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A rapid and strong apoptotic process is triggered by hyperosmotic stress in cultured rat cardiac myocytes

Abstract In all cell types, the maintenance of normal cell volume is an essential homeostatic function. Relatively little is known about the induction of apoptosis by hyperosmotic stress and its molecular mechanism in terminally differentiated cardiac myocytes. We compared the apoptotic response of cultured neonatal rat cardiomyoctes to hyperosmotic stress by sorbitol (SOR) with those induced by doxorubicin (Doxo) or angiotensin II (Ang II). We also examined the apoptotic-signaling pathway stimulated by the hyperosmotic stress. Apoptosis was assessed by the observation of: (1) cell viability, (2) DNA fragmentation detected by the TUNEL method and by agarose gel electrophoresis, and (3) poly(ADP-ribose)polymerase (PARP) degradation, and Bcl-X_S and Bcl-X_L levels by Western blot analysis. Exposure of cardiomyocytes to 0.3 M SOR for 24 h resulted in decreased cell viability and increased generation of oligosomal DNA fragments (2.5-fold of controls). At this time, 83±5% of SOR-treated myocytes were TUNEL-positive (vs 23.7±6.8% in controls; $P<0.01$). PARP levels also decreased by approximately 42% when cardiac myocytes were exposed to SOR. Hyperosmotic stress induced a more rapid and stronger apoptotic response in cardiomyocytes than Doxo or Ang II. In addition, SOR increased 3.2-fold Bcl-X_S proapoptotic protein without changes in Bcl-X_L antiapoptotic protein levels and in the p53-transactivating activity. Taken together, these results strongly suggest that hyperosmotic stress triggers cardiac myocyte apoptosis in a p53-independent manner, being earlier and stronger than apoptosis induced by Doxo and Ang II.

Keywords Osmotic stress · Angiotensin II · Doxorubicin · Cardiac myocyte · Apoptosis · Cell culture · Rat · Sprague Dawley

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Introduction

Apoptosis in the cardiac cells has been suggested only recently as a major mechanism of cardiac cell death (Brömme and Holtz 1996). Evidences of apoptosis have been reported from different aspects of cardiac pathology (James et al. 1996; Kajstura et al. 1996; Narula et al. 1996; Gottlieb and Engler 1999). This process is also activated in terminally differentiated cardiomyocytes exposed to hypoxia (Long et al. 1997), mechanical stretching (Cheng et al. 1995), cytokines (Pulkki 1997), doxorubicin (Doxo; Delpy et al. 1999), and angiotensin II (Ang II; Cigola et al. 1997). The apoptotic intracellular signaling pathways are not fully understood, but a critical component of this mechanism is the activation of a group of caspases (cysteine proteases) which cleave a set of proteins [i.e., poly(ADP-ribose)polymerase, PARP], leading to nucleosomal fragmentation of DNA and disassembly of the cell (Allen et al. 1998). The activities of caspases are regulated by the Bcl-2 family, and some of these proteins such as Bcl-X_S, Bax, Bak, and Bad promote apoptosis, whereas others such as Bcl-X_L and Bcl-2 block cell death. Neonatal cardiac myocytes expressed predominantly Bcl-X_L and Bak but low levels of Bcl-2 and Bax (Ing et al. 1999). Multiple pathways exist for inducing apoptosis. Depending on the cellular type, elevated levels of p53 after DNA damage can result in either growth arrest or apoptosis (Kastan et al. 1992; Lowe et

al. 1993). There are controversial findings about the role of p53 in cardiac myocyte apoptosis. Increased activity of p53 protein has been reported to accompany apoptosis in response to stretching (Leri et al. 1998). In this model, increased levels of Ang II induced expression of p53, promoting changes in the balance between Bcl-2 and Bax in favor of apoptosis (Leri et al. 1998). Cardiac myocyte apoptosis resulting from hypoxia-acidosis is, however, independent of changes in the p53 (Webster et al. 1999).

Osmotic changes can occur in pathological states such as ischemia, septic shock, and diabetic coma (Wright and Ress 1998). If the osmolarity around a cell is decreased, the cell swells, and if increased, it shrinks. In order to tolerate changes in osmolarity, cells have evolved volume-regulatory mechanisms, activated by osmotic challenge to normalize cell volume and maintain normal function. In the heart, osmotic stress is encountered during a period of myocardial ischemia (Wright and Ress 1998).

Hyperosmotic stress has been shown to induce apoptosis in cell lines such as human neuroblastoma (Matthews et al. 1997), Jurkat T lymphocytes (Juo et al. 1997), rat alveolar type II cells (Edwards et al. 1998), and, recently, cardiac myocytes (Hoover et al. 2000; Morales et al. 2000) and cardiac fibroblasts (Mockridge et al. 2000). However, relatively little is known about the apoptotic mechanism stimulated by hyperosmotic stress in terminally differentiated cardiac myocytes. We undertook the present study: (a) to compare hyperosmotic stress-programmed cell death response in cultured cardiac myoctes with other apoptotic stimuli previously reported such as Doxo (Delpy et al. 1999) and Ang II (Cigola et al. 1997); and (b) to determine the apoptotic signaling pathway activated by hyperosmotic stress. We studied the hyperosmotic stress effect on Bcl-X_S, Bcl-X_L, PARP, DNA fragmentation, and transactivation of p53-dependent gene expression in cultured neonatal rat cardiac myocytes. Hyperosmotic stress induced a more rapid and stronger cardiac myocyte apoptosis than the other studied stimuli. This response to osmotic stress results, in part, from the increase in Bcl-X_S levels and the activation of caspases but without participation of p53.

Materials and methods

Chemicals

Sorbitol (SOR), Ang II, Doxo, bafilomycin A1, RNase, proteinase K, Dulbecco's modified Eagle's medium (DMEM), medium 199 (M199), protease inhibitors, and other biochemicals were purchased from Sigma (St. Louis, Mo.) unless stated otherwise. Heat-inactivated fetal calf serum (FCS), newborn calf serum (NCS), and other tissue culture products were from Life Technologies (Gaithersburg, Md.). Antibodies raised against PARP, Bcl-X_S, Bcl-X_L, and peroxidase-conjugated anti-IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca.). ECL immunoblotting detection reagents, autoradiographic film, and prestained molecular mass standard proteins were from Amersham International. Protein assay reagents were from BioRad (Richmond, Va.). Plasmid pON249, a gift from Dr. K.R. Chien (Department of Medicine, University of California, San Diego), in which β-galactosidase (β-gal) expression is controlled by a constitutive cytomegalovirus (CMV) promoter, was cotransfected to control for transfection efficiency. PG13, MG15, and pBV luciferase reporter constructions were kindly provided by Dr. B. Volgestein (Johns Hopkins University, Baltimore).

Culture and treatment of ventricular myocytes

All procedures involving animals were performed in accordance with institutional guidelines for the care and use of animals. Neonatal ventricular myocytes were prepared from hearts of 1- to 3-day-old Sprague-Dawley rats (Animal Breeding Facility, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile) as described previously by Foncea et al. (1997). The myocytes, plated at a final density of $1.4\times10^3/mm^2$ on gelatin-precoated 60-mm dishes, were confluent and spontaneously beating after 18 h. This procedure yielded less than 5% of noncardiomyocytes in the cultures based on staining of sarcomeric apparatus with anti-α-actinin antibody. In addition, to inhibit the proliferation of noncardiomyocyte cells, bromodeoxyuridine was added throughout the culture period. To study cell death by apoptosis, different cell culture conditions have been used. Basically, cells have been cultured with or without different serum concentration in the culture medium. Since there is no consensus in the specialized literature with respect to this last point, and in order to avoid the interference of any undefined substances present in the fetal serum, our experiments were done under serum-free conditions. Cells were treated with or without the following agonists: 0.3 M SOR, 100 nM Ang II, or 1 µM Doxo in serum-free medium (DMEM-M199) at 37°C for 0–48 h. Cardiac myocytes incubated in DMEM-DME served as controls. In some experiments (TUNEL), cardiac myocytes (0.5×10^6 cells) were cultured on coated coverslip dishes and treated for 24 h with the agonists.

Cell viability

The number of viable cells were determined by trypan blue exclusion. Cells were rinsed once with phosphate-buffered saline (PBS) and then resuspended with trypsin and EDTA. The cells were immediately stained with 0.5% trypan blue, and the number of viable and nonviable cells were determined.

Detection of DNA ladder formation in apoptotic cultured cardiomyocytes

For the detection of DNA fragmentation, cardiomyocytes were washed three times with cold PBS and sedimented by centrifugation. Cellular 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), 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 reextracted from supernatants with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the upper aqueous phase using 0.1 vol of 3M sodium acetate (pH 5.2) with 2 vol of ice-cold ethanol and left at –20°C overnight before centrifugation. Pellets were resuspended in 200 µl TE buffer, followed by a 60-min incubation with DNase-free RNase A (2 mg/ml) at 37°C. Samples were reextracted, and DNA was precipitated as described. Pellets were resuspended in TE buffer, and DNA concentrations were quantified by measuring 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 of ethidium bromide.

Detection of apoptotic cardiomyocytes by the TUNEL method

Apoptotic cells were detected by the TUNEL method using an in situ detection kit (Promega) according to the manufacturer's recommended protocol. Briefly, cells were cultured on circular coverslips coated with 2% gelatin, fixed in 4% paraformaldehyde for 25 min at 4°C, and then washed in PBS three times for 5 min each. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then incubated with fluorescein-labeled dUTP for 60 min at 37°C to detect the free 3' hydroxyl fragmented DNA ends. After washing in PBS, apoptotic nuclei were visualized by immunofluorescence after all nuclei were stained with a propidium iodide solution.

Preparation of cell extracts

Culture medium was removed by aspiration and the cells were washed twice with cold PBS. Cardiac myocytes were scraped into 100 µl of cold lysis buffer (10 mM TRIS-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1% v/v Triton X-100). Samples were centrifuged at 10,000*g* for 15 min at 4°C, and the protein content of the supernatant was determined by BioRad Bradford assay (Bradford 1976). Soluble fractions from myocytes were heated at 95°C with 0.33 vol of SDS-PAGE sample buffer.

Western blot analysis

Proteins (20–50 µg) were separated by SDS-PAGE on an 8% (for PARP) or 15% (for Bcl-X_S and Bcl-X_L) polyacrylamide gel and were transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked with 5% (w/v) nonfat milk powder in PBS (pH 7.5) containing 0.05% (v/v) Tween-20 (PBST) for 60 min at room temperature. Primary antibodies were diluted in blocking solution (1:200 for anti-PARP, 1:100 for anti-Bcl-X_S, and 1:500 for anti-Bcl-X_L). Nitrocellulose was incubated with primary antibodies overnight at 4°C. After washing in PBST (3 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, bound antibody was detected by ECL with exposure to Hyperfilm for 0.5–30 min. Blots were quantified by scanning densitometry.

Transient transfection of cardiomyocytes in culture and the p53 transactivation

Transfections (by the calcium phosphate method) involved the addition of 14 µg of luciferase reporter plasmid and 2 µg of pON249 to 60-mm cell culture dishes containing 1×10^6 cells. SOR or bafilomycin A1 (positive control; see Long et al. 1997), when present, was added to a concentration of 0.3 M and 100 nM, respectively, 20 h after the transfection. After a further 6–24 h, cardiac myocytes were extracted and assayed for luciferase and β-gal activities as detailed previously by Long et al. (1997). p53 transactivating ability was assessed from the activity of the transfected reporter, PG13-luc, which carries the luciferase reporter gene driven by the minimal polyoma promoter and 13 copies of the wild-type p53-binding site. For controls, cardiac myocytes were transfected with pMG15-luc, a luciferase reporter plasmid driven by the minimal polyoma promoter and 15 copies of a mutated p53-binding site or pBV-luc (Long et al. 1997).

Expression of results and statistical analysis

Results are expressed as means ± SEM for the number of independent experiments indicated (*n*). Time-course analysis was performed using ANOVA, and comparisons between groups were performed using a protected Tukey's *t*-test.

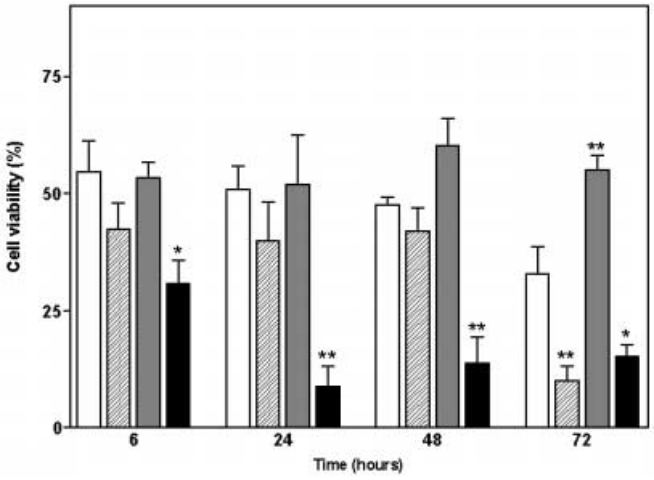

Fig. 1 Cell viability in the different experimental apoptotic models on cultured cardiac myocytes. Cells were incubated in serum-free media or in medium containing 1 µM doxorubicin (*crossed-hatch bar*), 100 nM angiotensin II (*gray bar*), or 0.3 M sorbitol (*black bar*) for the indicated times. Cardiac myocytes cultured in DMEM-DME served as controls (*white bar*). The cultures were harvested 6 h, 24 h, 48 h, and 72 h after exposure to the stimuli, and cell viability was assessed by trypan blue exclusion as described in Materials and methods. Values are means ± SEM (*n*=3–5 independent experiments). **P*<0.05; ***P*<0.01 vs control

Results

Effect of hyperosmotic stress on cardiac myocyte viability

Figure 1 shows that in control plates the number of viable cardiomyocytes maintained was almost 45% over 72 h of the number of plated cells. As depicted in Fig. 1, only cells cultured with SOR showed a significant decrease in cardiac myocyte viability throughout the study (6–72 h). Doxo induced a late cell death response which was detectable after 72 h. In contrast, no cell death was observed with Ang II. Relative to control plates, the addition of 0.3 M SOR further decreased the number of cells to 30% and 10% at 6 and 24 h, respectively (Fig. 1). Exposure to SOR for 48–72 h did not cause any further decrease in the cell number. Higher concentrations of SOR (i.e., 0.6 M) induced a deleterious acute response in cultured cardiac myocytes (data not shown). Cell viability assay using trypan blue exclusion, however, cannot differentiate cell death by apoptosis or necrosis. The remaining cells after treatment of primary cell cultures with SOR may be nonmyocyte cells, probably cardiac fibroblasts. Our data showed that cultured cardiac fibroblasts responded to SOR in a similar way to cultured cardiac myocytes (results not shown). This agreed with the recent report by Mockridge et al. (2000).

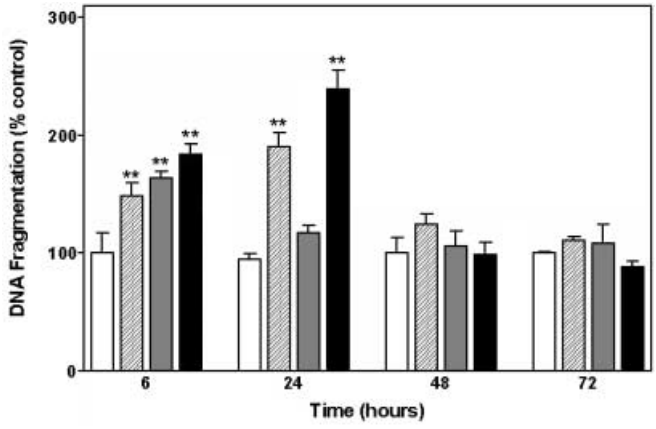

Fig. 2 Time-course of osmotic stress and other apoptotic stimuli-induced, internucleosomal DNA fragmentation in neonatal cultured cardiomyocytes. Genomic DNA was isolated from cardiac myocytes cultured in serum-free media or medium containing 1 µM doxorubicin (*crossed-hatch bar*), 100 nM angiotensin II (*gray bar*), or 0.3 M sorbitol (*black bar*) for the indicated times, subjected to electrophoresis on 2% agarose gels, and imaged by ethidium bromide staining and photography. Cardiac myocytes cultured in serum-free medium served as controls (*white bar*). Values (quantitation of DNA fragments) are means ± SEM of 3–5 independent experiments. ***P*<0.01 vs control

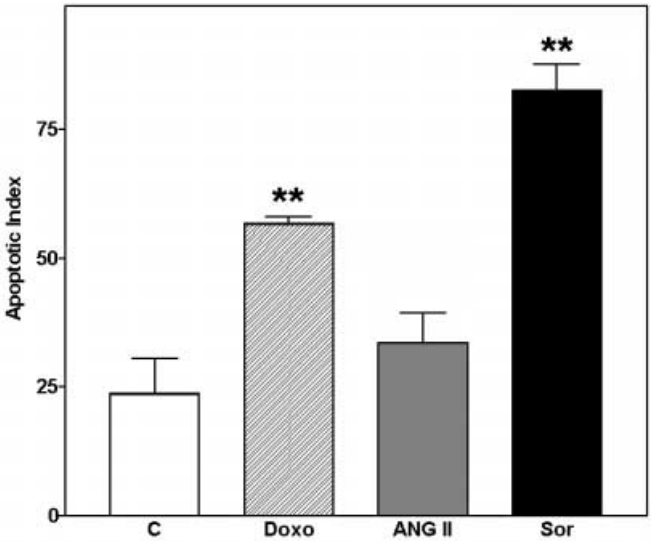

Fig. 3 In situ detection of DNA fragmentation induced by osmotic stress and other stimuli on cultured cardiac myocytes. Neonatal rat cardiomyocytes were cultured in serum-free media or medium containing 1 µM doxorubicin (*Doxo*), 100 nM angiotensin II (*Ang II*), or 0.3 M sorbitol (*Sor*) for 24 h. Cells cultured in serum-free medium served as controls (*C*). Cells were washed in PBS, fixed, permeabilized, and then labeled with fluorescent dUTP following the manufacturer's protocol described in Materials and methods. The labeled cells were analyzed by fluorescent microscopy. Quantitative apoptosis was determined by counting apoptotic cardiac myocytes and expressing them as percentage of total cell counted (apoptotic index). Values are means ± SEM for 5–12 independent experiments. ***P*<0.01 vs control

Effect of hyperosmotic stress on DNA fragmentation in cultured cardiomyocytes

DNA cleavage into nucleosome-sized fragments is a hallmark of apoptosis and results from the caspase-induced activator of a specific DNase. To investigate whether cardiomyocyte death after the stimuli was primarily due to apoptosis, DNA was isolated from cell cultures containing SOR, Ang II, Doxo, and control cells and analyzed on agarose gels as described in Materials and methods. Control and Ang II-treated cells exhibited a low level of DNA laddering, which was maintained over the course of the experiment. In contrast, SOR and Doxo clearly increased the intensity of 180- to 1,200-bp DNA fragments over 24 h (Fig. 2). The effect of SOR and Doxo on DNA fragmentation was maximal at 24 h and similar in magnitude. However, cell viability was significantly different in both stimuli at this time, indicating the coexistence of necrosis and apoptosis in cells cultured with SOR.

We further visualized DNA fragmentation in situ in individual cardiac myocytes by using the TUNEL method. The results are shown in Fig. 3. After 24 h, control cells exhibited nuclear labeling in 24±6% of cells. In contrast, treatment with SOR, Doxo, or Ang II for 24 h increased the fraction of apoptotic cells to 83±5%, 57±1%, and 34±6%, respectively (n=5–12). Cells treated with 1 mg/ml DNase I (used as a positive control) exhibited positive staining (data not shown). When the terminal deoxynucleotidyl transferase (TdT) was omitted from the reaction solution, no positively stained cardiac myocytes were detected (data not shown), indicating the specific labeling targets were the new 3' hydroxyl DNA ends generated by DNA fragmentation induced by SOR.

Effect of osmotic stress on PARP fragmentation in cultured cardiomyocytes

A well-characterized substrate of caspase-3 is PARP, which maintained the integrity of chromosomal DNA. To confirm the activation of the apoptotic pathway in SOR-treated cardiac myocytes, PARP degradation was analyzed in cell lysate by immunoblotting with an anti-PARP antibody (Fig. 4). In control cells, a 112-kDa band corresponding to PARP was identified. The PARP levels reduced markedly after treatment for 6–48 h with SOR, whereas Doxo decreased PARP levels in 50% of controls after 48 h. In contrast, Ang II did not stimulate PARP degradation throughout the study.

Response of Bcl-X_S and Bcl-X_L to hyperosmotic stress in cultured cardiomyocytes

The expression of the antiapoptotic (Bcl-X_L) and pro-apoptotic (Bcl-X_S) members of the Bcl-2 family was assessed in control and SOR-treated cardiomyocytes by Western blot analysis. In neonatal cardiac myocytes, the Bcl-X_S and Bcl-X_L antibodies detected bands of 26 kDa and 30 kDa, respectively. As shown in Fig. 5, SOR (0.3 M) for 24 h stimulated an increase in Bcl-X_S levels (3.2-fold of control), but without changes in Bcl-X_L levels in cardiac myocytes (P<0.01 vs control).

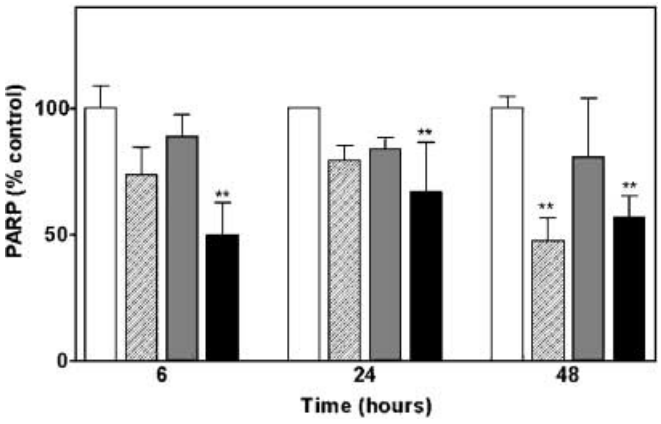

Fig. 4 Effect of osmotic stress and other stimuli on poly(ADP-ribose)polymerase (*PARP*) levels from cultured cardiomyocytes. PARP levels were assayed at various times after treatment of neonatal cardiac myocytes with serum-free media or medium containing 1 μM doxorubicin (*crossed-hatch bar*), 100 nM angiotensin II (*gray bar*) or 0.3 M sorbitol (*black bar*). Cells cultured in serum-free medium served as controls (*white bar*). Cardiomyocyte lysates were subjected to Western blot analysis as described in Materials and methods. Values are means ± SEM for three independent experiments. ***P*<0.01 vs control

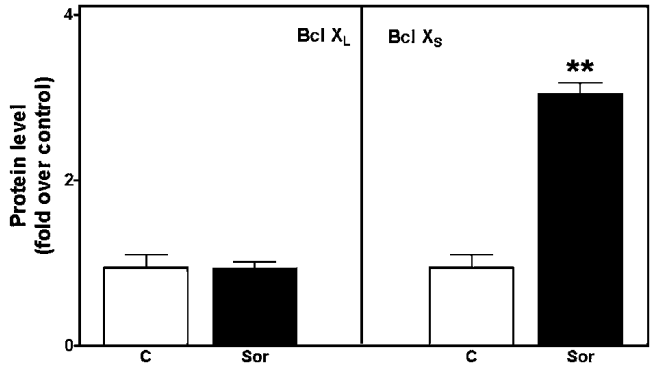

Fig. 5 Effect of hyperosmotic stress by sorbitol on Bcl-X_S and Bcl-X_L levels in cultured cardiomyocytes. Bcl-X_S and Bcl-X_L levels were assayed after treatment of neonatal cardiac myocytes with serum-free media (control, *C*) or 0.3 M sorbitol (*Sor*) for 24 h. Cardiomyocyte lysates were subjected to Western blot analysis as described in Materials and methods. Values are means ± SEM for three independent experiments. ***P*<0.01 vs control

Effect of osmotic stress on p53 transactivation in cultured cardiomyocytes

The measurement of p53-dependent gene transactivation was performed using a p53-dependent reporter gene. As shown in Fig. 6, the exposure of the cardiomyocytes to serum-free media for 12 h and 24 h (control cells) resulted in an increase in p53 activity. When the cultures were exposed to 0.3 M SOR for 6 h, 12 h, and 24 h, the p53 transactivating activity was less than 50% of the control condition, suggesting that SOR induced-apoptosis was p53-independent (Fig. 6; $P<0.01$ vs control). Nontransfected cells, as expected, gave a relative luciferase activity of almost zero, and the transfection with control

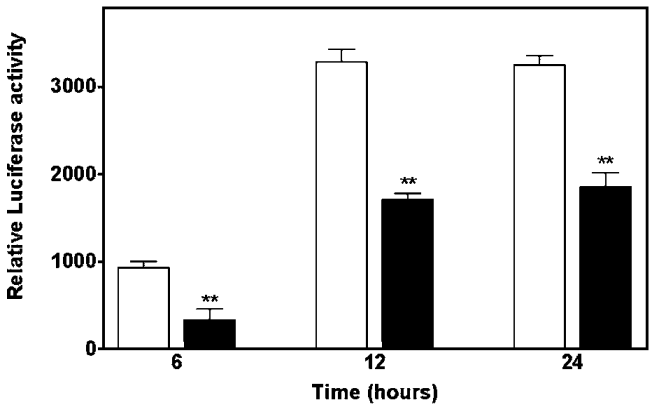

Fig. 6 Effect of hyperosmotic stress on p53-mediated sequence-specific transactivation in cultured cardiomyocytes. Cultured neonatal cardiac myocytes were transfected with pPG13-luc as described in Materials and methods. All cells were cotransfected with pON249 (to control β-galactosidase expression) to normalize variations in transfection efficiencies between experiments. Cardiac myocytes were harvested 6 h, 12 h, and 24 h after exposure to serum-free media (*white bar*) or medium containing 0.3 M sorbitol (*black bar*) and analyzed for luciferase and β-galactosidase activity. Untransfected cells were considered as a negative control (not shown). Values reported are the means ± SEM for at least three independent experiments and are expressed as luciferase activity normalized to β-galactosidase activity. ***P*<0.01 vs control

plasmids pBV-luc and pMG15-luc (see Materials and methods) showed a relative luciferase activity of the same magnitude as in the untransfected condition (data not shown).

Discussion

To our knowledge, this is the first report comparing different apoptotic stimuli in cardiac myocytes and determining the apoptotic signaling mechanism activated by hyperosmotic stress in this cell type. Our results showed that exposure to hyperosmotic stress stimulated apoptosis of neonatal rat cardiomyocytes in vitro in a p53-independent manner. This effect was more rapid and stronger in magnitude than the apoptosis induced by Doxo as indicated by typical nuclear and biochemical changes. Ang II did not stimulate an apoptotic process in cultured cardiac myocytes, which is not in agreement with Cigola et al. (1997). Both angiotensin II type 1 and 2 receptors (AT1 and AT2, respectively) have been described in cardiac myocytes as having antagonist effects on cell proliferation, hypertrophy, and apoptosis (Sadoshima 2000). The activation of both receptors may explain why, in our experiment, Ang II did not induce apoptosis in cultured cardiac myocytes.

It is now apparent that long-lived, terminally differentiated cardiomyocytes retain the ability to die via the apoptotic mechanism. Increased interest in apoptosis research in cardiology mainly stems from the hope that understanding the mechanisms of apoptosis in cardiac myocytes may provide new strategies to prevent cardio-

myocyte loss. Although the stimuli controlling programmed cardiac cell death remain unknown, there is evidence that hypoxia, mechanical stretching, cytokines, and anticancer drugs (Cheng et al. 1995; Long et al. 1997; Pulkki 1997; Delpy et al. 1999) can stimulate apoptosis in cardiac myocytes in vitro. We showed that SOR-induced hyperosmotic stress triggered a rapid and strong apoptosis in cultured cardiac myocytes. Detection of apoptosis was performed by three methods: (1) gel electrophoresis, searching for oligonucleosomal DNA fragmentation, (2) a nick-end labeling procedure with which to identify apoptosis in individual cells (TUNEL), and (3) by the assessment of Bcl-X_S, Bcl-X_L, and PARP levels as evidence of caspase activation. All the results were consistent, indicating that hyperosmotic stress is a strong stimulus for cardiac apoptosis. These results are consistent with previous reports that hyperosmotic stress induces cardiac myocyte apoptosis (Hoover et al. 2000; Morales et al. 2000), as well as inducing apoptosis in neuroblastoma cells (Matthews et al. 1997), alveolar type II cells (Edwards et al. 1998), and cardiac fibroblasts (Mockridge et al. 2000). They are also in agreement with other reports (Cigola et al. 1997; Delpy et al. 1999), which demonstrated the development of an apoptotic process in cardiac myocytes after exposure to Ang II and Doxo.

These results must be interpreted with caution and one must be prudent in extrapolating to the in vivo situation for the following reasons. First, although isolated cardiac myocyte preparations are well suited to study the apoptotic potential of osmotic stress and the molecular mechanisms involved, baseline rate of apoptosis is reported to be in the range of 5–10% in most of studies in cultured cardiomyocytes, which exceeds the true baseline apoptotic rate in cardiac tissue sections by approximately 1000-fold (Haunstetter and Izumo 2000). The in vivo relevance of osmotic stress-stimulated cardiomyocyte apoptosis remains to be determined in further studies. Second, we showed that hyperosmotic stress induces apoptosis in as many as 83% of the cells, which is certainly a lot more than the true incidence, even in physiopathological conditions. Third, although positive TUNEL staining has also been related to DNA repair and not to apoptotic DNA breakdown in cardiac tissue (Kanoh et al. 1999), SOR-induced PARP degradation was a clear experimental demonstration of caspase activation and execution of the apoptotic process.

There are potentially a large number of mechanisms by which hyperosmotic stress could induce cardiomyocyte apoptosis. One is the activation of caspases by an osmosensor-mediated pathway, which is an unsolved issue that needs to be addressed by future research. Another possibility is the increased expression of p53 as observed in a hypoxia model of apoptosis (Long et al. 1997) or during stretch-induced cardiomyocyte apoptosis and the resulting secretion of Ang II (Leri et al. 1998). Our results demonstrated, however, that there was no increased p53 transactivating activity in cardiac myocytes exposed to hyperosmotic stress, in agreement with Webster et al. (1999), who has reported that hypoxia-acidosis-mediated cardiomyocyte apoptosis is also independent of p53. Many lines of evidence have suggested that some mitogen-activated protein kinases (MAPKs), including extracellular regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 MAPK, play important roles in cell survival and cell death (Xia et al. 1995). Bogoyevith et al. (1995) have shown that osmotic stress induced by SOR produced sustained activation of JNKs, 10- to 20-fold for at least 4 h, in ventricular myocytes. It remains to be determined whether JNK activation and other signaling pathways are necessary for hyperosmotic stress-induced cardiomyocyte apoptosis.

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