GH prevents apoptosis in cardiomyocytes cultured *in vitro* through a calcineurin-dependent mechanism

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Abstract

The use of GH to treat heart failure has received considerable attention in recent years. Although the mechanisms of its beneficial effects are unknown, it has been implicated in the regulation of apoptosis in several cell types, and cardiomyocyte apoptosis is known to occur in heart failure. We therefore decided to investigate whether GH protects cardiomyocytes from apoptosis. Preliminary experiments confirmed the expression of the GH receptor (GHR) gene in primary cultures of neonatal rat cardiomyocytes (PC), the specific binding of GH by HL-1 cardiomyocytes, and the GH-induced activation of GHR and its classical downstream effectors in the latter. That GH prevented the apoptosis of PC cells deprived of serum for 48 h was shown by DNA electrophoresis and by Hoechst staining assays in which GH reduced the percentage of cells undergoing apoptosis. Similarly, the TUNEL-evaluated pro-apoptotic effect of cytosine arabinoside (AraC) on HL-1 cells was almost totally prevented by pre-treatment with GH.

Fluorescence-activated cell sorter (FACS) analysis showed apoptosis in 9.7% of HL-1 cells growing in normal medium, 21.1% of those treated with AraC and 13.9% of those treated with AraC+GH, and that GH increased the percentage of AraC-treated cells in the S/G₂/M phase from 36.9% to 52.8%. GH did not modify IGF-I mRNA levels or IGF-I secretion in HL-1 cells treated with AraC, and the protection afforded by GH against AraC-induced apoptosis in HL-1 cells was not affected by the presence of anti-IGF-I antibodies, but was largely abolished by the calcineurin-inhibiting combination cyclosporin+FK506. GH also reduced AraC-induced phosphorylation of mitogen-activated protein kinase p38 (MAPK p38) in HL-1 cells. In summary, GH protects PC and HL-1 cells from apoptosis. This effect is not mediated by IGF-I and may involve MAPK p38 as well as calcineurin.

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Introduction

Growth hormone (GH) is a pleiotropic hormone with lipolytic, anabolic and cardiovascular effects. The evidence that GH is essential for good cardiovascular function chiefly comprises three types of observation: (a) GH deficiency (Vance & Mauras 1999, Feinberg *et al.* 2003) or excess (Clayton 2003) can be associated with cardiovascular abnormalities; (b) some of the cardiovascular abnormalities derived from GH deficiency can be remedied by GH administration (Silverman & Friedlander 1997, Jallad *et al.* 2003); and (c) GH administration can also benefit dilated cardiomyopathy patients with normal GH levels (Bengtsson *et al.* 1997, Perrot *et al.* 2001). Also, the observation of a direct relationship between GH levels and left ventricle wall thickness and morphology in

humans (Fazio *et al.* 1997) and animals (Houck *et al.* 1999) has shown that GH has a role in the maintenance of myocardial structure. Most studies of the cardiac effects of GH have focused on its stimulation of cardiomyocyte hypertrophy and contractility (Lombardi *et al.* 1997, Tanaka *et al.* 1998, Ross 1999). The possibility of other mechanisms has received little attention.

Although cardiomyocytes were long thought not to undergo apoptosis, it is now known that they do, and it is believed that in certain circumstances apoptosis may play a key role in the development of heart failure. The fact that GH is thought to regulate apoptosis in a number of cell types, acting anti-apoptotically (Costoya *et al.* 1999, Haeflner *et al.* 1999, Sirotkin & Makarevich 1999, Jeay *et al.* 2000, Mylonas *et al.* 2000, Segard *et al.* 2003) or pro-apoptotically (Santos *et al.* 1999, Kiya *et al.* 1999)

according to cell type, raises the possibility that its beneficial effects on cardiac function may at least in part involve its reducing apoptosis among cardiomyocytes. In the work described here we investigated this possibility using both primary cultures of rat neonatal cardiomyocytes (PC) and HL-1 cells, a cardiomyocyte line derived from AT-1 mouse atrial tumour cells that retains morphological, biochemical and electrophysiological properties of differentiated cardiomyocytes, including the ability to contract (Claycomb *et al.* 1998).

In preliminary experiments we confirmed the expression of GH receptor (GHR) gene in PC cells, the binding of GH by HL-1 cells, and the GH-induced activation of GHR and its classical downstream effectors in the latter. Using multiple techniques (DNA electrophoresis, TUNEL assays, Hoechst dye vital staining and flow-cytometric cell cycle analysis), we next found that GH treatment inhibited apoptosis in both cell types, and that this effect was not accompanied by changes in insulin-like growth factor-I (IGF-I) mRNA levels or IGF-I secretion and was not affected by the presence of anti-IGF-I antibodies, but was largely prevented by inhibition of calcineurin. Finally, the involvement of calcineurin was further supported by the finding that GH diminished cytosine arabinoside (AraC)-induced phosphorylation of mitogen-activated protein kinase p38 (MAPK p38), a kinase that has been implicated in calcineurin-mediated apoptosis regulation.

Materials and Methods

All sera were from Life Technologies (Poole, UK), and all other products from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

PC cells

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The hearts of 1-day-old Sprague-Dawley rats were digested with collagenase and pancreatin at 37 °C in three 30-min digestion cycles, cells were centrifuged from the pooled supernatants, and fibroblasts were removed by differential seeding with incubation for 4 h at 37 °C in newborn calf serum. In experiments, the cardiomyocytes were seeded at a density of 50 000/cm² in gelatin-coated plates containing DMEM/M199 medium (from Life Technologies) supplemented with horse serum, foetal calf serum, L-glutamine, antibiotics and, to inhibit fibroblast proliferation, 10 µM AraC (Citarabina from Pharmacia & Upjohn, Barcelona, Spain). In experiments on the effects of GH, cells seeded 36 h previously were deprived of serum for 48 h in the

presence or absence of 1 µg/ml recombinant human GH (Saizen, from Serono, Madrid, Spain).

RT-PCR

RT-PCR for GHR was performed on PC cells and cells obtained by standard methods from neonatal rat liver or adult rat heart or liver. The total RNA of 10⁶ cells was prepared using TRIzol reagent (from Life Technologies), and 2 µg was transcribed into cDNA by incubation for 1 h at 42 °C with 200 U of murine leukaemia virus reverse transcriptase (from Life Technologies) in 20 µl of a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml BSA, deoxy-NTPs (each 1 mM) and 20 U of the ribonuclease inhibitor RNasin (from Promega, Madison, WI, USA). The resulting cDNA was used as a PCR template in a reaction mixture containing 5 U of *Taq* DNA polymerase (from Life Technologies), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml BSA, deoxy-NTPs (each 0.2 mM) and the rat-GHR-specific primers 5'-GAGGA GGTGACCACCATCTTGGGC-3' (sense) and 5'-ACG ACCTGCTGGTGTAAATGTC-3' (antisense) (each 0.2 mM). Thirty-five PCR cycles were performed, each consisting of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min, and extension at 72 °C for 1 min. DNA fragments were electrophoresed on 1% agarose gel and examined under u.v. light.

In experiments on whether IGF-I mediated the observed anti-apoptotic action of GH, IGF-I mRNA levels were determined by RT-PCR as described previously (Kamai *et al.* 1996). Briefly, total RNA was treated with 10 U of RNAase-free DNAase (Life Technologies) for 1 h at 37 °C, and 3 µg were used to reverse transcription by using 400 U of murine leukaemia virus reverse transcriptase (Life Technologies) in 30 µl of a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml BSA, deoxy-NTPs (each 1 mM) and 30 U of the ribonuclease inhibitor RNasin (Promega). From reverse transcription product, 5 µl were used in PCR. For both IGF-I class I/I *del* and IGF-I class II mRNA 32 cycles were performed, each consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min.

HL-1 cells and apoptosis experiments thereon

HL-1 cardiomyocytes (a generous gift from Dr W. C. Claycomb, Louisiana State University Medical Center, New Orleans, LA, USA) were cultured on fibronectin-covered plates containing ExCell 320 medium (from JRH Biosciences, Andover, UK) supplemented with foetal bovine serum (FBS), insulin, noradrenaline, endothelial cell growth supplement (from Upstate Biotechnology, Lake Placid, NY, USA), and retinoic acid (Claycomb *et al.* 1998).

In experiments testing for a possible anti-apoptotic action of GH and its requirements, cells were maintained for 12 h in starvation medium (ExCell 320 with 10% FBS but no other supplements) with or without 1 µg/ml GH, with or without 3 µM cyclosporin + 150 ng/ml FK506 and with or without 10 µg/ml anti-IGF-I antibody capable of blocking IGF-I action (Baixeras *et al.* 2001) (from Santa Cruz Biotechnology, Delaware, CA, USA), and were then treated for 12 or 24 h with 100 µM AraC as pro-apoptotic agent.

GH binding by HL-1 cells

HL-1 cells (5×10^5) were deprived of serum for 4 h and then incubated overnight at 4 °C in PBS containing 0.1% of BSA, [125 I]hGH (10^5 c.p.m./ml) and various concentrations of unlabelled hGH. Cells were then washed and lysed in 0.1 M NaOH, and total associated radioactivity was measured in a γ -counter. Scatchard analysis was performed using the program Ligand (Munson & Rodbard 1980).

Immunoprecipitation and Western blotting

HL-1 cells (3×10^6) were deprived of serum for 4 h in DMEM, treated for 5, 15 or (in some cases) 30 min with 1 µg/ml hGH, and then lysed with Triton X-100 (1% in a buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor and 1 mM NaVO₄). The supernatant was incubated overnight with protein A Sepharose beads and antibodies against GHR (Sotiropoulos *et al.* 1996), JAK2 (Janus kinase 2; from Upstate Biotechnology), or STAT5 (signal transducer and activator of transcription 5; from Santa Cruz Biotechnology), and after thorough washing with lysis buffer the incubation products were resuspended in 20 µl of sample buffer, separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (from BioRad Laboratories, Hercules, CA, USA), which were then successively incubated for 1 h in TBS-T (50 mM Tris-HCl, 200 mM NaCl and 0.1% Tween 20) with 4% BSA, for 1 h in TBS-T containing 1% BSA and antibody against phosphotyrosine (from Upstate Biotechnology), and for 1 h with (horseradish peroxidase)-conjugated anti-IgG antibodies (from Amersham Pharmacia Biotech, Little Chalfont, UK). After visualization using an ECL chemiluminescence detection system (from Amersham Pharmacia Biotech), the membranes were re-incubated for 1 h in TBS-T containing 1% BSA and the antibody used for immunoprecipitation and for 1 h with (horseradish peroxidase)-conjugated anti-IgG antibodies, and were visualized as before. MAPK p38 was detected from total lysate using an antibody from Santa Cruz Biotechnology.

Cell cycle analysis

HL-1 cells (10^6) treated as described above ('HL-1 cells and apoptosis experiments thereon') were then treated

with trypsin, washed with PBS, treated with 4% para-formaldehyde for 10 min, and incubated overnight at -20 °C in 70% ethanol. Cell pellets were resuspended in 1 ml of PBS containing 1 mg/ml RNAase and 5 µg/ml propidium iodide, and after 1 h this suspension was analysed by flow cytometry in a FACSCALIBUR (from Becton & Dickinson, San José, CA, USA) using the program Cell Quest. Apoptosis was determined as the percentage of DNA in the hypodiploid (sub-G₀/G₁) peak.

TUNEL assays

TUNEL assays were performed using the *In Situ* Cell Death Detection Kit (Fluorescein) from Boehringer Mannheim (Indianapolis, IN, USA) and an Olympus X-FLA fluorescence microscope (from Olympus Optical, Hamburg, Germany).

Hoechst dye vital staining

HL-1 or PC cells (10^4) were seeded in 24-well plates and incubated for 45 min at 37 °C in Hoechst 33258 dye. Then HEPES (pH 7.8) was added to a final concentration of 5 mM, and the cells were fixed with 0.4% para-formaldehyde for 30 min and examined by fluorescence microscopy.

Analysis of DNA fragmentation by electrophoresis

Cells (5×10^5 per treatment) were lysed in a buffer comprising 50 mM Tris-HCl, 10 mM EDTA and 0.1% SDS, and were then incubated with 3 mg/ml proteinase K for 30 min at 65 °C. After phenol-chloroform extraction and precipitation with 0.3 M sodium acetate (pH 5.3), 2 µg of DNA per lane was subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide, and examined under u.v. light.

MTT viability assay

HL-1 cardiomyocytes (10^4 per treatment) were deprived of serum for 12 h, and then treated for 48 h with 10% foetal calf serum with or without 3 µM cyclosporin + 150 ng/ml FK506. Four hours before the expiry of this period, 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added. After overnight incubation at 37 °C, absorbance at 550–600 nm was measured.

IGF-I radioimmunoassay

IGF-I levels in HL-1 cell culture medium were determined using a kit from DRG Diagnostics (Marburg, Germany).

Statistical analysis

Results shown are the means $\pm$ S.E.M. of at least three independent experiments. The significance of differences

Figure 1 (A) Results of RT-PCR tests for expression of rat GHR gene in PC cells (lanes 1–3), neonatal rat liver (lanes 7–9), adult rat heart (lane 13) and adult rat liver (lane 14); beta-actin was used as a housekeeping gene (lanes 4–6, 10–12, 15 and 16). (B) Competition curve for GHR in HL-1 cells, using ¹²⁵I-labelled human GH as radioligand and various concentrations of unlabelled GH as competitor.

was estimated by ANOVA followed by Student–Newmann–Keuls multiple comparison tests; $P < 0.05$ was considered significant.

Results

Expression of the GHR gene by PC cells, and GH binding by HL-1 cells

RT-PCR showed PC cells to express the GHR gene at levels similar to those observed in adult rat heart (Fig. 1A). Scatchard analysis of GH binding by HL-1 cells yielded values of 1.98 nM for K_d , 0.012 nM for B_{max} , and 15 000 for the approximate number of binding sites per cell (Fig. 1B), showing efficient constitutive production of long-form GHR at the cell surface. Binding assays and Scatchard analysis were performed twice with similar results.

GH-induced activation of JAK2 and STAT5 in HL-1 cells

Immunoprecipitation and Western blotting showed that a 5-min incubation of HL-1 cells with GH sufficed to

Figure 2 Upper panels: immunoprecipitation and Western blotting shows that GH induces tyrosine phosphorylation of (A) GHR, (B) JAK2 and (C) STAT5 in HL-1 cardiomyocytes. Lower panels: the results of reprocessing the blots with the immunoprecipitating antibody (loading controls). Abbreviations: IP, immunoprecipitation; PY, phosphotyrosine; WB: Western blot. Figure is representative of three independent experiments.

induce the tyrosine phosphorylation of GHR and its classical downstream effectors JAK2 and STAT5 (upper photographs in Fig. 2A, B and C respectively; lower photographs show reprocessing of the same blots with anti-GHR, anti-JAK2 or anti-STAT5 confirming that equal amounts of protein had been loaded for each incubation time).

Figure 3 The hallmark of apoptosis is the fragmentation of genomic DNA into multiples of an approximately 180 bp subunit. GH prevented extensive fragmentation of DNA in PC cells deprived of serum for 48 h.

Prevention of apoptosis of PC and HL-1 cells by GH, and non-involvement of IGF-I

Electrophoresis detected DNA fragmentation in PC cells deprived of serum for 48 h in the absence of GH, but not in those starved in the presence of 1 µg/ml GH (Fig. 3). GH reduced the number of apoptotic starved PC cells detected by Hoechst dye staining analyses (Fig. 4). Experiments were performed three times with similar results.

Fluorescence-activated cell sorter (FACS) analysis showed apoptosis rates of 9.6% in untreated HL-1 cultures, 21.1% in cells treated with AraC for 24 h, and 13.9% in cells pre-treated with GH for 12 h before AraC treatment. The percentage of cells in the S/G₂/M phase was 56.9% in untreated cultures, 36.9% in AraC-treated cells in the absence of GH, and 52.8% in AraC-treated cells pre-treated with GH (Fig. 5).

Similarly, TUNEL analyses (Fig. 6A) showed that AraC treatment for 12 h increased the rate of apoptosis in HL-1 cells by approximately twofold ($P < 0.01$), which was blocked when the cells had received 12 h of pre-treatment with GH ($n = 3$).

In AraC-treated HL-1 cultures, administration of 10 µg/ml anti-(rat IGF-I) antibody with GH did not significantly alter the anti-apoptotic effect of the hormone (Fig. 6A) ($n = 3$), and pre-treatment for 12 h with GH did not significantly alter either secretion of IGF-I into the culture medium (radioimmunoassays measured concentrations of 83 ± 6 ng/ml with AraC and 96 ± 8 ng/ml

48 h serum deprivation

48 h serum deprivation + GH

Figure 4 Fluorescence microphotographs (200 ×) (each representative of three independent experiments) of representative fields observed in Hoechst dye vital staining assays of PC cells deprived of serum for 48 h in the absence (above) or presence (below) of GH. Arrows indicate nuclei with higher fluorescence intensity reflecting greater nuclear fragmentation.

with AraC+GH; $n = 3$) or IGF-I mRNA levels (Fig. 6B) ($n = 3$).

Inhibition of the anti-apoptotic effect of GH by calcineurin inhibitors

Addition of the calcineurin-inhibiting combination cyclosporin+FK506 with GH almost totally prevented its anti-apoptotic action, resulting in FACS-evaluated percentages of apoptotic and S/G₂/M-phase cells (21.4% and 41.6% respectively) that were very similar to those observed among AraC-treated cells without 12 h of GH

Figure 5 Effect of GH and the calcineurin-inhibiting combination cyclosporin+FK506 on the cell cycle phase of HL-1 cells. Apoptosis was induced by exposure to AraC for 24 h. GH pre-treatment, with or without cyclosporin+FK506, started 12 h before AraC administration. Cell DNA content was evaluated by flow cytometry after propidium iodide staining. The Figure shows FACS plots (each representative of three independent experiments) of cell number versus DNA content, together with the percentages of cells that are apoptotic (Apo), or in the G₀/G₁ (2n), or S/M (4n) phases.

pre-treatment (Fig. 5). This effect of cyclosporin+FK506 on the action of GH was confirmed by Hoechst dye vital staining (Fig. 7A.1) ($P < 0.05$; $n = 3$). Cyclosporin+FK506 treatment for 48 h did not affect the viability of HL-1 cells as assessed by MTT viability assays (Fig. 7A.2) ($n = 3$).

Influence of AraC, with and without GH pre-treatment, on phosphorylation of MAPK p38

AraC treatment for 24 h induced the tyrosine phosphorylation of MAPK p38 in HL-1 cells, and this effect was partially prevented by pre-treatment for 12 h with GH (Fig. 7B). Experiments were performed twice with similar results.

Discussion

Cardiomyocyte apoptosis is currently known to occur in the early stages of myocardial dysfunction, when it impairs left ventricular performance by reducing contractile cardiomyocyte mass (Haunstetter & Izumo 1998), and to contribute to progressive cardiomyocyte loss in heart failure (Kang & Izumo 2000). Substances that may prove capable of halting or reverting these apoptotic processes include growth factors and other hormones and cytokines that are known to regulate cell death (Pulkki 1997, Kiess & Gallaher 1998), many of which act via receptors of the cytokine superfamily, which includes GHR (Horseman & Yu-Lee 1994). The role of GH in the regulation of apoptosis depends on the type of cell in question, and possibly on cell status. GH has been shown to be anti-apoptotic in myoblasts and in lymphoid, intestinal and ovarian granulosa cells (Costoya *et al.* 1999, Haeffner *et al.* 1999, Sirotkin & Makarevich 1999, Jeay *et al.* 2000, Mylonas *et al.* 2000, Segard *et al.* 2003), to stimulate proliferation of pro-B Ba/F3 cells (Baixeras *et al.* 2001), and to act as a neuronal rescue factor during recovery from injury (Scheepens *et al.* 2001), but it has also been reported to induce structural luteolysis through apoptotic mechanisms (Kiya *et al.* 1999) and to inhibit the proliferation of primary pre-adipocyte cultures (Gerfault *et al.* 1999). One study failed to find any significant effect of GH on apoptosis (Santos *et al.* 1999).

In patients with dilated cardiomyopathy or congestive heart failure, GH has been reported to have beneficial effects such as an increase in myocardial contractility (Giustina *et al.* 1999). Although many clinical and basic studies of these effects (and those of IGF-I) have focused on hypertrophic actions (Genth-Zotz *et al.* 1999, Colao *et al.* 2001), it has also been reported that GH lowers the levels of circulating proinflammatory cytokines and soluble components of the Fas/Fas-ligand system (Adamopoulos *et al.* 2002), that in rats with experimental myocardial infarction GH improves cardiac function (Isgaard *et al.* 1997, Jin *et al.* 2002), and that GH treatment reduces hypertension and improves cardiovascular function in animals exposed to adverse environmental conditions during foetal or postnatal life (Vickers *et al.* 2002).

The cells used in this study were primary *in vitro* cultures of neonatal rat heart and cultures of the murine heart cell line HL-1, which has been used by many groups to study cardiomyocyte biology (McWhinney *et al.* 2000,

Figure 6 (A) Effect of pre-treatment with GH on DNA damage in HL-1 cells treated for 12 h with AraC. Quantitative results of three independently performed TUNEL assays of untreated control cultures, AraC-treated cultures, AraC-treated cultures pre-treated for 12 h with GH, and AraC-treated cultures pre-treated for 12 h with GH and 10 µg/ml anti-IGF-I antibody. AraC increased the number of cells with green fluorescence, and pre-treatment with GH prevented this increase, even in the presence of anti-IGF-I. Results are displayed as y-fold increase in the incidence of apoptosis with respect to the controls; ** $P < 0.01$. (B) RT-PCR-assayed levels of IGF-I mRNA (class I or class I *del*, and class II) in HL-1 cells treated with AraC with or without pre-treatment for 12 h with GH. (B.1) Statistical analysis of three independently performed experiments showing no significant differences between the results of the two treatments; (B.2) a representative experiment (lanes 1 and 4: controls; lanes 2 and 5: AraC-treated cells; lanes 3 and 6: AraC-treated cells pre-treated for 12 h with GH). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Results in (A) and (B.1) are shown as means $\pm$ S.E.M.

Kitta *et al.* 2001). We began by confirming that these cells expressed the GHR gene. Although it has recently been reported that adult and cultured neonatal rat heart cells differ as regards GHR gene expression (Lu *et al.* 2001), our semi-quantitative RT-PCR experiments showed no such difference. We have no conclusive explanation for this discrepancy, but it may be due to differences in cell culture protocol. The presence of GHR on HL-1 cells was confirmed by binding studies, Scatchard analysis showing a K_d of 1.98 nM and a binding site density of about 15 000 per cell.

We next performed tests of whether our HL-1 cells were competent as regards the well-established biochemical pathways mediating 'classical' actions of GH such as somatic cell growth. These mechanisms involve receptor dimerization leading to activation of the GHR-associated

tyrosine kinase JAK2, transphosphorylation of the latter, and tyrosine phosphorylation of the receptor and numerous cytoplasmic proteins, including STAT proteins (Kelly *et al.* 2001). The finding that GH induced the tyrosine phosphorylation of JAK2, GHR and STAT5 is evidence of the integrity of these mechanisms in HL-1 cells.

Hoechst dye vital staining and the observation of DNA fragmentation showed that PC cells were protected from serum-deprivation-induced apoptosis if GH was present in the serumless medium, and TUNEL assays confirmed that DNA damage induced by serum deprivation was prevented by GH. FACS and TUNEL analyses showed that HL-1 cells were protected from AraC-induced apoptosis by 12 h of pre-treatment with GH.

Many of the effects of GH are mediated by IGF-I produced in response to GH by the liver or by specific

Figure 7 (A.1) Results of Hoechst dye vital staining assays of apoptosis in HL-1 cells treated with AraC 24 h with or without pre-treatment with GH or GH plus the calcineurin-inhibiting combination cyclosporin+FK506 for 12 h. Results are displayed as y-fold increase in the incidence of apoptosis with respect to the controls. * $P < 0.05$; ** $P < 0.01$ ($n = 3$). (A.2) MTT viability assay results showing that treatment for 48 h with cyclosporin+FK506 did not affect HL-1 cell viability. *** $P < 0.001$ ($n = 3$). Results in (A.1) and (A.2) are shown as means $\pm$ S.E.M. (B) GH pre-treatment for 12 h partially prevented 24 h AraC-induced phosphorylation of MAPK p38. Experiments were performed twice. Abbreviations: WB: Western blot, PY: phosphotyrosine.

target tissues. However, effects of GH on cardiomyocytes are known that are not mediated by IGF-I (Lu *et al.* 2001), and the anti-apoptotic effects observed in our study appear

to be among them: the administration of anti-IGF-I antibody together with GH did not reduce the TUNEL-assayed anti-apoptotic effect of GH on HL-1 cells, and

neither the IGF-I mRNA levels of AraC-treated HL-1 cells nor the concentration of secreted IGF-I in their culture medium were modified by co-treatment with GH.

Although the mechanisms of the classical actions of GH have been clear for some years (Kelly *et al.* 2001), those mediating its apoptosis-related effects are far from being well understood. Relevant partial results that have been published include the finding that GH retards the colchicine-induced depolymerization of microtubules, thereby decreasing colchicine-induced apoptotic cell death in Chinese hamster ovary cells (Goh *et al.* 1998); the finding that in cultured bovine granulosa cells GH regulates apoptosis through activation of the cAMP/PKA system (Sirotkin & Makarevich 1999); indications that the anti-apoptotic effects of GH may be mediated by Bcl-2 (Haeflner *et al.* 1999, Mitsunaka *et al.* 2001, Kolle *et al.* 2002) and/or Akt (Costoya *et al.* 1999, Segard *et al.* 2003); and proof that GH favours mammary carcinoma proliferation by transcriptional repression of proteins that promote cell cycle arrest and apoptosis (Graichen *et al.* 2002). Also, a protective effect of GH against oxidative stress damage in cardiomyocytes has been attributed at least in part to the activation of ERKs through Ras and protein kinases such as Src and epidermal growth factor (EGF) receptor tyrosine kinase (Gu *et al.* 2001).

In this work, as a first step towards elucidating the mechanism of the observed anti-apoptotic action of GH in our cells, we investigated whether it was affected by inhibition of calcineurin, a calcium-dependent phosphatase that is known to be involved in apoptosis regulation in a number of cells (Lotem *et al.* 1999), including cardiomyocytes (Molkentin 2001). The targets regulated by calcineurin include transcription factors such as NFAT, MEF2 and NF- κ B: activation of NF- κ B has been shown to be involved in prevention of the apoptosis of lymphoid cells by GH (Jeay *et al.* 2000, 2002), and NFAT transcription factors have been reported to be critical survival factors that inhibit cardiomyocyte apoptosis during phenylephrine stimulation *in vitro* (Pu *et al.* 2003).

In this study, flow cytometry and Hoechst dye vital staining showed that the anti-apoptotic effect of GH on HL-1 cells was largely abolished by inhibition of calcineurin with cyclosporin+FK506. Furthermore, Western blot studies showed that AraC treatment of HL-1 cells led to tyrosine phosphorylation of MAPK p38, and that GH reduced this effect; MAPK p38 is known to be involved in calcineurin-mediated regulation of apoptosis in cardiomyocytes and other cells (Lotem *et al.* 1999, Yue *et al.* 2000, Molkentin 2001), and in the coordination of the pleiotropic effects of GH (Zhu & Lobie 2000).

In conclusion, our results show that GH protects PC and HL-1 cells from apoptosis, that this effect is not mediated by IGF-I, and that its mechanism involves calcineurin, possibly through prevention of phosphorylation of p38. On the basis of these findings, we hypothesize that this prevention of cardiomyocyte

apoptosis through calcineurin may be one of the mechanisms by which GH acts beneficially on the heart. Further studies are needed to explore this hypothesis and, more generally, to evaluate the effects of GH in cardiac pathologies that involve apoptosis.

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