Aldose Reductase Induced by Hyperosmotic Stress Mediates Cardiomyocyte Apoptosis

DIFFERENTIAL EFFECTS OF SORBITOL AND MANNITOL*

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Cells adapt to hyperosmotic conditions by several mechanisms, including accumulation of sorbitol via induction of the polyol pathway. Failure to adapt to osmotic stress can result in apoptotic cell death. In the present study, we assessed the role of aldose reductase, the key enzyme of the polyol pathway, in cardiac myocyte apoptosis. Hyperosmotic stress, elicited by exposure of cultured rat cardiac myocytes to the nonpermeant solutes sorbitol and mannitol, caused identical cell shrinkage and adaptive hexose uptake stimulation. In contrast, only sorbitol induced the polyol pathway and triggered stress pathways as well as apoptosis-related signaling events. Sorbitol resulted in activation of the extracellular signal-regulated kinase (ERK), p54 c-Jun N-terminal kinase (JNK), and protein kinase B. Furthermore, sorbitol treatment resulting in induction and activation of aldose reductase, decreased expression of the antiapoptotic protein Bcl-xL, increased DNA fragmentation, and glutathione depletion. Apoptosis was attenuated by aldose reductase inhibition with zopolrestat and also by glutathione replenishment with *N*-acetylcysteine. In conclusion, our data show that hypertonic shrinkage of cardiac myocytes alone is not sufficient to induce cardiac myocyte apoptosis. Hyperosmolarity-induced cell death is sensitive to the nature of the osmolyte and requires induction of aldose reductase as well as a decrease in intracellular glutathione levels.

Programmed cell death culminating in apoptosis is responsible for normal tissue homeostasis and has increasingly been implicated in mediating pathological cell loss (1). Apoptosis is accompanied by characteristic morphological changes, including cell shrinkage, nuclear condensation, plasma membrane blebbing, chromatin condensation, and the formation of apoptotic bodies (2). In recent years, molecular signaling pathways leading to apoptosis have been elucidated, and the central role of caspases in initiation of events characteristic of this form of cell death, such as DNA fragmentation, is well established (3). Dysregulated apoptosis has been implicated in the genesis and development of several human diseases, including cardiovascular conditions such as congestive heart failure and myocardial ischemia (4, 5).

Cardiac myocyte apoptosis can be induced by varied stimuli, including mechanical stretching, tumor necrosis factor, angiotensin II, doxorubicin, hypoxia, myocardial infarction, hypertension and ischemia/reperfusion (6–14). We have recently shown that hyperosmotic stress induced by sorbitol rapidly stimulated apoptosis in cultured cardiomyocytes (15). However, other osmotically active substances such as mannitol are intravenously administered in the treatment of a number of clinical conditions including myocardial reperfusion injury (16, 17), and its beneficial clinical effects have been mainly attributed to the hyperosmotic properties (18, 19) as well as the scavenging of hydroxyl radicals (20). In contrast to these beneficial applications, little is known about the effects of mannitol on cardiomyocyte apoptosis. The primary mechanism of action of hyperosmotic stress appears to be mechanical and related to shrinkage of these cells. However, the underlying mechanisms by which hyperosmotic stress triggers apoptosis in the cardiomyocyte remain unknown. Maeno *et al.* (21) have shown that apoptotic volume decrease, which is caused by disordered cell volume regulation, is an early prerequisite for events leading to apoptotic cell death.

To avoid excessive alterations in volume, cells have developed regulatory mechanisms including ion transport across the membrane and changes in metabolism. The ability of cells to resist osmotic shrinkage by cell volume regulation parallels their resistance to apoptosis after osmotic shock (22). Cells adapt to hyperosmotic stress by a variety of mechanisms that restore cell volume by restoring intracellular salt and osmolyte concentrations (23). Aldose reductase (AR[1]; EC 1.1.1.21) is the first enzyme in the polyol pathway, which helps to promote resistance of cells to anisotonic perturbations. AR catalyzes the formation of sorbitol from glucose using NADPH as a cofactor (24–26). Sorbitol accumulation is considered an adaptive re-

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[1] The abbreviations used are: AR, aldose reductase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKB, protein kinase B; NAC, *N*-acetylcysteine; SDH, sorbitol dehydrogenase; BSO, DL-buthionine-(*S*,*R*)-sulfoximine; Sor, sorbitol; Man, mannitol; LY, LY-294002; PD, PD-98059; SB, SB-203580; SP, SP-600125; KRPH, Krebs Ringer phosphate HEPES buffer; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RVI, regulatory volume increase.

sponse to hypertonicity that is observed in many cells (27, 28).

Although the induction of AR has been associated with compensatory responses to hyperosmotic stress, it also plays an important role in the development of complications in diabetes (29–31) and myocardial ischemia-reperfusion injury (32–35). The relationship between AR and apoptosis has not been previously examined in the cardiomyocyte. However, in lens epithelial cells and pancreatic β-cells, AR activation induced apoptosis, possibly causing an imbalance in redox status (36, 37). In cultured retinal pericytes, glucose-induced apoptosis is mediated through the AR pathway, involving increased oxidative stress characterized by reduced GSH contents (38). Whether osmotically active nonionic substances such as sorbitol or mannitol induce AR and whether this enzyme mediates apoptosis as a consequence of hyperosmotic stress in cardiac myocytes has not been investigated. In this study, we addressed such issues in cardiomyocytes and asked whether sorbitol- and mannitol-mediated hyperosmotic stress activate different signaling pathways. We also investigated whether induction of AR and increased intracellular sorbitol levels mediates hyperosmotic stress-induced apoptosis.

We demonstrate here in cultured rat cardiomyocytes that mannitol or sorbitol induced hyperosmotic stress, reduced cell volume, and stimulated 2-deoxyglucose uptake to a similar degree. Treatment with sorbitol, but not mannitol, induced and activated AR, decreased sorbitol dehydrogenase levels, stimulated intracellular sorbitol accumulation, increased DNA fragmentation, and decreased the levels of the antiapoptotic Bcl-xL protein. Phosphorylation of the mitogen-activated protein kinase ERK was enhanced in the presence of sorbitol but not with mannitol. Both JNK isoforms (p46 and p54) were differentially activated by sorbitol and mannitol. p38-MAPK and protein kinase B (PKB) were activated to a lesser extent by both sorbitol and mannitol at hyperosmotic concentrations. Increases in AR protein levels and activity by sorbitol-induced hyperosmotic stress were differentially regulated by ERK, p38-MAPK, and phosphatidylinositol 3-kinase (PI3K)/PKB pathways. GSH intracellular levels were decreased by hyperosmotic sorbitol but not by mannitol. Hyperosmotic sorbitol-induced apoptosis was attenuated by zopolrestat, a specific AR inhibitor. DNA fragmentation stimulated by sorbitol was also prevented by *N*-acetylcysteine (NAC; a GSH precursor). Our data demonstrate that AR contributes to apoptosis triggered by hyperosmotic stress in cultured cardiac myocytes. These data also indicated that our current understanding of the protective mechanisms of mannitol in myocardial reperfusion injury needs to be reevaluated.

EXPERIMENTAL PROCEDURES

All studies were performed following the guidelines approved by the bioethics committee of the Faculty of Chemical and Pharmaceutical Sciences at University of Chile, Santiago. The experiments were performed as stipulated in Ref. 88.

Materials—Polyclonal antibodies against AR and sorbitol dehydrogenase (SDH; NAD^+ oxidoreductase; EC 1.1.1.14) were kindly provided by Dr. N. Taniguchi (Osaka University Medical School, Osaka, Japan) (39). Dulbecco's modified Eagle's medium, medium 199, SDH, agarose, β-NAD, NADPH, GSH, glutathione reductase, NAC, DL-buthionine-(*S*,*R*)-sulfoximine (BSO), sorbitol (Sor), mannitol (Man), and other biochemicals were purchased from Sigma unless stated otherwise. ECL, autoradiographic films, and 2-deoxy-D-[^{3}H]glucose (8.1 Ci/mmol) were from PerkinElmer Life Sciences. Prestained molecular mass standard proteins were purchased from Invitrogen. Protein assay reagents were from Bio-Rad. LY-294002 (LY), PD-98059 (PD) and SB-203580 (SB) were from Calbiochem. SP-600125 (SP) was from Tocris (Ellisville, MO). Polyclonal antibodies against phosphorylated and total ERK, p38-MAPK, JNK, and PKB used in Western blot analysis were purchased from Cell Signaling Technology Inc. (Beverly, MA). Polyclonal antibody against Bcl-xL was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Zopolrestat was kindly provided by Pfizer (Groton, CT). Calcein-acetoxymethylester was from Molecular Probes, Inc. (Eugene, OR).

Culture and Treatment of Cardiomyocytes—Cardiac myocytes were prepared from hearts of 3-day-old Sprague-Dawley rats as described previously (40). Rats were bred in the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). Cardiomyocytes were plated at a final density of $1.4 \times 10^3/mm^2$ on gelatin-coated 35-, 60-, or 100-mm Petri dishes. For fluorescence measurements, cells were plated on gelatin-coated 25-mm glass coverslips in 35-mm Petri dishes. Cardiac myocytes were cultured in the absence or presence of different concentrations of sorbitol or mannitol dissolved in serum-free Dulbecco's modified Eagle's medium/medium 199. The final osmolarities of culture media containing different amounts of sorbitol or mannitol were determined using a freezing point osmometer (Osmet). Cultured cardiomyocytes were identified using an anti-β-myosin heavy chain antibody. Cell cultures were at least 95% pure.

Preparation of Cell Extracts—Cardiomyocytes were scraped into 100 μl of cold lysis buffer: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride containing 10% (v/v) glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1% (v/v) Triton X-100. Samples were centrifuged at $12,000 \times g$ for 10 min at 4 °C, and the protein content of supernatants was determined by a Bio-Rad Bradford assay using bovine serum albumin as a standard (41). Soluble fractions were heated at 95 °C with 0.33 volumes of 4× SDS-PAGE sample buffer for Western blot analysis.

Western Blot Analysis for Aldose Reductase and Sorbitol Dehydrogenase—Cell lysates were matched for protein (15 μg), separated by SDS-PAGE on 12% (w/v) polyacrylamide gels, and electrotransferred to nitrocellulose (0.45 μm) using a Trans-blot unit (Bio-Rad) for 1.5 h at 100 V. Membranes were blocked with 3% (w/v) bovine serum albumin in PBS (pH 7.4) containing 0.1% (v/v) Tween 20 (PBST) overnight at 4 °C. Anti-AR or -SDH primary antibodies were diluted in blocking solution (1:5,000). Nitrocellulose membranes were incubated with primary antibody for 1 h at 25 °C. After washing in PBST (4×15 min each), blots were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody (1:5,000 in 3% (w/v) bovine serum albumin in PBST). Blots were washed again in PBST (4×15 min each), and specific binding was detected using ECL with exposure to Kodak film for 2–10 s. Each blot was quantified by scanning densitometry.

Aldose Reductase Activity—Cultured cardiomyocytes were exposed to culture media containing different concentrations of sorbitol or mannitol at the indicated times. Cells were then rinsed three times with PBS and lysed with 200 μl of lysis buffer (10 mM potassium phosphate, pH 7.0, containing 5 mM β-mercaptoethanol). Cell extracts were centrifuged at $10,000 \times g$ for 1 h at 4 °C, and the resulting supernatants were dialyzed in 2 liters of lysis buffer overnight. The dialyzed samples were incubated with 1 ml of DEAE-cellulose pre-equilibrated with lysis buffer. AR was eluted with increasing NaCl concentrations (0–300 mM). The fraction with the highest AR activity (50 mM NaCl) was used throughout the study. Enzyme activity was determined spectrophotometrically at 37 °C by monitoring the decrease in absorbance of NADPH at 340 nm for 3 min in the absence or presence of 5 mM D-glucose as a substrate (25). Briefly, the assay, in a total volume of 1 ml, contained 50 mM potassium phosphate, pH 6.0, 5 mM β-mercaptoethanol, 0.4 M Li_2SO_4, 5 mM D-glucose, enzyme (1–3 μg of protein); the reaction was started by the addition of 100 μM NADPH. One unit of the enzyme was defined as 1 μmol of NADPH oxidized per min at 37 °C.

Determination of Intracellular Sorbitol Levels—Intracellular sorbitol level was determined essentially as described by Malone (42). Briefly, cultured cardiac myocytes (4×10^6 cells/60-mm Petri dish) were lysed with 200 μl of 6% (w/v) $HClO_4$ and then centrifuged at $10,000 \times g$ for 10 min at 4 °C. Supernatants were neutralized with 62 mM glycine (pH 9.4). Sorbitol was determined using a 40-μl sample in glycine buffer 50 mM (pH 9.4), 0.64 units of SDH, and 0.2 mM β-NAD, in a final volume of 0.5 ml. After incubation for 30 min at 37 °C, samples were analyzed in a spectrofluorometer (emission, 366 nm; excitation, 452 nm). The acid pellets were resuspended in 150 μl of 1 M NaOH and incubated for 30 min at 50 °C. Protein content was determined by the Lowry method (43). Results were expressed as mg of sorbitol per mg of protein.

Assessment of Cell Volume—Cardiomyocyte relative volume was determined by calcein measurements using a modified protocol (44). Briefly, cardiomyocytes grown on glass coverslips were loaded at room temperature with 5 μM calcein-acetoxymethyl ester for 5–10 min in Krebs Ringer phosphate HEPES buffer (KRPH; 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 5 mM Na_2PO_4, 20 mM HEPES, pH 7.4) supplemented with 25 mM glucose (KRPH-glc). Cells were imaged

at room temperature every 30 s at 488-nm excitation/510–545-nm emission using a Zeiss LSM 410 confocal microscope. Under these conditions, dye bleaching was negligible. In order to compare values from different cells, data were standardized by assigning base-line fluorescence (F_0) the value of 1.

Glucose Transport Assay—Cardiomyocytes were placed in Dulbecco's modified Eagle's medium/medium 199 containing 25 mM glucose for 2 h at 37 °C. Cells were then washed with KRPH buffer and either untreated or stimulated as described in the legend to Fig. 4. Uptake of 0.2 mM 2-deoxy-D-[^{3}H]glucose was estimated over a period of 2 min in KRPH buffer at 4 °C by a protocol modified from a previous communication (45). Control experiments showed that at these temperatures intracellular hexose concentrations were less then 20% of the extracellular values. Carrier-mediated uptake rates were obtained by subtracting nonspecific uptake. The latter was measured in the presence of 20 μM cytochalasin B and was less than 10% of the basal uptake. Transport was stopped with three washes of 50 μM phloretin in ice-cold PBS. Radioactivity was released with 1% Triton X-100 and measured by liquid scintillation counting (45). Samples were normalized for protein content using the Bradford protein assay.

ERK, p38-MAPK, JNK, and PI3K/PKB Signaling Pathway Activation—Western blots were performed as described above. The membranes were subjected to immunoblot analysis with anti-phospho-ERK antibody, anti-phospho-p38-MAPK antibody, anti-phospho-JNK antibody, or anti-phospho-PKB antibody. Membranes were stripped and reprobed with anti-ERK antibody, anti-p38-MAPK antibody, anti-JNK antibody, or anti-PKB antibody. Blots were quantified by laser-scanning densitometry. Results were expressed as the ratio of phosphorylated protein kinase to total protein kinase levels. All blots were controlled for equal loading.

Bcl-xL Levels—Bcl-xL levels were determined by Western blot analysis for Bcl-xL. Proteins (20–50 μg) were separated by SDS-PAGE on a 15% polyacrylamide gel and were transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked with 3% (w/v) nonfat milk powder in PBS (pH 7.5) containing 0.1% (v/v) Tween 20 (PBST) for 60 min at room temperature. Primary antibody was diluted 1:1,000 in blocking solution. Nitrocellulose was incubated with primary antibody overnight at 4 °C. After washing in PBST (three times for 10 min each), nitrocellulose was incubated for 1.5 h at room temperature with horseradish peroxidase-linked secondary antibody (1:5,000 in 1% (w/v) nonfat milk powder in PBST). After repeating the washing procedure described above, bound antibody was detected by ECL with exposure to Hyperfilm for 0.5–30 min. Blots were quantified by scanning densitometry.

DNA Fragmentation—For the detection of DNA fragmentation, cells were washed with cold PBS. DNA was prepared by scraping the cells into 1 ml of lysis buffer consisting of 0.8 mM EDTA (pH 8.0), 8 mM Tris-HCl (TE; pH 8.0) and 4% SDS. The DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. The resulting DNA was incubated with proteinase K (50 μg/ml, Sigma) for 1 h at 50 °C to facilitate protein disruption. DNA was re-extracted from supernatants with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). DNA, precipitated from the upper aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol, was left at −20 °C overnight before centrifugation. Pellets were resuspended in 200 μl of TE buffer, followed by a 60-min incubation with DNase-free RNase A (2 mg/ml; Sigma) at 37 °C. Samples were re-extracted, and DNA was precipitated as described above. Pellets were resuspended in TE buffer, and DNA concentrations were quantified from the absorbance at 260 nm. DNA samples were analyzed by electrophoresis on 2% agarose and visualized by staining with a solution containing 0.2 μg/ml ethidium bromide (46).

Measurement of Intracellular Glutathione—GSH levels were determined using an enzymatic assay according to Anderson and Meister (47).

Expression of Results and Statistical Analysis—Data shown are the means ± S.E. of the number of independent experiment indicated (n) or representative experiments performed on at least three separate occasions with similar outcome. Data were analyzed by analysis of variance, and comparisons between groups were performed using a protected Tukey's test. A value of $p < 0.05$ was set as the limit of statistical significance.

RESULTS

Effect of Hyperosmotic Solutions of Sorbitol and Mannitol on Polyol Pathway in Cultured Cardiomyocytes—AR is the first enzyme in the polyol pathway, and different reports have shown that a hyperosmotic environment stimulates the expression and activity of AR in many cell types (48, 49). The time course of hyperosmotic AR induction by sorbitol is depicted in Fig. 1*A*. Induction of AR was slow and only detectable after 8 h of hyperosmotic exposure. Maximum AR levels were reached after 24 h; however, after 32 h, AR levels decreased again.

To assess whether hyperosmolarity affects the levels of AR in cardiac myocytes, we compared the amount of AR protein in lysates obtained from isosmotically or hyperosmotically treated cardiomyocytes with sorbitol and mannitol for 24 h. AR was detected as a single band with M_r 36,000 in isosmotically and hyperosmotically treated cells. Hyperosmotic challenge with sorbitol (400 and 600 mosmol/kg water) caused an increase (1.4- and 2.5-fold, respectively) in the amount of AR protein after 24 h of treatment (Fig. 1*B*). Mannitol did not significantly change AR protein levels in hyperosmotically treated cardiomyocytes when compared with control cells maintained under isosmotic conditions (270 mosmol/kg water) (Fig. 1*B*). Thus, AR induction in cardiac myocyte was dependent on the nature of the osmolyte.

Hyperosmolarity is also a known regulator of AR activity in some cell types (23). Hence, we next determined whether the increases in AR protein levels were paralleled by increases in AR activity levels. Most human tissues have three forms of aldo-keto reductases, namely aldose reductase and aldehyde reductases I and II (26). Since there is some overlap in the substrate specificity of various aldo-keto reductases, AR is usually purified by DEAE column chromatography (26). Despite the fact that aldehyde reductase is not expressed in cardiac muscle (50), this partial purification step ensured that measurements in our assay were specific for AR activity. As shown in Fig. 1*C*, sorbitol-supplemented hyperosmotic medium increased AR activity. In general, AR protein content paralleled the changes in AR activity induced by sorbitol (Fig. 1, *B* and *C*). Under all hyperosmotic conditions tested, mannitol failed to increase AR activity in cardiac myocytes (Fig. 1*C*). Collectively, these results suggest that mannitol neither activated AR nor induced AR expression in rat cardiac myocytes.

Several studies in other cell types have suggested that the osmoprotective effect of AR is due to intracellular accumulation of sorbitol. To demonstrate this in cardiac myocytes, sorbitol levels were measured in these cells. Fig. 1*D* shows that sorbitol-induced hyperosmotic stress increases intracellular content of sorbitol from 0.096 (control cells) to 1.25 mg/mg protein (sorbitol-treated cells). In contrast, mannitol did not have any effect. Although these results are consistent with the observed increase in AR expression and activity, intracellular sorbitol levels depend on both AR and SDH, an enzyme responsible for sorbitol catabolism (51). Therefore, intracellular sorbitol accumulation may also be due to a decrease in SDH. To test this possibility, cardiac myocytes were incubated for 24 h in medium containing sorbitol or mannitol (600 mosmol/kg water) and SDH protein levels were determined. As shown in Fig. 1*E*, sorbitol (600 mosmol/kg water) decreased SDH protein levels by 40%. As was observed with AR, mannitol also failed to affect SDH levels (Fig. 1*E*). Collectively, these data suggest that intracellular sorbitol accumulation by hyperosmotic sorbitol is the result of both AR induction and SDH down-regulation.

Effect of Hyperosmotic Sorbitol and Mannitol on Cardiomyocyte Volume—Hypertonic stress causes rapid cell dehydration, the degree of which is determined by the effective reflection coefficient of the osmolyte applied. In order to measure the permeability properties of the two sugars compared in this study, we loaded cardiomyocytes with the inert

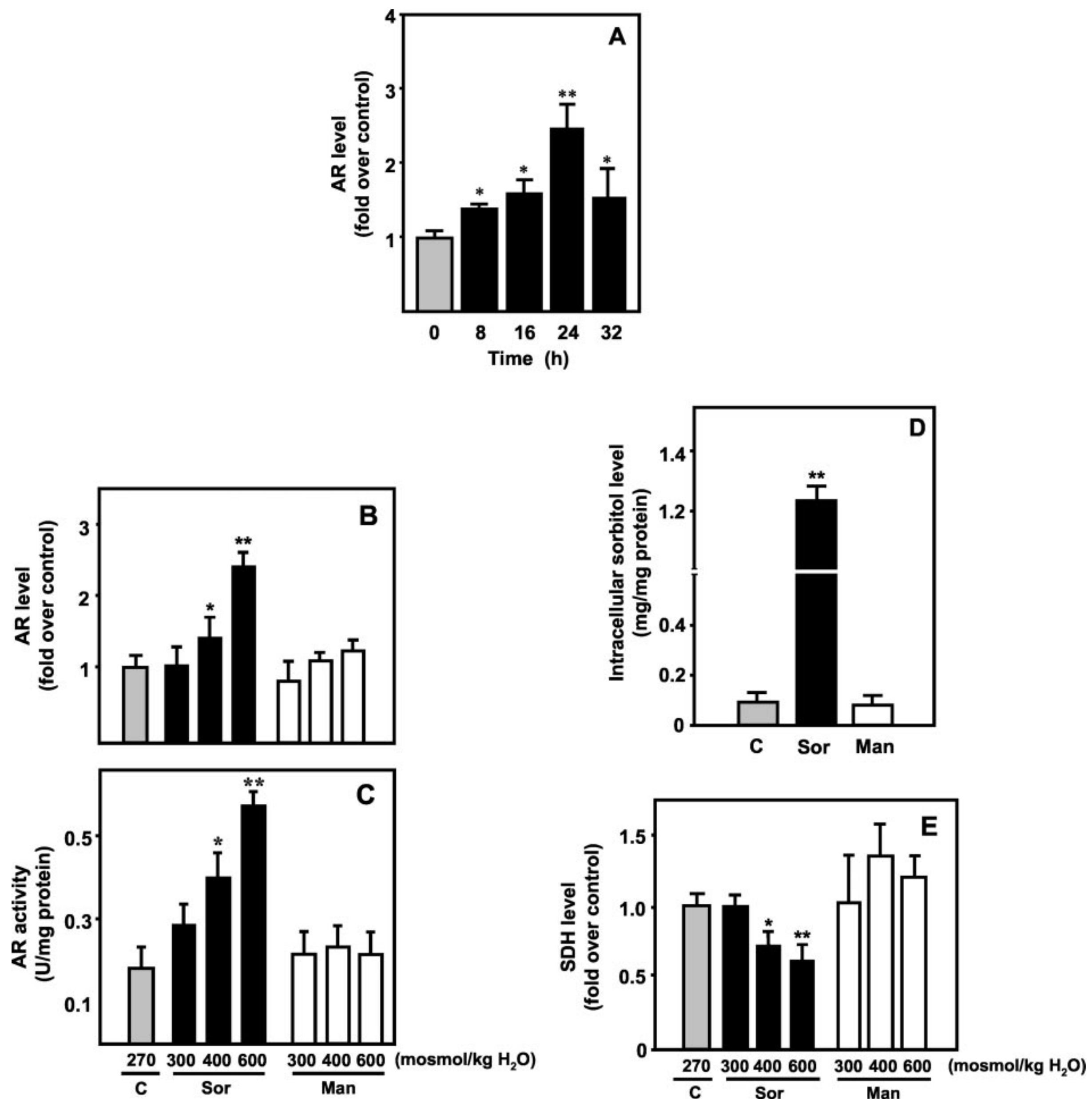

FIG. 1. **Hypertonic sorbitol and mannitol differentially activated polyol pathway in cultured cardiac myocytes.** *A*, time course of the effect of sorbitol hyperosmotic solution on AR protein levels. Cells were incubated for the indicated times with sorbitol (600 mosmol/kg water; *black bars*) before being processed by Western blot analysis for AR using an anti-AR polyclonal antibody, followed by densitometric analysis. *B* and *C*, effect of hyperosmolarity on AR protein levels and activity. Cultured cardiomyocytes were incubated in isotonic serum-free medium (control (*C*); 270 mosmol/kg water; *gray bar*) or solutions of Sor (300–600 mosmol/kg water; *black bars*) or Man (300–600 mosmol/kg water; *white bars*) for 24 h. After stimulation, cell protein extracts were obtained as described under "Experimental Procedures." *B*, cell lysates were matched for protein and separated by SDS-PAGE, and AR protein levels were determined by Western blotting. *C*, AR was semipurified by DEAE-cellulose chromatography using increasing NaCl concentrations. AR activity was assessed by monitoring the decrease in absorbance of NADPH in the absence or presence of 5 mM D-glucose and expressed as units/mg protein. 1 unit = 1 μmol NADPH oxidized per min at 37 °C. *D*, effect of hyperosmolarity on intracellular sorbitol levels in cultured cardiomyocytes. Cells were exposed to isosmotically serum-free culture media (control, 270 mosmol/kg water; *gray bar*) or hyperosmotic solutions of sorbitol (600 mosmol/kg water; *black bars*) or mannitol (600 mosmol/kg water; *white bars*) for 24 h. Cultured cardiomyocytes were exhaustively washed and lysed with perchloric acid. Supernatants were neutralized, and sorbitol levels were determined by an enzymatic assay as described under "Experimental Procedures." Data are expressed as mg of Sor/mg of protein. *E*, effect of hyperosmolarity on SDH protein levels. Cells were incubated as described above, and SDH protein levels were determined by Western blotting, using an anti-SDH polyclonal antibody, followed by densitometric analysis. Results are means ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* control.

fluorescent probe calcein and measured changes in its intracellular concentration by confocal microscopy. We have previously shown that this probe behaves as a linear reporter of relative cell volume in HeLa cells using up to 700 mosmol/kg water (44). Exposure of rat cardiomyocytes to 600 mosmol/kg water solutions of sorbitol or mannitol in KRPH-glc resulted in a sudden, osmometric increase in calcein fluorescence, consistent with the high water permeability typical of most mammalian cells (Fig. 2). In four independent experiments, the deflections (F/F_0) triggered by sorbitol and mannitol were 1.32 ± 0.05 and 1.34 ± 0.04, respectively. No additional changes were observed after 10 min. These results demonstrated that the two sugars display similar reflection coefficients of ~1, indicating that their net uptakes were negligible at the concentrations tested. Thus, cardiomyocytes do not display a measurable regulatory volume increase (RVI) re-

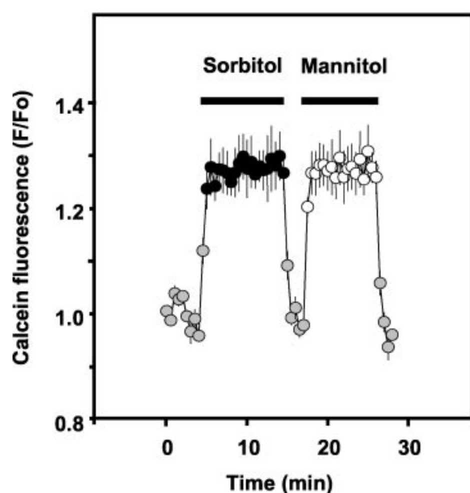

FIG. 2. **Sorbitol and mannitol were equally effective at inducing osmotic shrinkage in cardiac myocytes.** Cultured rat cardiomyocytes were ester-loaded with calcein by 5 min, washed with KRPH-Glc, and then incubated with a hyperosmotic solution of sorbitol (600 mosmol/kg water). After 10 min, cells were washed with KRPH-Glc, followed by a 10-min incubation with mannitol (600 mosmol/kg water), and finally washed again with KRPH-Glc. Relative cell volume was estimated as described under "Experimental Procedures." Changes in intracellular calcein concentration, as an indicator of relative cell volume, were determined using confocal microscopy, and F/F_0 was calculated for each point. Data are mean ± S.E. (n = 3 independent experiments).

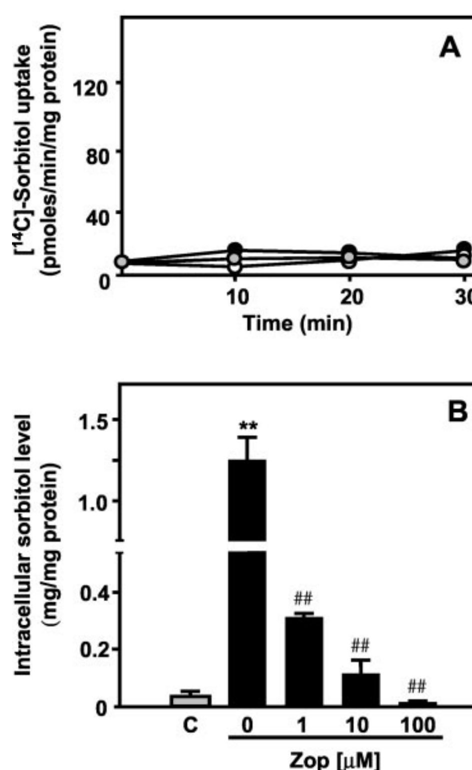

FIG. 3. **Intracellular sorbitol accumulation resulted from *de novo* synthesis by aldose reductase and was not due to sorbitol uptake in cultured cardiac myocytes.** *A*, [^{14}C]sorbitol uptake by cardiomyocytes incubated with isosmotic and hyperosmotic solutions of sorbitol or mannitol. Cultured rat cardiomyocytes were incubated with serum-free culture media (control; 270 mosmol/kg water; *gray circles*), sorbitol (600 mosmol/kg water; *black circles*), or mannitol (600 mosmol/kg water; *white circles*) supplemented with [^{14}C]sorbitol. At the times shown, cells were exhaustively washed with PBS and then lysed. Released [^{14}C]sorbitol was determined by liquid scintillation as described under "Experimental Procedures." *B*, effect of AR inhibition on intracellular sorbitol content in cardiomyocytes. Cultured cardiomyocytes were incubated in isotonic serum-free medium (control (*C*); 270 mosmol/kg water; *gray bar*) or treated with or without different concentrations of zopolrestat (*Zop*; a specific AR inhibitor) and then incubated with sorbitol (600 mosmol/kg water; *black bars*) for 24 h. Cardiomyocytes were exhaustively washed with PBS, protein extracts were obtained, and sorbitol was determined as described under "Experimental Procedures." Results are the average of at least three independent experiments ± S.E. **, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* 0 μM zopolrestat.

sponse. RVI was also not detected after a 50-min exposure to hypertonic sorbitol (data not shown), as has been reported in a previous study with these cells (52).

External Sorbitol Does Not Enter into Cultured Cardiac Myocytes—The differential effect of hyperosmotic solutions of sorbitol and mannitol on the polyol pathway described above raised the question of whether external sorbitol could enter into cultured cardiac myocytes as previously shown in rat glial primary cultures (53) and human erythrocyte (54). To investigate directly this point, uptake of [U-^{14}C]sorbitol was studied in cultured cardiomyocytes. Time courses of sorbitol uptake by cardiomyocytes under isosmotic conditions or in the presence of sorbitol or mannitol (600 mosmol/kg water) are shown in Fig. 3*A*. We did not detect ^{14}C-labeled sorbitol in cardiomyocytes following incubation up to 30 min in isosmotic media. Similar results were obtained under hyperosmotic stress induced either by the presence of sorbitol or mannitol (600 mosmol/kg water) (Fig. 3*A*). Thus, no measurable uptake of extracellular sorbitol was detectable under isoosmotic or hyperosmotic conditions in cultured rat cardiomyocytes.

To investigate the source of intracellular sorbitol levels in sorbitol-stressed cardiac myocytes, we studied the effect of zopolrestat, a well characterized specific AR inhibitor (55), on intracellular sorbitol levels. As depicted in Fig. 3*B*, inhibition of AR by zopolrestat blocked the intracellular sorbitol increase stimulated by sorbitol-dependent hyperosmotic stress. These results indicate that the increases in intracellular sorbitol levels observed in cultured cardiomyocytes exposed to hyperosmotic sorbitol solutions are dependent on the activity of the polyol pathway rather than on the uptake of external sorbitol.

Effect of Hyperosmotic Solutions of Sorbitol and Mannitol on Hexose Uptake in Cultured Cardiac Myocytes—Exposure to hypertonicity caused a rapid increase in the rate of hexose uptake by cardiomyocytes (Fig. 4). Both with sorbitol and mannitol, the effect was rapid, reaching plateaus of 2.6 ± 0.1- and 2.2 ± 0.2-fold stimulation and requiring 2.6 ± 0.5 and 1.8 ± 1.4 min, respectively, to reach half-maximal stimulation. The comparable efficiency of the two osmolytes is consistent with published data obtained using 3T3-L1 adipocytes and Clone 9 cells, in which stimulation of hexose uptake by sorbitol was similar to that elicited by several other nonpermeant solutes (56). In 3T3-L1 adipocytes, which express densities of GLUT4 and GLUT1 comparable with those detected in cardiac myocytes, hyperosmotic stress stimulates hexose transport by a complex signaling pathway, involving the tyrosine kinase PYK2 and many other downstream mediators, whose precise role in these events remains unclear (57, 58). The data presented here suggest that signaling events triggered by hyperosmotic stress in which glucose transporters play a major role do not provide the molecular basis that allows cardiac myocyte to distinguish between sorbitol and mannitol.

Effect of Hyperosmotic Solutions of Sorbitol and Mannitol on Stress Signaling Pathways in Cultured Cardiac Myocytes—Hyperosmotic stress has been shown to activate at least three different mammalian MAPKs: ERK, JNK, and p38-MAPK (23). The PI3K/PKB pathway is also stimulated by hyperosmotic stress in certain types of cells (59, 60). Fig. 5*A* shows that sorbitol, but not mannitol, resulted in strong phosphorylation of both ERK-1 and ERK-2, which was rapidly detectable (3- and

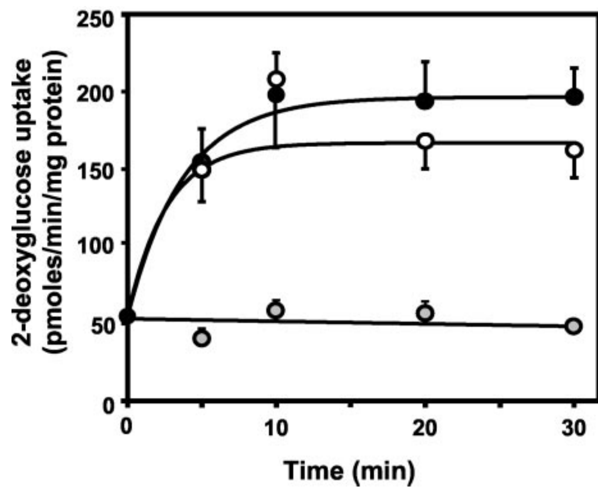

FIG. 4. **Hyperosmotic sorbitol and mannitol were equally effective at inducing hexose transport stimulation in cardiac myocytes.** Cells were exposed for 30 min at 37 °C to sorbitol (600 mosmol/kg water; *black circles*), mannitol (600 mosmol/kg water; *white circles*), or serum-free culture media (control; 270 mosmol/kg water; *gray circles*). Initial rates of 0.2 mM 2-deoxy-D-[^{3}H]glucose were measured for 2 min at 4 °C as described under "Experimental Procedures." Data are means ± S.E. (3–8 separate experiments performed in duplicate). For stimulated cells, continuous lines were obtained by fitting the following three-parameter nonexponential function: uptake (t) = basal uptake + maximum uptake × ($1 - e^{-b \times t}$).

5.5-fold, respectively, at 5 min) and maximal at 15 min (7.5- and 8-fold, respectively). In contrast, sorbitol stimulated phosphorylation of p38-MAPK that reached maximal values at 45–60 min, whereas for mannitol, maximal values were observed after 5 min (Fig. 5*B*). JNK phosphorylation was also analyzed in similar samples. Sorbitol caused a strong phosphorylation of JNK, whereby the effect observed was more pronounced for p54-JNK than p46-JNK (Fig. 5*C*). In contrast, mannitol induced a stronger phosphorylation of the p46-JNK than of the p54-JNK isoform (Fig. 5*C*). Sorbitol-induced hyperosmotic stress, but not mannitol, caused a minor transient phosphorylation of PKB (Fig. 5*D*). Taken together, these results suggest that signaling pathways involving a variety of kinases are differentially activated by hyperosmotic stress in response to increased sorbitol or mannitol in cultured rat cardiomyocytes. The most significant differences triggered in response to mannitol or sorbitol were observed for phosphorylation of ERK and JNK.

Involvement of Stress Signaling Pathways in AR Protein Level and Activity—To test whether ERKs, p38-MAPK, JNKs, and PI3K/PKB are involved in the signaling pathway of AR induction by hyperosmotic condition, cardiomyocytes were preincubated with PD (50 μM, MEK-1 inhibitor), SB (10 μM, p38-MAPK inhibitor), SP (10 μM, JNK inhibitor) (61), and LY (50 μM, PI3K inhibitor) for 1 h and then treated with sorbitol for 24 h. The effects of such inhibitors on mannitol-treated cells were not assessed because of their inability to alter AR protein and activity levels as shown above. Cells cultured with isotonic serum-free medium served as controls. As shown in Fig. 6*A*, PD and SB, but not SP, blocked the increase in hyperosmotic stress-induced AR protein level in cardiac myocytes. LY has a minor effect that is not statistically significant. AR enzymatic activity correlated with AR protein levels (Fig. 6*B*). None of the inhibitors tested produced alterations either in cell viability or basal AR levels at the concentrations indicated. These experiments suggest that ERK and p38-MAPK activation are involved in events leading to increased AR function in response to hypertonic sorbitol. These results are consistent with the notion that phosphorylation-dependent activation of the osmotic response element-binding protein represents a key step in the induction of AR transcription by hyperosmolarity (62).

Effects of Hyperosmotic Solutions of Sorbitol and Mannitol on Cardiac Myocyte Apoptosis—We have previously reported that sorbitol-induced hyperosmotic stress is a rapid and effective inducer of cardiac myocyte apoptosis (15). The following study extends these findings by showing that mannitol did not induce similar apoptotic responses in cultured cardiomyocytes. The expression of the antiapoptotic protein Bcl-xL, a member of the Bcl-2 family, was compared in sorbitol- and mannitol-treated cardiomyocytes by Western blot analysis. As depicted in Fig. 7*A*, exposure of cardiac cells to sorbitol (400 and 600 mosmol/kg water) decreased Bcl-X_L levels (25 and 45%, respectively), whereas mannitol (300–600 mosmol/kg water) did not. To extend this finding, DNA was isolated from cardiomyocyte cultures exposed to sorbitol or mannitol and analyzed on agarose gels as described under "Experimental Procedures." In mannitol-treated cardiomyocytes, DNA laddering was not detectable (Fig. 7*B*), whereas sorbitol significantly increased the intensity of 180–1,200-bp DNA fragments at 24 h (Fig. 7*B*). Together these results indicate that apoptosis induced by hyperosmotic stress in cardiac myocyte depends on the nature of the osmolyte rather than the osmolarity itself.

Effect of Hyperosmotic Solutions of Sorbitol and Mannitol on Glutathione Levels—In order to explore a mechanism responsible for the differential effect of sorbitol and mannitol on cardiac myocyte death, intracellular GSH levels were determined in cardiomyocytes exposed to hyperosmotic stress (600 mosmol/kg water) with sorbitol or mannitol. Sorbitol decreased significantly GSH levels, whereas mannitol had no effect during the studied times (Fig. 8). In order to evaluate whether GSH levels were decreased as a consequence of GSH consumption, the effects of BSO (an irreversible inhibitor of γ-glutamyl cysteine synthetase) and NAC (a GSH precursor) were tested after a 6-h incubation with or without sorbitol. The presence of BSO or NAC did not modify basal GSH levels in cultured cardiac myocytes. However, after 6 h of exposure to sorbitol (600 mosmol/kg water) GSH levels decreased from 29.2 ± 2.3 to 15.5 ± 2.1 nmol/mg protein. This effect was either reduced by 44% or increased by 55% upon preincubation with NAC or BSO, respectively (data not shown). Collectively, these results suggest that intracellular GSH levels are reduced by hyperosmotic stress in the presence of sorbitol and can be restored by NAC.

A Role for Aldose Reductase Involvement in Cardiac Myocyte Apoptosis—Finally, having observed that hypertonic sorbitol decreased intracellular GSH levels and increased both AR and apoptosis, we investigated the possible link between these three events. Using the specific inhibitor zopolrestat at a concentration that abolished more than 90% of AR activity (Fig. 3*B*), both the increase in DNA fragmentation and the decrease in Bcl-xL levels, observed in response to sorbitol (600 mosmol/kg water), were prevented (Fig. 9, *A* and *B*). NAC also attenuated the increase in DNA fragmentation stimulated by hyperosmotic sorbitol (Fig. 9*B*). These data suggest that AR induction and GSH depletion are at least part of the cellular response leading to cardiomyocyte apoptosis upon exposure to hyperosmotic sorbitol.

DISCUSSION

Apoptosis occurs in response to a variety of stimuli under physiological and pathological circumstances. A visible decrease in cell body volume was recognized as a hallmark of apoptosis in the pioneering studies of Wyllie *et al.* (63). Other studies suggested that cell shrinkage (apoptotic volume decrease) developed relatively quickly in the course of apoptosis (64). Despite the universal prominence of cell volume loss in

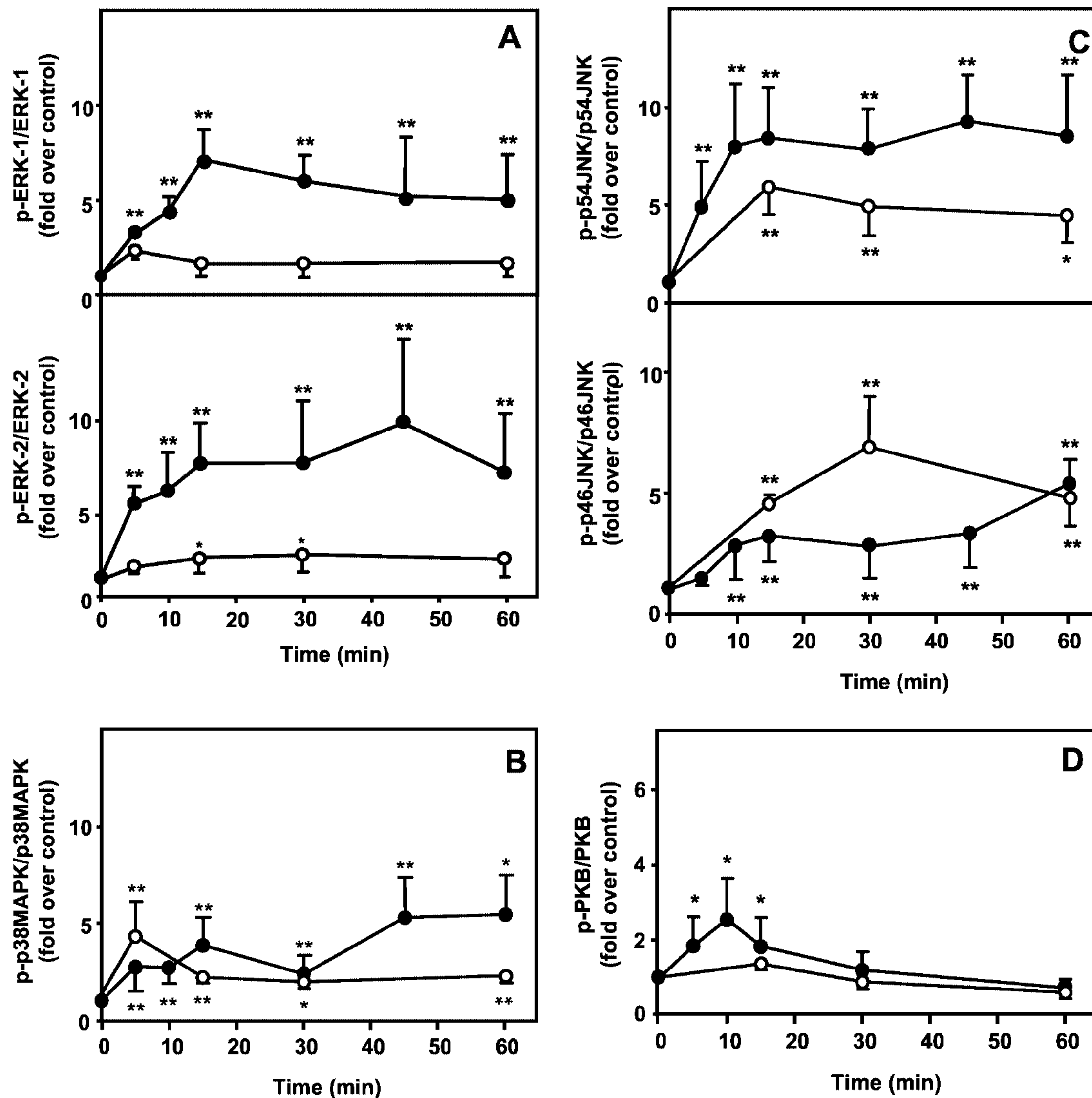

FIG. 5. **Different signaling pathways were activated by hyperosmotic sorbitol or mannitol in cultured cardiac myocytes.** Cells were exposed to sorbitol (600 mosmol/kg water; *black circles*) or mannitol (600 mosmol/kg water; *white circles*) for the indicated times. Then cells were lysed, and the phosphorylated and total levels of ERK-1 and ERK-2 (*A*), p38-MAPK (*B*), p46-JNK and p54-JNK (*C*), and PKB (*D*) were analyzed by Western blot as indicated under "Experimental Procedures." Values are means ± S.E. (n = 3–5 independent experiments) and are expressed as relative increase in the ratio of phosphorylated kinase/total kinase. *, $p < 0.05$; **, $p < 0.01$ *versus* time 0 min.

cells undergoing apoptosis, the relationship between this event and ensuing cell death remains uncertain. The ability of cells to resist osmotic shrinkage by cell volume regulation paralleled the resistance to apoptosis after osmotic shock (65). Interestingly, cardiac myocytes and thymic lymphocytes provide an exception to the volume regulatory response after exposure to hypertonic conditions in that they do not compensate for an initial reduction in cell volume caused by hypertonic conditions (22, 49, 66, 67). In thymocytes, the absence of RVI contributes to rapid activation of apoptosis (22). In contrast, we demonstrate here that cell volume decrease is not sufficient to trigger apoptosis by hyperosmotic stress in cultured cardiac myocytes.

We also show that exposure of cardiac myocytes to hyperosmotic solutions of sorbitol and mannitol resulted in cell shrinking to a similar degree. This decrease was maintained up to 50 min of continuous exposure, suggesting that cardiac myocytes did not regulate their volume under our hyperosmotic stress conditions. The lack of RVI shows that there was no net gain of either Na^+ or K^+ during the experiment. Therefore, although $Na^+K^+2Cl^-$ symporter (68), Na^+/H^+ exchanger (69), and nonselective cation channels (70) are present in the heart, these three mechanisms responsible for RVI in other cells were not activated in cardiac myocytes by hyperosmotic stress. The observed absence of regulation of those three systems in cardiomyocyte is consistent with the literature (52).

The chronic phase of the adaptive response to hyperosmolarity in the compatible osmolyte hypothesis is characterized by enhanced expression of several genes encoding proteins that mediate the accumulation of organic osmolytes; such as betaine, *myo*-inositol, taurine transporters, and the enzyme AR (23). The transporters serve to increase the intracellular concentration of organic osmolytes by facilitating uptake from the external medium. In contrast, AR metabolizes glucose to the nonpermeant osmolyte sorbitol. Therefore, osmoregulation may be the primary physiological function of AR. Expression of the genes for AR and betaine, *myo*-inositol, and taurine transporters has been shown to be stimulated at the transcriptional level by hyperosmotic stress, and osmotic response elements have been identified in their promoter regions; however, post-transcriptional regulatory mechanisms have also been described in some instances (59). In addition, tissues differ in the way they utilize usage of organic osmolytes during the adaptive

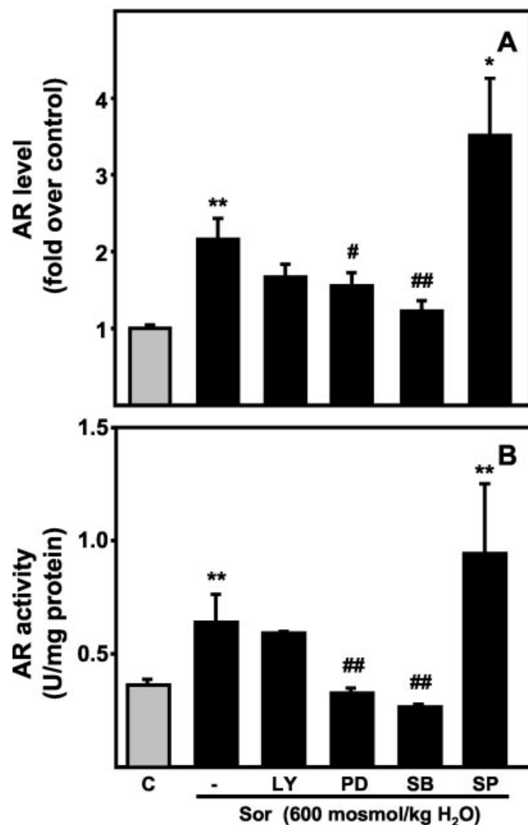

FIG. 6. **Effects of different inhibitors on induction of aldose reductase by sorbitol-dependent hyperosmotic stress.** Cultured rat neonatal cardiomyocytes were preincubated with LY (50 μM; PI3K inhibitor), PD (50 μM; MEK-1 inhibitor), SP (10 μM; JNK inhibitor), or SB (10 μM; p38-MAPK inhibitor) for 1 h before hypertonicity treatment with sorbitol (600 mosmol/kg water; *black bars*) for 24 h. Cells cultured with isotonic serum-free medium served as a control (270 mosmol/kg water; *gray bars*). After stimulation, cell protein extracts were obtained as described under "Experimental Procedures." *A*, expression of AR protein by Western blot analysis. Cell lysates were matched for protein, separated by SDS-PAGE, and analyzed by Western blotting using an anti-AR polyclonal antibody. Relative AR protein levels were obtained by densitometric analysis. *B*, AR activity. After stimulation, AR was semipurified by DEAE-cellulose chromatography as described under "Experimental Procedures." AR activity was assessed by monitoring the decrease in absorbance of NADPH in the absence or presence of 5 mM D-glucose and expressed as units/mg protein. Values are means ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* control (*C*). #, $p < 0.05$; ##, $p < 0.01$ *versus* Sor.

response. For example, the concentration of osmolytes is generally up-regulated in kidney medullary cells in response to hyperosmolarity. On the other hand, all, except sorbitol, accumulate in the rat brain during adaptation to acute and chronic hypernatremia (23).

In this study, we also demonstrate the presence of a functional polyol pathway in cardiomyocyte that is characterized by slow accumulation of sorbitol. Quite unexpectedly, AR activation was found to be dependent of the nature of the osmolyte used. Sorbitol and mannitol, two epimeres with identical osmotic properties, did not stimulate the same signaling mechanisms and cell responses in cultured cardiac myocytes. When cardiomyocytes were exposed to hypertonic solutions of sorbitol, the levels of AR protein and its enzyme activity were increased and correlated with the intracellular content of sorbitol. In contrast, mannitol employed under similar conditions did not have any effect. The different cellular events triggered by sorbitol and mannitol are not a generalized phenomenon, as demonstrated by their equivalent ability to stimulate cell shrinkage and glucose uptake. The accumulation of sorbitol

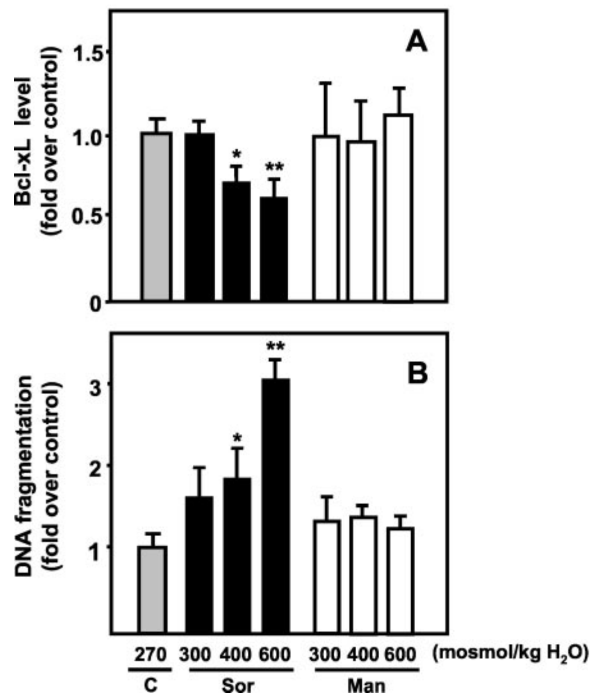

FIG. 7. **Effect of sorbitol or mannitol-dependent hyperosmotic stress on the induction of cardiac myocyte apoptosis.** Cultured cardiomyocytes were incubated in isotonic serum-free medium (control (*C*), 270 mosmol/kg water; *gray bar*) or solutions of Sor (300–600 mosmol/kg water; *black bar*) or Man (300–600 mosmol/kg water; *white bar*). After stimulation, Bcl-xL levels (*A*) and DNA fragmentation (*B*) were determined. *A*, cardiomyocyte lysates were subjected to Western blot analysis for Bcl-xL as described under "Experimental Procedures." *B*, genomic DNA was isolated and subjected to electrophoresis on 2% agarose gels and imaged by ethidium bromide staining and photography. DNA fragments were quantified by densitometry. Values are means ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ *versus* control.

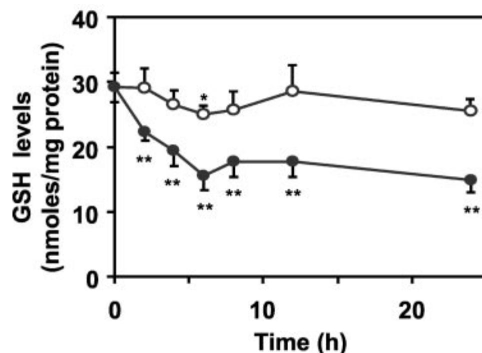

FIG. 8. **Hypertonic sorbitol and mannitol solutions differentially induced glutathione depletion in cultured cardiac myocytes.** Cells were incubated for the indicated times with isotonic serum-free medium (control; 270 mosmol/kg water) or sorbitol (600 mosmol/kg water; *black circles*) or mannitol (600 mosmol/kg water; *white circles*). After stimulation for the indicated times, total intracellular GSH levels were determined as described under "Experimental Procedures." Values are means ± S.E. of 4–7 independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* time 0 h.

over 24 h corresponds to 1.25 mg/mg protein. Considering that the volume of an individual cardiomyocyte is 2 pl (71), the intracellular sorbitol concentration amounts to 210 mM, which accounts for a substantial part of full volume regulation. The cell culture medium used in this study (Dulbecco's modified Eagle's medium/medium 199), did not contain taurine or betaine but did contain *myo*-inositol, a substrate for the Na^+-dependent co-transporter found in cardiac cells (68–70, 72, 73). Thus, it is possible that *myo*-inositol may accumulate in car-

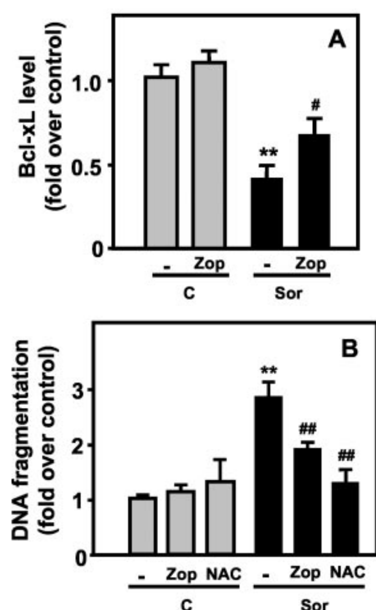

FIG. 9. **Effect of aldose reductase inhibition or replenishing of intracellular GSH levels on sorbitol-induced cardiac myocyte apoptosis.** Cultured cardiomyocytes were preincubated with zopolrestat (*Zop*), an AR inhibitor, or NAC (a GSH replenisher) for 1 h before hypertonicity treatment with Sor (600 mosmol/kg water; *black bars*) for 24 h. Cells cultured with isotonic serum-free medium served as control (*C*; 270 mosmol/kg water; *gray bars*). After stimulation, cell protein extracts and genomic DNA were obtained as described under "Experimental Procedures." Bcl-xL levels (*A*) and DNA fragmentation (*B*) were determined as described in Fig. 7. Results are means ± S.E. (n = 3–5 independent experiments). **, $p < 0.01$ *versus* control; #, $p < 0.05$ *versus* Sor; ##, $p < 0.01$ *versus* Sor.

diomyocytes. Currently, we do not know whether cells actually recovered their original volume after exposure to sorbitol or mannitol for 24 h. Since calcein-based methods cannot be employed for long term measurements, we are currently devising three-dimensional reconstruction methods to investigate such events. If, indeed, cell volume is restored, we will seek to identity the osmolytes (Na^+, K^+, *myo*-inositol, etc.) involved in this process.

Increases in AR protein levels and its activity could be protective or harmful depending on cell type, nature, and concentration of the stimuli. In renal cells, after increases in osmolarity up to 500 mosmol/kg water, an increase in AR activity results in sorbitol accumulation (27). This event has been associated with protection against apoptosis (27). However, in the same cells, extreme acute hypertonicity (700 mosmol/kg water) causes initiation of apoptosis (74). In cardiac tissue, a recent study has shown an increase in AR activity during low flow ischemia and shown that the pharmacological inhibition of aldose reductase is cardioprotective (75). This last finding suggests that AR plays a critical role in myocardial ischemic injury (75). Interestingly, AR inhibition also protected diabetic hearts from ischemic injury (33, 76). Our present results agree with these last findings, showing that AR triggers cell death by apoptosis in response to sorbitol-dependent hypertonicity.

Mannitol has been widely used because of its beneficial properties in clinical treatment of several pathologies including cardiac injury and cranial trauma. This beneficial effect has been associated with its osmotic properties and also its properties as an antioxidant (18–20). The 200–300 mosmol/kg water increment used in our study corresponded to the maximal mannitol concentrations (final osmolarity 500–600 mosmol/kg water) achieved transiently in clinical applications, assuming equilibration throughout the vasculature. Much higher local concentrations can be reached transiently (17, 76).

TABLE I
Different cellular events triggered by hyperosmotic solutions of sorbitol and mannitol in cultured cardiac myocytes

Parameter	Sorbitol	Mannitol
Cell volume decrease	+++	+++
Stress-activated pathways		
ERKs	++	+/−
p38-MAPK	+	+
p54-JNK	+++	++
p46-JNK	+	++
PKB	+	−
Hexose uptake stimulation	+++	+++
GSH depletion	+++	−
Polyol pathway		
Aldose reductase protein increase	+++	−
Aldose reductase activity increase	+++	−
Sorbitol dehydrogenase protein	++	−
Intracellular sorbitol increase	+++	−
Apoptosis		
Bcl-xL decrease	++	−
DNA fragmentation	+++	−

Our results show that mannitol is an osmoactive compound that did not induce cell damage. Hyperosmotic mannitol solution has been shown to decrease myocardial reperfusion injury (78). Because AR seems to play an essential role in cardiac ischemic injury, the induction of AR by hypertonic solutions could be considered deleterious. The cardioprotection of hyperosmotic mannitol solution observed by Harada *et al.* (78) may, in part, be explained according to our results by its incapacity to induce AR and hence lead to accumulation of intracellular sorbitol in cardiac myocytes. However, there is evidence that mannitol can induce AR and increase the intracellular sorbitol content in other type of cells. For example, in human retinal pigment epithelial cells, myoinositol and sorbitol behaved as compensating intracellular osmolytes by accumulating markedly in response to hyperosmolarity with mannitol (79). In agreement with this last observation, L-929 cells grown in hyperosmotic media (600 mosmol/kg water) containing NaCl or mannitol showed markedly enhanced expression of a protein corresponding to AR as well as accumulation of intracellular sorbitol (80). These data establish that mannitol has differential effects on AR depending on the type of cells under investigation.

The mechanisms whereby hyperosmolarity regulates AR induction are not fully understood. The control of osmoprotective genes is an extremely complex process, involving MAPKs and PI3K pathways (23, 27). Because hyperosmolarity has been shown to affect these signaling pathways, their involvement in the regulation of the osmotic effects was studied. Hypertonicity increases synthesis of AR mRNA 15-fold in 24 h without a detectable change in the rate of degradation of the AR protein (81). Although the activation of ERK, JNK, and p38-MAPK by hyperosmotic stress has been demonstrated in mammalian cells (82–84), ERK activity was not found to be essential for transcriptional regulation of betaine and inositol transporters. Studies in the Madin-Darby canine kidney renal epithelial cell line showed that a specific p38-MAPK inhibitor, SB-203580, blocked the induction of betaine/γ-amino-*n*-butyric acid transporter mRNA in response to hyperosmotic medium (85), suggesting that the p38-MAPK pathway is essential for the hypertonicity response. However, our pharmacological findings support a role for ERK and p38-MAPK in the modulation of both AR protein levels and activity. Consistent with this finding, Nadkarni *et al.* (86) showed that the hypertonic induction of AR mRNA in HepG2 cells is regulated by p38-MAPK and MEK1. Inhibition of either protein kinase independently results in loss of the hypertonic effects, suggesting that both signal pathways are necessary for the hyperosmotic response

(86). In a rat aortic smooth cell line, activation by phosphorylation and/or increased synthesis of osmotic response element-binding protein(s) are key steps in induction of transcription of the rat AR gene by hyperosmolarity (62). Future studies should define whether these protein kinases affect the nuclear translocation and activation of specific transcription factors and/or their binding to the osmotic response element.

Finally, we also explored the mechanism involved in the cardiomyocyte apoptosis triggered by hyperosmotic stress. Our data show that zopolrestat, a specific and potent inhibitor of AR (56), almost completely inhibited sorbitol-dependent AR activation and apoptosis. These results suggest that AR plays an important role in the induction of apoptosis by sorbitol-dependent hyperosmotic stress in cardiomyocytes. This result is also in agreement with data obtained in lens epithelial cells and pancreatic β-cells, where apoptosis was dependent on increased AR activation (36, 37). Our results also suggest that GSH depletion may be involved in processes leading to induction of apoptosis by hyperosmotic stress in cardiac myocytes. Sorbitol, but not mannitol, caused a significant decrease in intracellular GSH levels, whereas pharmacological GSH supplementation with NAC resulted in decreased DNA fragmentation. These data agree with those reported in cultured retinal pericytes, where glucose-induced apoptosis is mediated through an AR-sensitive pathway, involving increased oxidative stress characterized by reduced GSH content (38). In diabetic rat lens cells, increased activity of the polyol pathway was also associated with changes in NAD^+/NADH and $NADP^+$/NADPH levels (87).

In summary, the results show that hyperosmotic stress induced by sorbitol and mannitol triggers different cellular events in cultured cardiac myocytes (Table I). Our studies provide evidence that (*a*) physical shrinkage of cardiac myocytes is not sufficient to induce apoptosis, (*b*) a functional polyol pathway is present in cardiomyocytes, but its induction by hypertonicity is dependent of the nature of the osmolyte, (*c*) different signaling pathways regulate AR protein expression and activity in cardiac myocytes, and (*d*) AR and/or GSH depletion mediate apoptosis induced by hyperosmotic sorbitol concentrations.

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