^{-1}$, grey bar), media containing 248 (black bar) or 202 mosmol (kg water)$^{-1}$ culture (white bar). As a positive control, cardiomyocytes were treated with H_2O_2 (1 mM) for 10 min (dark grey bar). At different times cells were lysed and fluorescence was determined as indicated in Materials and methods. (B) Cardiomyocytes were incubated in isosmotic medium or media containing 248 or 202 mosmol (kg water)$^{-1}$ in the presence of DMPO (200 mM) for 30 min. Cells were lysed and extracts were analysed by ESR. Spectrometer conditions: microwave frequency 9.83 GHz microwave power 20 mW, modulation amplitude 0.2 G, scan rate 1.25 G/s, time constant 0.5 s, and number of scans: 20. (C) Effect of over-expression of antioxidant enzymes on ROS induction by hyposmotic stress. Cardiomyocytes were transduced with adenovirus β-galactosidase (AdLacZ), catalase (AdCAT), cytosolic CuZn-superoxide dismutase (SOD1), mitochondrial Mn-superoxide dismutase (SOD2) and incubated for 24 h. Cardiomyocytes were incubated in isosmotic medium or media containing 248 or 202 mosmol (kg water)$^{-1}$ in the presence of DMPO (200 mM) for 30 min. Cells were lysed and extracts were analysed by ESR using DMPO as spin trap. Arrows indicate the position of the signals corresponding to the DMPO-OH adduct. Data are means ± SEM and figures are representative of 3 independent experiments. *$p < 0.05$ vs control.

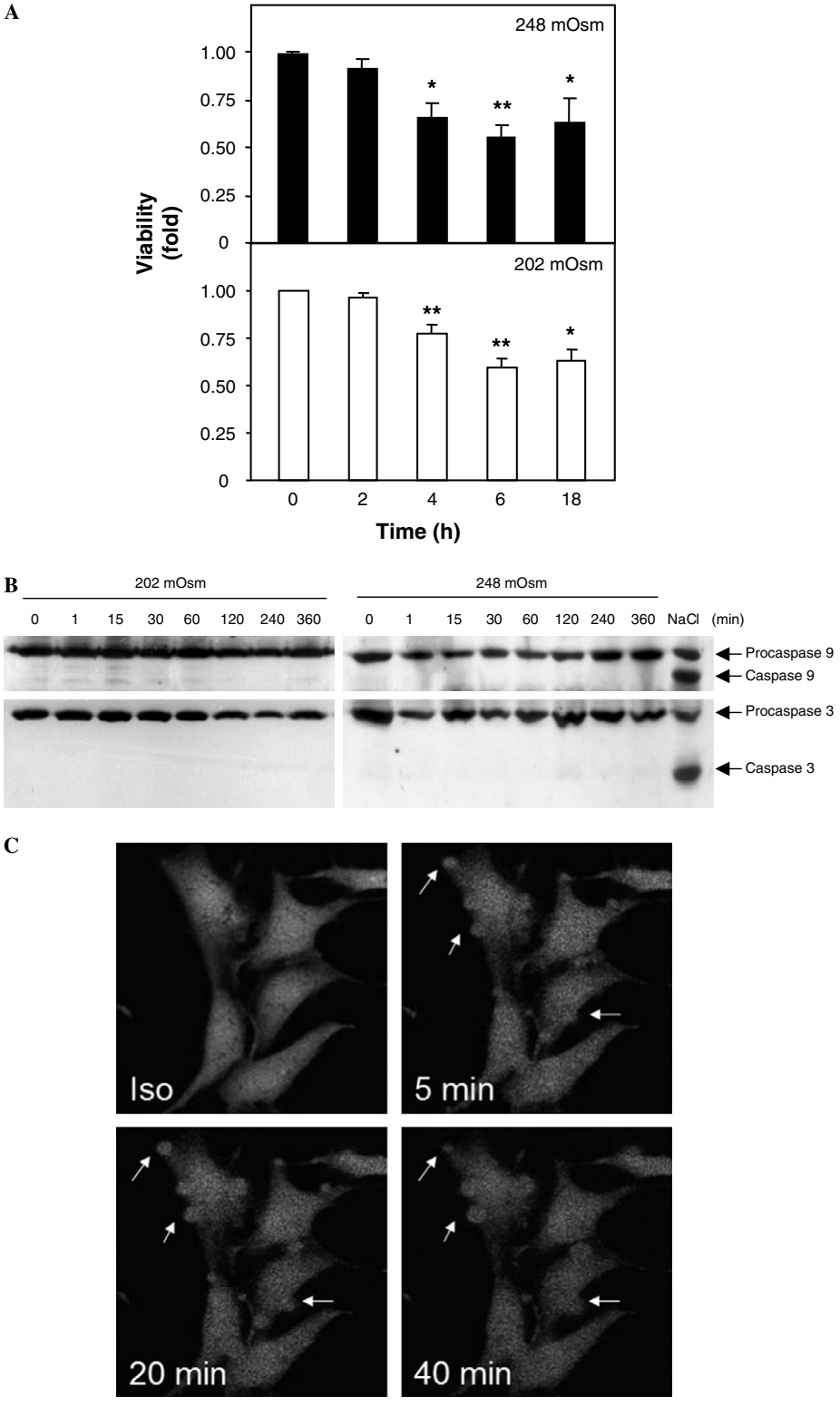

Fig. 3. Effect of hyposmotic stress on cardiomyocyte viability. (A) Cardiomyocytes were incubated with media containing 248 (black bar) or 202 mosmol (kg water)$^{-1}$ (white bar). At different times cells were trypsinized and cell viability was determined by trypan blue exclusion. Data are means ± SEM ($n = 5$ independent experiments). *$p < 0.05$ and **$p < 0.01$ vs control 0 h. (B) Cardiomyocytes were incubated with media containing 248 or 202 mosmol (kg water)$^{-1}$. At different times protein extracts were obtained. Procaspases and caspases 9 and 3 were determined by Western blotting as described in Materials and methods. As a positive control, cardiomyocytes were treated with NaCl (300 mM). Gels are representative of at least 3 independent experiments. (C) Cardiomyocytes were loaded with calcein-AM and were treated with 202 mosmol (kg water)$^{-1}$ culture media. Cell necrotic-like bleb structures were visualized at indicated times by confocal microscopy.

animals are less clearcut. Cultured chick embryo cardiomyocytes, newborn rat cardiomyocytes, as well as adult rabbit, canine, and guinea pig ventricular myocytes display RVD in response to hyposmotic swelling [18–22]. In contrast, guinea pig, rabbit, and canine ventricular myocytes, and canine atrial cells swelled when exposed to hyposmotic media, without evidence of RVD [23–25]. It may be that spontaneous RVD in cardiomyocytes may depend on the species and/or developmental status.

Hyposmotic stress induced ROS in cardiomyocytes

Hyposmotic stress induced by exposure of cardiomyocytes to media containing 248 or 202 mosmol (kg water)$^{-1}$ caused a 2.7 ± 0.9 or 3.4 ± 0.5-fold increase in ROS (Fig. 2A). Hydrogen peroxide, used as a positive control, induced a 3.9 ± 1.1-fold increase in ROS (Fig. 2A). ROS formation was confirmed by ESR using the spin trap DMPO. Treatment of cardiomyocytes as above in the presence of DMPO generated a four-line (1:2:2:1) ESR spectrum with $aN = aH = 14.9$ G (Fig. 2B). These ESR spectral characteristics are consistent with the formation of a relatively stable nitroxide-hydroxyl radical spin adduct (DMPO-OH) [26].

Exposure to both hyposmotic media significantly decreased GSH concentrations. After 8 h of exposure to 248 or 202 mosmol (kg water)$^{-1}$ GSH concentrations decreased from an initial 19.1 ± 2.4 to 9.9 ± 2.4 or 8.1 ± 1.1 nmol (mg protein)$^{-1}$, respectively (data not shown). Taken together, these results suggest that hyposmosis induces oxidative stress in cultured cardiomyocytes.

ESR measurements showed that adenoviral over-expression of catalase and cytosolic superoxide dismutase decreased the extent of $OH^{\cdot}$ formation induced by exposure to both 248 and 202 mosmol (kg water)$^{-1}$. Over-expression of mitochondrial superoxide dismutase was less effective than that of the cytosolic enzyme (Fig. 2C). These results suggest that $OH^{\cdot}$ was formed from H_2O_2, which in turn was synthesized mainly from cytosolic $O_2^{\cdot -}$.

With an osmolyte concentration of 248 mosmol (kg water)$^{-1}$, apocinine (an inhibitor of NADPH oxidase) and rotenone (an inhibitor of complex I of the mitochondrial respiratory chain) inhibited $OH^{\cdot}$ formation by 100% and 75%, respectively ($n = 3$). When the hyposmotic stress was greater (202 mosmol (kg water)$^{-1}$), however, apocinine was still totally effective but rotenone inhibited $OH^{\cdot}$ formation by only 12% ($n = 3$). These results suggest that, with limited hyposmosis, ROS were produced by both NADPH oxidase and the mitochondrial respiratory chain, but that, under greater stress conditions, ROS were produced principally by the NADPH oxidase.

Hyposmotic stress and cardiomyocyte viability

Incubation with 248 or 202 mosmol (kg water)$^{-1}$ resulted in a fall in cell viability at 6 h to 60.1 ± 4.3 or 55.4 ± 7.3% of control, respectively (Fig. 3A). We could not detect any activation of either caspase-9 or caspase-3 (Fig. 3B). After a 6 h period with either hyposmotic medium, lactic dehydrogenase activity in the supernatants was increased by 2-fold (not shown). 202 mosmol (kg water)$^{-1}$ hyposmotic cultured media induced in cardiomyocytes necrotic bleb-like structures as early as 5 min incubation, which persisted even after 40 min incubation (Fig. 3C). These results suggest that hyposmosis kills these cells by a caspase-independent pathway, probably necrosis.

Overexpression of catalase protected cardiomyocyte from hyposmotic death (Fig. 4A). Moreover, cardiomyocytes transduced with AdCAT, but not those transduced with AdLacZ, exhibited RVD when exposed to an osmolarity of 202 mosmol (kg water)$^{-1}$. Cells over-expressing catalase recovered 58 ± 5% of the swelling after incubation for 20 min with the hyposmotic solution (Fig. 4B). These results suggest that ROS, and in particular H_2O_2, is involved in the regulation of RVD, and that RVD is a significant factor in minimizing hyposmotic death in cardiomyocytes.

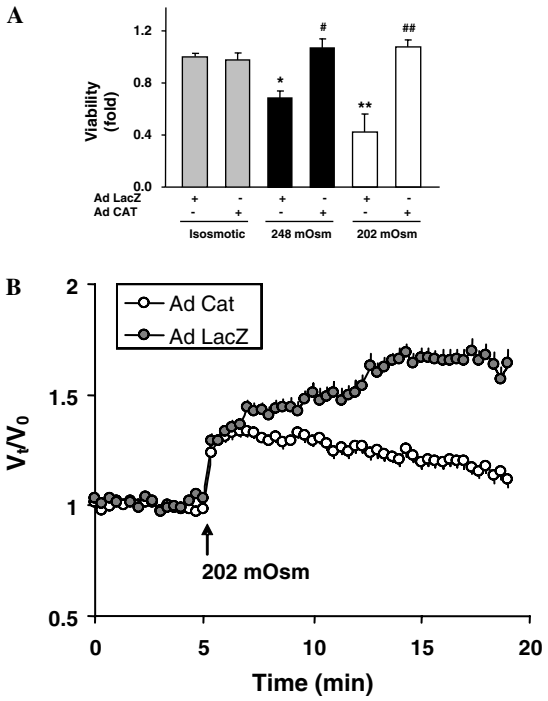

Fig. 4. Overexpression of catalase protects cardiomyocyte from hyposmotic death and activates a regulatory volume decrease. (A) Cardiomyocytes were transduced with adenovirus catalase (AdCAT) or adenovirus β-galactosidase (AdLacZ) and incubated for 24 h. Transduced cells were treated with isosmotic medium(gray bar), or media containing 248 (black bar) or 202 mosmol (kg water)$^{-1}$(white bar) for 6 h. Cell were trypsinized and cell viability was determined with trypan blue. Values are means ± SEM ($n = 5$ independent experiments). *$p < 0.05$ and **$p < 0.01$ vs. isosmotic condition. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. control AdLacZ. (B) Cardiomyocytes were transduced with adenovirus catalase (AdCAT, white circles) or adenovirus β-galactosidase (AdLacZ, grey circles) and incubated for 24 h. Transduced cells were preincubated with calcein-AM (5 μM) for 10 min, washed with isosmotic medium, and then treated with medium containing 202 mosmol (kg water)$^{-1}$ for 10 min. Changes in intracellular calcein concentrations were determined using confocal microscopy; Vt/Vo was calculated for each point as described in Materials and methods. Data are means ± SEM ($n = 3$ independent experiments).

There have been few studies of the mechanisms involved in RVD regulation in cardiomyocytes. [20,21,23,25]. In adult guinea pig cardiomyocytes, spontaneous RVD was not observed. RVD can however be induced by β-catecholamine/cAMP-dependent activation of a Cl^- channel [25]. Although Cl^- channels [7], K^+–Cl^- co-transport [28], K^+ channels [29,30], and taurine efflux [27] can be regulated by ROS, especially H_2O_2, there have been no reports of RVD inhibition by ROS. Moreover, insensitivity to gramicidin suggests that neonatal rat cardiac myocytes exposed to hyposmotic solution did not display a classical RVD mechanism, as compared to HeLa cells. Chloride channel activated by swelling could be not involved, in spite of this channel being described to be implicated in RVD of cardiac myocytes isolated from guinea pig ventricles [25].

This is the first report which describes the induction of oxidative stress by exposure of cardiomyocytes to hyposmotic solutions. ROS generated by the swelling preventing RVD suggests that ROS inhibits swelling-induced K^+ or Cl^- conductance in a manner specific to cardiomyocytes. This aspect will require further exploration.

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