IGF-1 Regulates Apoptosis of Cardiac Myocyte Induced by Osmotic-Stress

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Insulin-like growth factor-1 (IGF-1) is a natural protectant of cardiac myocytes that has been shown to improve cardiac function. The role of IGF-1 in attenuating apoptosis induced by osmotic stress (sorbitol, SOR) or by other known apoptotic stimuli (doxorubicin, angiotensin II, and serum withdrawal) was determined in cultured cardiac myocytes. After 6 h of exposure to SOR, apoptosis was initiated, concomitant with a decrease in cell survival and increases in poly-[ADP-ribose] polymerase (PARP) degradation and DNA fragmentation. These effects were maximal after 24 h. IGF-1 partially attenuated apoptosis induced by sorbitol but not that induced by angiotensin II, doxorubicin, or serum withdrawal. In cells preincubated with IGF-1 before the addition of SOR, we detected an increase in the number of viable cells, a decrease in the generation of DNA fragments on agarose gel electrophoresis and in the percentage of positive TUNEL cells, and a reduction on PARP levels. These results suggest that IGF-1 prevents apoptosis induced by osmotic stress in cardiac myocytes but not apoptosis induced by doxorubicin and angiotensin II.

Key Words: **insulin-like growth factor-1; apoptosis; osmotic stress; angiotensin; doxorubicin.**

The mechanisms responsible for the death of cardiac tissue cells in many heart diseases have been of increasing interest in recent years. It has recently been proposed that apoptosis plays a major role in cardiac cell death (1). Apoptosis is a form of cell death that involves discrete genetic and molecular programs, de novo protein expression and a unique cellular phenotype. This process also represents an active, ubiquitous, evolutionarily conserved, physiological and energy-dependent process in which cells participate in their own destruction (2). Apoptosis differs from necrosis in that there is condensation of nuclear chromatin, cell shrinkage with preservation of intracellular organelles and by extensive degradation of genomic DNA into oligonucleosomal fragments (3, 4). There is evidence of a role for apoptosis in several important cardiac pathologies, such as dilated cardiomyopathy and heart failure, cardiac allograft rejection, acute myocardial infarction, right ventricular dysplasia, ischemia–reperfusion injury (5–9). Apoptosis is also activated in terminally differentiated cardiac myocytes by many pathological conditions and chemical agents (10–15). Because cardiac myocytes possess minimal capacity to proliferate (16), the regulation of cardiac myocyte loss through suppression of apoptotic pathways represents a novel therapeutic strategy to prevent heart failure.

Insulin-like growth factor-1 (IGF-1) regulates several pleiotropic cellular responses and mediates the cardiovascular effects of growth hormone *in vivo* (17, 18). Although IGF–1 controls apoptosis and promotes hypertrophy by growth and differentiation in many types of cells (19), we know little about its action on cardiac myocytes where IGF-1 may act in an autocrine or paracrine manner (20). IGF-1 is also known to play important roles in the initiation and development of left ventricular hypertrophy and heart failure (21–24). Transgenic mice overexpressing IGF-1 in cardiac myocytes leads to a physiological, then pathological, cardiac hypertrophy (25) and also display less myocyte apoptosis after myocardial infarction (26). More recently, it has been shown that IGF-1 is a critical factor for the function and survival of cultured cardiac myocytes exposed to doxorubicin (DOX), angiotensin II (AII) or serum withdrawal (SW) (27).

Osmotic stress has also been shown to induce apoptosis in cell lines (28–30) and cardiac myocytes (Gálvez *et al.*, unpublished results), but the preventive actions of IGF-1 on osmotic stress-induced cardiac apoptosis are largely unknown. To resolve this issue and

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better characterize the role of IGF-1 in cardiac apoptosis, we investigated the effects of IGF-1 on DNA fragmentation and PARP degradation induced by sorbitol in neonatal cultured cardiac myocytes. In addition, these data have been compared to the IGF-1 effect with other known apoptotic stimuli in cardiomyocytes such as DOX, AII or SW.

MATERIALS AND METHODS

Reagents. Sorbitol, doxorubicin, angiotensin II, RNase, proteinase K and other biochemicals were 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 Inc. (Gaithersburg, MD). Human recombinant IGF-1 was a kind gift of Dr. C. George-Nascimento (Chiron Corp., CA). Antibodies raised against PARP and peroxidase-conjugated anti IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL immunoblotting detection reagents, autoradiographic film and prestained molecular mass standard proteins were from Amersham Pharmacia Biotech (Piscataway, NJ). Protein assay reagents were from Bio-Rad (Richmond, VA).

Animals. Sprague–Dawley rats obtained from our animal breeding facility (Faculty of Chemical & Pharmaceutical Sciences, University of Chile, Santiago). The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No 85-23, revised 1985).

Culture and treatment of cardiac myocytes. Neonatal ventricular myocytes were prepared from hearts of 1- to 3-day-old rats as reported previously (31). Cardiac myocytes were plated at a final density of $1.4 \times 10^3/mm^2$ on gelatin-pre-coated 60 mm dishes. Serum was withdrawn before the cells were pre-exposed for 1 h with 100 nM IGF-1 and further treated with or without 0.3 M SOR, 100 nM AII or 1 μM DOX in serum-free medium (DMEM-M199) at 37°C for 0–48 h. AII and DOX concentrations used here has been previously reported as those inducing maximal apoptotic effects on cardiac myocytes (13, 14). Cells incubated in the presence of DMEM-M199 containing 10% FBS served as controls. Cardiac myocytes (0.5×10^6 cells) were plated on NUNC-8 well coverslip dishes for TUNEL studies and treated for 24 h with the apoptotic agonists.

Cardiomyocyte viability. The number of viable cells was determined by trypan blue exclusion. Briefly, cells were rinsed once with 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 was determined by counting in a microscope.

Poly-[ADP-ribose]polymerase (PARP) degradation. PARP catalyzes the ADP-ribosylation of nuclear proteins at the sites where DNA strands break spontaneously and thereby facilitates the repair of this DNA damage (32). A critical step in the regulation of apoptosis is the proteolytic inactivation of PARP by caspases (32). Degradation of PARP in cardiac myocytes was analyzed by immunoblotting with an anti-PARP antibody. Briefly, medium was removed by aspiration and the cells were washed twice with cold PBS. Cells were scraped into 100 μl of cold lysis buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM EDTA, 1 mM PMSF, 1% (v/v) Triton X-100, 2 μg/mL leupeptin and 2 μg/mL aprotinin). The protein content of the lysate was determined according to Bradford (33) and equal amounts of protein were separated by SDS-PAGE on 8% gels, and then transferred electrophoretically to nitrocellulose membrane (0.45 μm). PARP was probed with a polyclonal anti-PARP antibody and detected with horseradish peroxidase-conjugated anti-goat immunoglobulin using an ECL system.

TUNEL staining. Apoptotic cells were detected by the TUNEL method using an *in situ* detection kit (Promega) and the manufacturer's recommended protocol. Briefly, cells were cultured on circular cover-slips coated with 2% gelatin, fixed in 4% paraformaldehyde for 25 min at 4°C, and then washed in PBS. 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'-OH fragmented DNA ends. After washing in PBS, the cells were analyzed by fluorescent microscopy. Apoptotic nuclei were visualized by incubation with propidium iodide for 5 min, and was used for nuclear counterstaining.

DNA fragmentation. For the detection of DNA fragmentation, cells were washed with cold PBS and sedimented by centrifugation. DNA was prepared by scraping the cells into a 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 vol of 3 M sodium acetate (pH 5.2) and 2 vol of ice-cold ethanol, was left at −20°C overnight before centrifugation. Pellets were resuspended in 200 μl TE buffer, followed by 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 absorbency 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.

Statistical analysis. Results are means ± SEM for the number of independent experiments indicated (n). Mean differences were compared by ANOVA; P values less than 0.05 were regarded as significant.

RESULTS

IGF-1 enhances cardiac myocyte viability. To assess the protective effects of IGF-1 under our experimental conditions, neonatal cardiac cells were exposed to SOR, AII, DOX or cultured in serum free medium in the presence or absence of IGF-1, and cell viability was determined by trypan blue exclusion at various time points (Fig. 1). In controls (cells cultured in the presence of serum), more than 70% of cardiac myocytes remained viable 72 h after plating. In the absence of IGF-1, cardiac myocytes exposed to SOR or DOX or SW displayed a time-dependence decrease in cell viability (Fig. 1). Exposure to SOR rapidly decreased the number of cardiac myocytes by 57 and 86% at 6 and 24 h, respectively (Fig. 1). There was only a small reduction in the cell number by AII (Fig. 1). As depicted in Fig. 1, IGF-1 partially prevented the decrease in cell survival induced by DOX, AII, SOR or SW. This protective effect of IGF-1 (near 50% increase in cell survival) was early evident in those cells exposed to SOR (6 and 24 h). In contrast, IGF-1 attenuated cell death induced by DOX after 72 h.

IGF-1 prevents osmotic stress-induced PARP fragmentation in cultured cardiac myocytes. A critical step in the control of the apoptotic DNA fragmentation

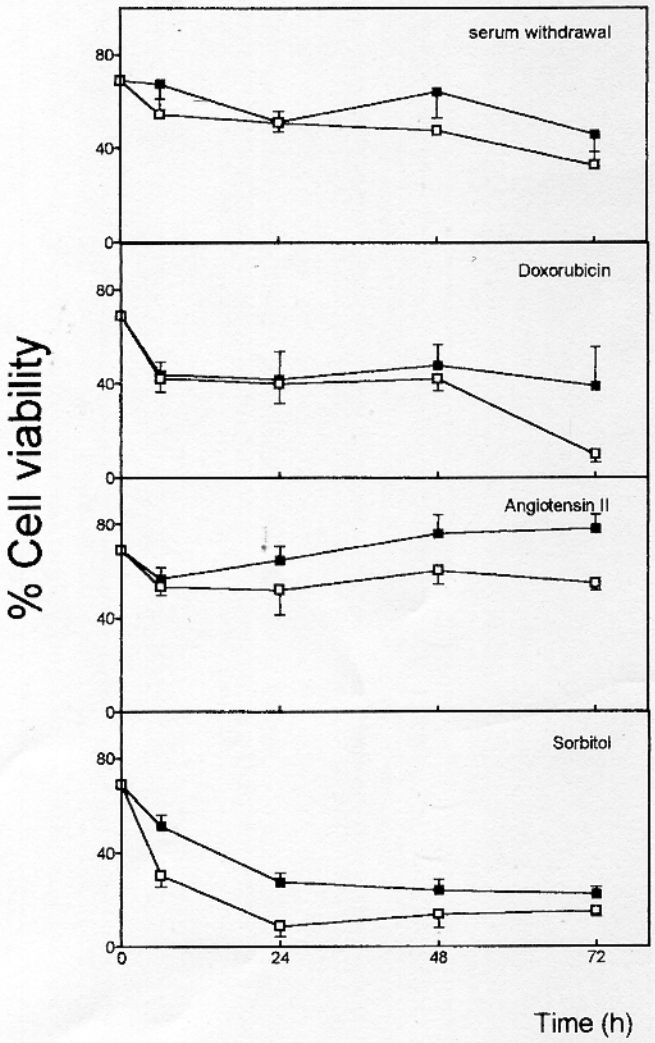


FIG. 1. Time course of cell survival effects of IGF-1 on cultured cardiac myocytes. Cells were preincubated with (■) or without (□) 100 nM IGF-1 in serum-free media and further exposed to 1 μM doxorubicin, 100 nM angiotensin II, or 0.3 M sorbitol for the indicated times, and surviving cells were counted by trypan blue exclusion assay as described under Materials and Methods. Cardiac myocytes cultured in DMEM-M199 containing 10% FBS served as controls, presenting a 70% of cell viability throughout the study. Data are shown as means ± SEM for three independent experiments.

is the proteolytic inactivation of poly-[ADP-ribose]-polymerase (PARP) by caspases. PARP catalyzes the ADP-ribosylation of nuclear proteins at the sites where DNA strands break spontaneously and thereby facilitates the repair of this DNA damage (32). To document the activation of the apoptotic pathway at this level, cardiac myocytes were exposed to SOR, AII, and DOX or cultured in serum free medium in the presence or absence of IGF-1, and we examined the cell lysate for PARP at various time points (Fig. 2). In the absence of IGF-1, the PARP amount was markedly reduced after treatment with DOX or SOR whereas both AII and SW lightly decreased PARP levels. As shown in Fig. 2, the pre-exposure of IGF-1 to cardiac myocytes further incubated with SOR markedly attenuated PARP degradation. This last effect was selective because IGF-1 did not prevented PARP degradation in cells exposed to DOX, AII or SW (Fig. 2).

IGF-1 attenuates osmotic stress-induced DNA fragmentation in cultured cardiac myocytes. DNA cleavage into nucleosome-sized fragments is a hallmark of apoptosis. To investigate the occurrence of apoptosis *in situ*, cardiac myocytes were exposed to SOR, AII, DOX or cultured in serum free medium for 24 h in the presence or absence of 100 nM IGF-1, and broken DNA in the nucleus was labeled with TUNEL method and visualized by fluorescence microscopy (Fig. 3A). Con-

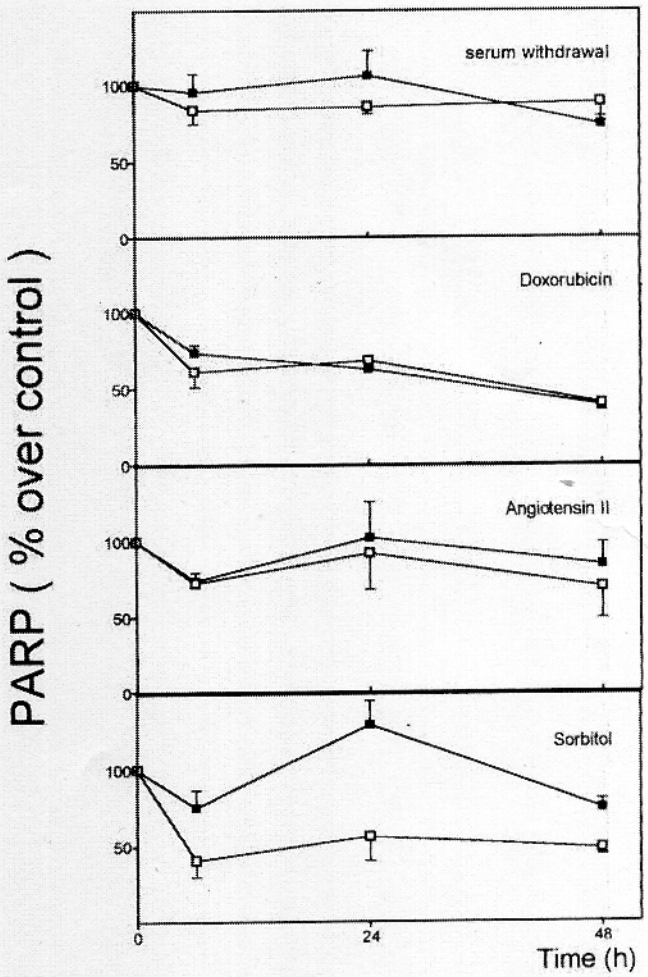


FIG. 2. Time course of PARP degradation effects of IGF-1 on cultured cardiac myocytes. Cells were preincubated with (■) or without (□) 100 nM IGF-1 in serum-free media and further exposed to 1 μM doxorubicin, 100 nM angiotensin II, or 0.3 M sorbitol for the indicated times, cardiomyocyte lysates were subjected to Western blot analysis to PARP as described under Materials and Methods. Cardiac myocytes cultured in DMEM-M199 containing 10% FBS served as controls and displayed PARP levels about 100 ± 5% throughout the study. Data are shown as means ± SEM for three independent experiments.

trol cells displayed nuclear labeling in 0.5 ± 0.2% of cells. In absence of IGF-1, SOR, DOX, AII or SW increased the number of positive apoptotic nuclei (Fig. 3A). As depicted in Fig. 3A, IGF-1 treatment significantly decreased the number of positive cells after SW or SOR incubation. However, IGF-1 did not change the number of apoptotic nuclei in cells exposed to DOX or AII for 24 h. On the other hand, when the TdT was omitted in the reaction, no positively stained cardiac myocytes were detected (data not shown). In contrast, cells treated with 1 mg/mL DNase I (used as positive control) exhibited positive staining. Cardiac myocytes, rather than contaminating fibroblasts, were the predominant contributor to the apoptosis since our cell cultures were treated with bromodeoxyuridine (BrdU) to avoid fibroblast proliferation. BrdU did not induce cardiac myocyte apoptosis assessed by DNA fragmentation (data not shown).

DNA fragmentation was also analyzed on agarose gels as described under Materials and Methods. Fig. 3 B showed that control cells displayed a low level of DNA laddering. In contrast in cells exposed to SOR and DOX for 24 h, DNA fragmentation was significantly increased (Figs. 3B and 3C). As shown in the same figure, moderate DNA laddering was found in the cardiac myocytes grown for 24 h in serum-free medium or AII. IGF-1 partially attenuated the DNA fragmentation induced by SOR or DOX (Figs. 3B and 3C). These data suggested that IGF-1 can prevent osmotic-stress-induced apoptosis of cardiac myocytes.

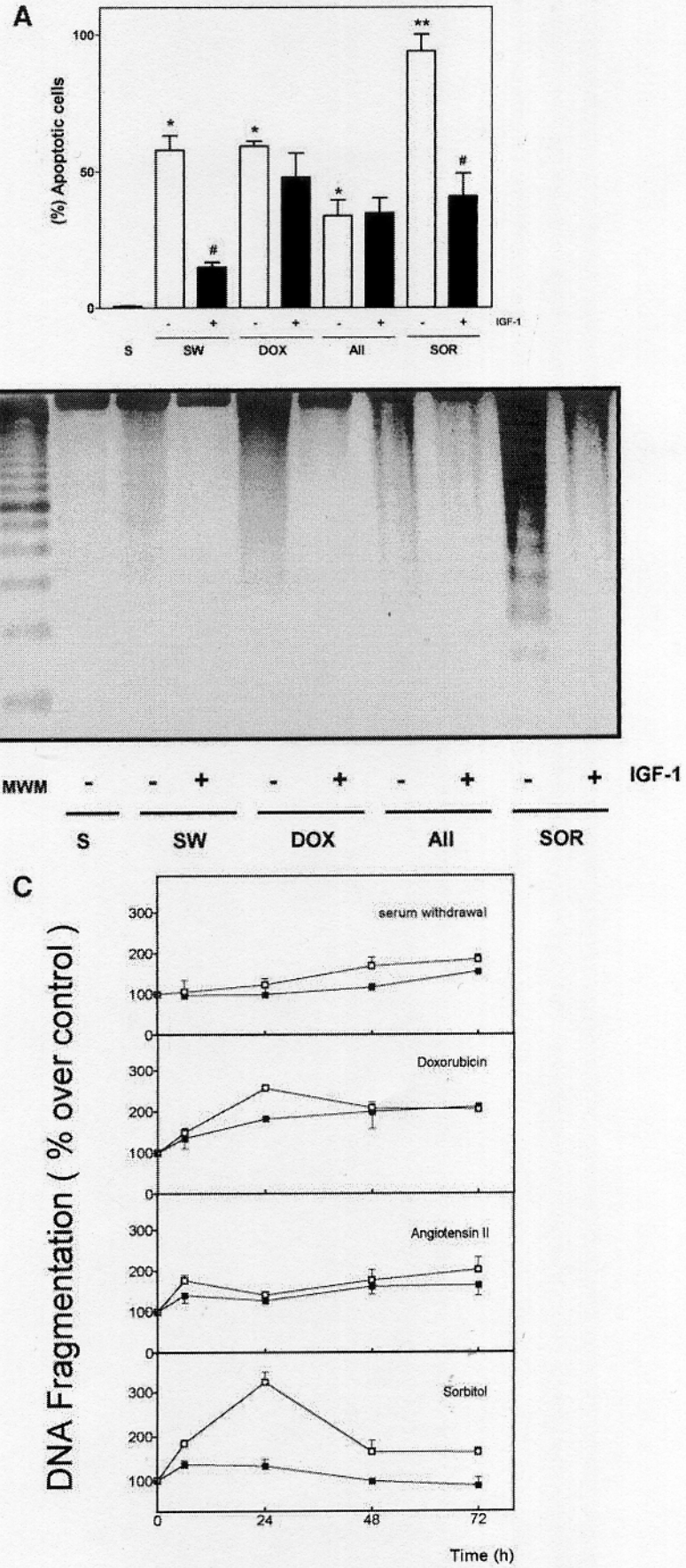
DISCUSSION

Cardiac myocytes are terminally differentiated cells which rarely proliferate after birth but have retain the ability to die via the apoptotic mechanism. As a result, the maintenance of cardiac myocyte survival is critical for the preservation of normal heart function. IGF-1 is a major cellular survival factor, protecting cells from apoptosis induced by a wide variety of agents, including growth withdrawal, etoposide, oncogene overexpression, and overexpression of caspases (34–37). We showed here that the exposure of cardiac myocytes to osmotic stress resulted in strong and early increase of apoptosis, as indicated by PARP degradation and DNA fragmentation. This effect was also rapid and stronger in magnitude than the apoptosis induced by AII, DOX and serum withdrawal. IGF-1 has also shown to protect cardiac myocytes from apoptosis induced by AII (38), by DOX (39), by stretch (40), by hypoxia (41) or by serum withdrawal (39, 42). The data presented here suggest that IGF-1 can also regulate apoptosis induce by osmotic stress in cultured cardiac myocytes. IGF-1 clearly attenuated both PARP degradation and DNA fragmentation induced by sorbitol in cardiac myocytes. This antiapoptotic action of IGF-1 on cultured cardiac myocytes is consistent with the results of recent *in vivo* studies (25, 26). In our experimental system, DOX and AII induced an intense and weak apoptotic response, respectively, which was only marginally inhibited by IGF-1. Delpy *et al.* (13) and Leri *et al.* (40) have demonstrated that both AII and DOX induced apoptosis in cultured rat ventricular myocytes (13) which was prevented by IGF-1 (27, 40).

Feuerstein and Young have proposed five possible pro-apoptotic signaling pathways in cardiac myocytes which can provide opportunities for specific pharmacological interventions (43). These are: (a) redox-regulated systems (activated by reactive oxygen species and NO/ONOO), (b) the Fas/TNFα family of cytokines receptors operating via unique "death domains" that are linked to several intracellular signaling pathways, (c) caspases that are activated either by receptor originating signals or mitochondrial-associated cytochrome *c*, (d) G-protein-coupled receptor (GPCR)-dependent stimulation induced by ligands/agonists such as AII, and (e) phospholipase-C type biochemical reactions linked to sphingomyelinase activation and generation of ceramide. The stimuli studied here (osmotic stress, AII, DOX and SW) may activate multiple signal transduction pathways and cross-talk between various pathways. This can also explain the time-course and difference in magnitude in cardiac myocyte apoptosis induced by SOR respect to other stimuli.

While the effectiveness of IGF-1 on inhibition of apoptosis in different cell types has been well established, the signaling pathways leading apoptosis and

FIG. 3. Effect of IGF-1 on DNA fragmentation induced by osmotic stress and other stimuli on cultured cardiac myocytes. (A) Cells were preincubated with (solid bars) or without (open bars) 100 nM IGF-1 in serum-free media and further cultured in serum free media (WS) or containing 1 μM doxorubicin (DOX), 100 nM angiotensin II (AII), or 0.3 M sorbitol (SOR) for 24 h. Broken DNA in the nucleus was labeled with TUNEL method and visualized by fluorescence microscopy as described under Materials and Methods. Quantitative apoptosis was expressed as percentage of total cell counted (apoptotic index). Data are shown as means ± SEM for three independent experiments. $^{**}P < 0.01$ vs Control and $\#P < 0.05$ vs cells cultured under serum-free conditions or SOR. (B and C) Cells were preincubated with (■) or without (□) 100 nM IGF-1 in serum free media and further exposed to 1 μM doxorubicin, 100 nM angiotensin II or 0.3 M sorbitol for 24 h (B) or for the indicated times (C). Genomic DNA was isolated from cardiac myocytes, subjected to electrophoresis on 2% agarose gels, and imaged by ethidium bromide staining and photography (B) or quantitated as described under Materials and Methods (C). Cardiac myocytes cultured in medium containing 10% FBS served as controls (S) and presented 0.5% of positive cells (TUNEL assay) or basal level near 100 ± 4% (DNA fragmentation assay on agarose gels) throughout the study. MWM: molecular weight markers. Data are shown as means ± SEM of three independent experiments.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DOX
AII
SOR
C
DNA Fragmentation (% over control)
serum withdrawal
Doxorubicin
Angiotensin II
Sorbitol
300
200
100
0
0
24
48
72
Time (h)

the mechanisms of action by which IGF-1 and other agents prevent apoptosis are largely unknown in cardiac myocytes. The activation of apoptotic signaling pathways may be intercepted and aborted by IGF-1 by a large number of mechanisms either enhancing anti-apoptotic mechanisms (e.g., up-regulation of bcl-2) or inhibition of key targets in the pro-apoptotic pathways such as stress activated protein kinases (reviewed in 43). We and others have found that IGF-1 activates multiple signal transduction pathways in cardiac myocytes (31, 40, 44) and some of these may be relevant to the antiapoptotic responses of the heart (45). The up-regulation of IGF-1 in myocytes of FVB.Igf+/− transgenic mice induces Mdm2 and, by this mechanism, may attenuated the function of p53, down-regulating the expression of several p53-inducible genes implicated in the modulation of apoptosis and myocyte renin-angiotensin system (40). However, there is no increase in p53 transactivating activity in cardiac myocyte exposed to osmotic stress (Gálvez *et al.*, unpublished data). Another potential targets are some members of MAPK family, including extracellular regulating kinases (ERKs), c-Jun-NH_2-terminal kinases (JNKs) and p38 MAPK (46). Whereas activation on the ERK signaling pathway protects cells from a variety of extracellular stresses, the JNK and p38-MAPK pathways may induce apoptosis (46). Daunomycin has shown to activate all three members of MAPKs in different ways in cardiac myocytes, ERKs protected from daunomycin-induced apoptosis whereas p38-MAPK promotes apoptosis. Phosphatidylinositol 3′-kinase (PI-3K) is another critical component in signal transduction pathways linked to cell survival. Treatment of cardiac myocytes with IGF-1 increased activity of both phosphatidylinositol 3′-kinase (PI-3K) (31, 41) and its downstream target, Akt or protein kinase B (PKB) (41). Constitutively active forms of PI-3 kinase or Akt inhibited apoptosis of hypoxic cardiac myocytes and subjected to hypoxia (41). It remains to be seen whether the importance of ERK and PI-3K pathways in the preventive effect of IGF-1 on osmotic stress-induced apoptosis of cardiac myocytes.

In summary, we provide evidence that IGF-1 prevents cardiac myocyte apoptosis induced by osmotic stress. These results reinforce the suggestion that IGF-1 is potential therapeutic agent in controlling cardiac myocyte apoptosis. The exact mechanisms of sorbitol-induced apoptosis in cardiac myocytes and how is modulated by IGF-1 are still not known and need to be elucidated

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REFERENCES

1. Brömme, H. J., and Holtz, J. (1996) *Mol. Cell. Biochem.* **163/164,** 261–275.
2. Goldstein, P. (1997) *Science* **275,** 1081–1082.
3. Kerr, J. F. R., Willie, A. H., and Currie, A. R. (1995) *Br. J. Cancer* **26,** 239–257
4. Wyllie, A. H. (1980) *Nature* **284,** 555–556.
5. Narula, J., Haider, N., Virmani, R., DiSalvo, T. G, Kolodgie,F. D., Hajjar, R. J., Schmidt, U., Semigran, M. J., Dec, G. W., and Khaw, B. A. (1996) *N. Engl. J. Med.* **335,** 1182–1189.
6. Kajstura, J., Cheng, W., Reiss, K., Clark, W. A., Sonnenblick, E. H., Krajewski, S., Reed, J. C., Olivetti, G., and Anversa, P. (1996) *Lab Invest.* **74,** 86–107.
7. James, T. N., St. Martin, E., Willis, P. W., III, and Lohr, T. O. (1996) *Circulation* **93,** 1424–1438.
8. Gottlieb, R. A., and Engler, R. L. (1999) *Ann. NY Acad. Sci.* **874,** 412–426
9. Szabolcs, M., Michler, R. E., Yang, X., Aji, W., Roy, D., Athan, E., Sciacca, R. R., Minanov, O. P., and Cannon, P. J. (1996) *Circulation* **94,** 1665–1673.
10. Long, X., Boluyt, M. O., Hipolito, M. L., Lundberg, M. S., Zheng, J. S., O'Neill, L., Cirielli, C., and Lakatta, E. G. (1997) *J. Clin. Invest.* **99,** 2635–2643.
11. Cheng, W., Li, B., Kajstura, J., Li, P., Wolin, M. S., Sonnenblick, E. H., Hintze, T. H., Olivetti, G., and Anversa, P. (1995) *J. Clin. Invest.* **96,** 2247–2259.
12. Pulkki, K. J. (1997) *Ann. Med.* **29,** 339–343.
13. Deply, E., Htem, S., Andrieu, N., De Vaumas, C., Henaff, M., Rucker-Martin, C., Jaffrézou, J., Laurent, G., Levade, T., and Mercadier, J. (1999) *Cardiovasc. Res.* **53,** 398–407.
14. Cigola, E., Kajstura, J., Li, B., Meggs, L., and Anversa, P. (1997) *Exp. Cell. Res.* **231,** 363–371.
15. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) *J. Biol. Chem.* **272,** 20490–20494.
16. Chien, K. R., Knowlton, K. U., Zhu, H., and Chien, S. (1991) *FASEB J.* **5,** 3037–3046.
17. LeRoith, D., and Roberts, C. T. (1993) *Ann. NY Acad. Sci.* **692,** 1–9
18. Froesch, E., Schmid, C., Schwander, J., and Zapf, J. (1985) *Annu. Rev. Physiol.* **47,** 443–467.
19. Butler, A. A., Yakar, S., Gewolb, I. H., Karas, M., Okubo, Y., and LeRoith, D. (1998) *Comp. Biochem. Physiol. B* **121,** 19–26.
20. Engelmann, G. L., Poem, K. D., Haskell, J. F., Khairallah, P. A., and Ilan, J. (1989) *Mol. Cell. Endocrinol.* **63,** 1–14.
21. Ito, H., Hiroe, M., Hirata, Y., Tsujino, M., Adashi, S., Shichiri, M., Koike, A., Nogami, A., and Marumo, F. (1993) *Circulation* **87,** 1715–1721.
22. Ebensperger, R., Acevedo, E., Meléndez, J., Corbalán, R., Acevedo, M., Sapag-Hagar, M., Jalil, J. E., and Lavandero, S. (1998) *Biochem. Biophys. Res. Commun.* **243,** 20–24.
23. Cittadini, A., Strömer, H., Hatz, S. E., Clark, R., Moses, A. C., Morgan, J. P., and Douglas, P. S. (1996) *Circulation* **93,** 800–809.
24. Donohue, T. J., Dworkin, L. D., Lango, M. N., Friegner, K., Lango, R. P., Benstein, J. A., Slater, W. R., and Catanese, V. M. (1994) *Circulation* **89,** 799–809.
25. Delaughter, M. C., Taffet, G. E., Fiorotto, M. L., Entman, M. L., and Schwartz, R. J. (1999) *FASEB J.* **13,** 1923–1929.
26. Li, Q., Li, B., Wang, X., Leri, A., Jana, K. P., Liu, Y., Kajstura, J., Baserga, R., and Anversa, P. (1997) *J. Clin. Invest.* **100,** 1991–1999.

27. Wang, L., Ma, W., Markovich, R., Chen, J. W., and Wang, P. H. (1998) *Circ. Res.* **83,** 516–522.
28. Edwards, Y. S., Sutherland, L. M., Power, J. H., Nicholas, T. E., and Murray, A. W. (1998) *Am. J. Physiol.* **275,** 670–678.
29. Juo, P., Kuo, C. J., Reynolds, S. E., Konz, R. F., Raingeaud, J., Davis, R. J., Biemann, H. P., and Blenis, J. (1997) *Mol. Cell. Biol.* **17,** 24–35.
30. Edwards, Y. S., Sutherland, L. M., Power, J. H., Nicholas, T. E., and Murray, A. W. (1998) *Am. J. Physiol.* **275,** 670–678.
31. Foncea, R., Andersson, M., Ketterman, A., Blakesley, V., Sapag-Hagar, M., Sugden, P. H., LeRoith, D., and Lavandero S. (1997) *J. Biol. Chem.* **272,** 19115–19124.
32. Allen, R. T., Cluck, M. W., and Agrawal, D. K. (1998) *Cell. Mol. Life Sci.* **54,** 427–445.
33. Bradford M. (1976) *Anal. Biochem.* **72,** 248–254.
34. Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992) *Cell* **70,** 31–46.
35. Sell, C., Baserga, R., and Rubin, R. (1995) *Cancer Res.* **55,** 303–306.
36. Harrington, E. A., Bennett, M. R., Fanidi, A., and Evan G. I. (1994) *EMBO J.* **13,** 3286–3295.
37. Jung, Y. K., Miura, M., and Yuan, J. (1996) *J. Biol. Chem.* **271,** 3229–3237.
38. Leri, A., Liu, Y., Wang, X., Kajstura, J., Malhotra, A., Meggs, L. G., and Anversa, P. (1999) *Circ. Res.* **84,** 752–62
39. Wang, L., Ma, W. Markovich, R., Chen, J. W., and Wang, P. H. (1998) *Circ. Res.* **83,** 516–522.
40. Leri, A., Liu, Y., Claudio, P. P., Kajstura, J., Wang, X., Wang, S., Kang, P., Malhotra, and Anversa P. (1999) *Am. J. Pathol.* **154,** 567–80
41. Matsui, T., Li, L., del Monte, F., Fukui, Y., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (1999) *Circulation* **100,** 2373–2379.
42. Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., and Walsh, K. (2000) *Circulation* **101,** 660–667.
43. Feuerstein, G. Z., and Young, P. R. (2000) *Cardiovasc. Res.* **45,** 560–569.
44. Takahashi, T., Fukuda, K., Pan, J., Kodama, H., Sano, M., Makino, S., Kato, T., Manabe, T., and Ogawa, S. (1999) *Circ. Res.* **85,** 884–891.
45. Lavandero, S., Foncea, R., Pérez, V., and Sapag-Hagar, M. (1998) *FEBS Lett.* **422,** 193–196.