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# IGF-1 Regulates Cardiac Fibroblast Apoptosis Induced by Osmotic Stress

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**In this study we have determined the ability of IGF-1 to protect cardiac fibroblasts against osmotic-induced apoptosis and investigated the potential mechanism(s) underlying this protection. Treatment with IGF-1 (1–100 ng/ml) promoted a dose dependent increase in cell survival against osmotic cell death. Both Akt and ERK1/2 were rapidly phosphorylated by IGF-1 and blocked by wortmannin and PD98059, inhibitors of their upstream activators respectively. However, IGF-1-induced protection was mediated via a wortmannin-dependent but PD98059-independent pathway as determined by cell survival assay suggesting a role of PI3-K/Akt. Furthermore, IGF-1 appeared to reduce the activation of a number of early components in the apoptotic pathway in a wortmannin dependent manner including the osmotic stress-induced perturbation in mitochondrial membrane potential, cleavage and activation of caspase-3 and DNA fragmentation. Thus, the results suggest that IGF-1 regulates osmotic stress-induced apoptosis via the activation of the PI3-K/Akt pathway at a point upstream of the mitochondria and caspase-3.** 

*Key Words:* **apoptosis; osmotic stress; cardiac fibroblasts; IGF-1; cytoprotection; phosphatidylinositol 3-kinase; wortmannin; Akt; ERK1/2; caspases.**

Insulin-like growth factor 1 (IGF-1) is a single chain peptide initially characterized as having mitogenic actions as well as insulin-like activities in muscle and adipose tissues. In addition to these effects, IGF-1 has also been shown to protect a variety of cells against apoptosis (1–4). Apoptosis, or programmed cell death, is thought to be involved not only in normal physiological processes but also in pathogenesis of many disease states that result from an imbalance between positive and negative regulators of cell survival. This process is regulated and executed by a number of different cellular proteins and is characterized by a series of distinct morphological and biochemical changes within the cell including condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation and formation of apoptotic bodies (5, 6). Although this process plays an important role during development, it can also be triggered by a variety of stresses including ischemia/reperfusion (7, 8) and sorbitol-induced osmotic stress (3, 9–11). Depending on the specific trigger and particular cell type, any one of a number of different signaling pathways can be activated. However, these pathways appear to converge into a common death pathway involving the activation of caspases, a family of aspartate-specific cysteine proteases (12). In particular caspase-3 has been proposed to be the major caspase co-ordinating the process of apoptotic cell death via cleavage of cytoskeletal and nuclear proteins.

Within the cardiovascular system IGF-1 has been shown to have a number of beneficial effects on the heart including protection against apoptosis (13–16; reviewed by 17). However, most of these cytoprotective studies have focussed on the effect of IGF-1 on cardiomyocyte apoptosis whilst the effects on the other cell types that make up the heart have generally been neglected. Indeed, fibroblasts are the most abundant cell type in the heart and these cells play an important role in maintaining the architecture of the heart (18). However, although a recent study by Morales *et al.* (3) has described the ability of IGF-1 to protect cardiomyocytes against osmotic stress-induced apoptosis, no studies have investigated the potential cytoprotective properties of IGF-1 in cardiac fibroblasts undergoing osmotic stress-induced apoptosis. Therefore, in this study we have determined the ability of IGF-1 to protect cardiac fibroblasts against osmotic stress-induced apoptosis and investigated the potential mechanism(s)

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underlying this protection. The results suggest that IGF-1 promotes cell survival against osmotic stress-induced cell death in a dose dependent manner via the activation of the PI3-K/Akt pathway, but not MEK/ERK pathway. Activation of this pathway leads to protection by blocking the cell death pathway at a point upstream of the mitochondria and effector caspase-3.

## MATERIALS AND METHODS

*Isolation and culture of neonatal rat fibroblasts.* Neonatal rat cardiac fibroblasts were isolated by serial pre-plating during the enzymic isolation of cardiomyocytes and grown to confluence on 90 mm dishes in full growth media (FGM; DMEM + 10% FCS + 1% Pen/Strep). Cells were passaged and grown until confluent on 60 mm dishes for cell signaling or 12 well plates for cell survival studies respectively. When confluent, serum was reduced to 1% FCS followed by 24 h incubation in serum free medium (SFM) prior to treatment. Cells were treated with 500 mM sorbitol dissolved in SFM in the presence/absence of IGF-1 for up to 8 h.

*Determination of cell viability.* Cell viability following sorbitol treatment was determined by methyl thiazoyltetrazolium (MTT) metabolism. Following treatment, cells were washed with warm PBS and incubated with 0.5 mg/ml MTT in PBS for 1 h at 37°C. The reaction was stopped by the addition of an equal volume of stop solution (acidified isopropanol; 1% Triton X-100) and the absorbance read at 570 nm.

*Polyacrylamide gel electrophoresis and Western blot analysis.* Following treatment, fibroblasts were rinsed with ice cold phosphate buffered saline (PBS) and harvested in 2× concentrated SDS-PAGE sample buffer (19). Proteins were separated on either 10% or 12.5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Following transfer, membranes were blocked for 2 h with 1% nonfat milk in Tris buffered saline (pH 7.6) containing 0.1% Triton (TBST) and probed overnight at 4°C with primary antibody (antibodies recognizing PKCδ [Transduction Labs, Oxford, UK], caspase-3 [Upstate Biochemicals, Botolph Claydon, UK], phospho-specific Akt Ser473 [1:500 dilution; New England Biolabs, Hitchin, UK] or phospho-specific ERK1/2 [1:1000 dilution; NEB]). Membranes were washed three times for 5 min in TBST containing 0.1% non-fat milk (TBSTM) prior to addition of secondary antibody (1:2500 dilution in TBSTM; Dako, Cambridge, UK) for 2 h at room temperature. Membranes were washed three times for 5 min with TBSTM and the antibody-antigen complexes were visualized by enhanced chemiluminescence.

*Analysis of mitochondrial membrane potential and DNA fragmentation by flow cytometry.* Changes in inner mitochondrial membrane potential and DNA fragmentation were studied during osmotic stress using either 3-3′-dihexyloxacarbocyanine ($DiOC_6(3)$; Sigma, Poole, UK) or propidium iodide (Sigma) respectively. Fibroblasts ($1 \times 10^5$) were treated with sorbitol in the presence/absence of IGF-1 and wortmannin either for 3 h to assess mitochondrial membrane potential ($\Delta\psi_m$) or for 5 h for DNA fragmentation and collected by trypsinisation. For determination of $\Delta\psi_m$, cells were then incubated in 250 μl PBS containing 1 nM $DiOC_6(3)$ for 20 min at 37°C and $DiOC_6(3)$ membrane potential-related fluorescence recorded on FL1-H by flow cytometry (Becton Dickinson, Oxford, UK). Data was analyzed using Win MIDI software and the loss of membrane-related fluorescence determined and expressed as a proportion of the total number of cells counted. For DNA fragmentation, cells were fixed in 500 μl of 70% ethanol overnight at −20°C. Following fixation, cells were centrifuged, washed with PBS and incubated in 500 μl PBS containing 50 μg/ml propidium iodide (PI) and 20 μg/ml RNase for 30 min at room temperature. PI-related fluorescence recorded on FL2-H by flow cytometry (Becton Dickinson, Oxford, UK). Data was analyzed using Win MIDI software and the proportion of fragmented DNA in the sub $G_1$ peak determined and expressed relative to control cells.

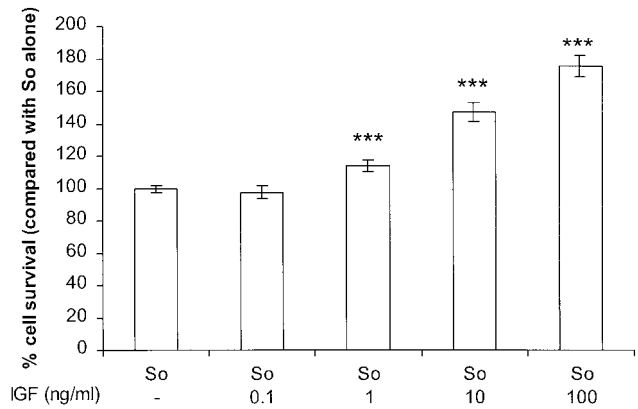


**FIG. 1.** IGF-1 promotes a dose-dependent protection against osmotic stress-induced cell death in cardiac fibroblasts. Cells were treated with IGF-1 (0.1–100 ng/ml) during 500 mM sorbitol for 8 h and cell viability determined by MTT metabolism. Results are expressed as mean ± S.E.M. from 3 independent experiments as percentage of cell survival relative to sorbitol treated cells; $***P < 0.001$ compared with sorbitol alone.

*Statistical analysis.* Data are expressed as mean ± S.E.M. from at least 3 independent experiments. Statistical analysis was performed using ANOVA with $P < 0.05$ being statistically significant.

## RESULTS

### *IGF-1 Promotes Protection against Osmotic-Induced Cell Death*

To determine the protective effect of IGF-1 against osmotic-induced cell death in neonatal cardiac fibroblasts, cells were treated for 8 h with 500 mM sorbitol in the presence/absence of increasing concentrations of IGF-1 (0.1–100 ng/ml) and cell survival determined using MTT bioreductive capacity. Figure 1 shows that IGF-1 promoted a dose-dependent increase in cell survival with IGF-1 concentrations between 1–100 ng/ml with significant protection being observed with doses of IGF-1 at 1 ng/ml ($P < 0.001$) and above.

### *Effect of Akt and ERK1/2 Activation on IGF-1-Mediated Protection*

Apoptosis induced by a variety of stresses can be blocked by growth factors via the activation of two major cytoprotective pathways incorporating PI3-K (20, 21) and its downstream target the serine/threonine kinase Akt (also known as protein kinase B or RAC-PK; 22–24) and the classical mitogen activated protein kinases (MAPK, also known as extracellular signal regulated protein kinase 1 and 2; ERK1/2; 20, 25, 26). Thus, the activation of Akt and ERK1/2 was determined upon IGF-1 treatment in fibroblasts. Figure 2A shows that treatment for 10 min with IGF-1

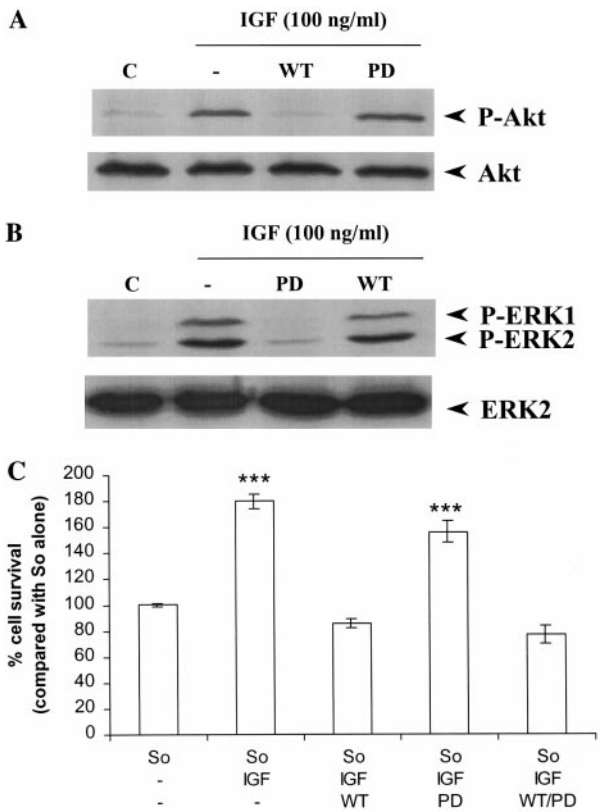


**FIG. 2.** IGF-1 induces phosphorylation of Akt and ERK1/2 but IGF-1 protects in a wortmannin sensitive but PD98059 insensitive manner. Cardiac fibroblasts were treated with IGF-1 (100 ng/ml) alone for 10 min or with wortmannin (WT; 100 nM) or PD98059 (PD; 50 $\mu$M) for 30 min prior to and for 10 min during IGF-1 treatment. Following treatment cells were lysed with 2× sample buffer and resolved by 10% SDS–PAGE and analyzed by Western blotting using antibodies recognizing either phosphorylated (A) Akt (S473) or (B) ERK1/2. Lower panels show total Akt and ERK2 respectively. In (C), cardiac fibroblasts were treated with IGF-1 (100 ng/ml) during treatment with sorbitol (500 mM) for 8 h in the presence/absence of wortmannin (100 nM) or PD98059 (50 $\mu$M). Cell viability was determined by MTT metabolism at the end of the treatment period and results expressed as a percentage of cell survival relative to sorbitol alone. Results expressed as mean ± S.E.M. from 3 independent experiments; $***P < 0.001$ compared with sorbitol alone.

(100 ng/ml) induced the phosphorylation of Akt on Ser473 and this was abolished by pre-treatment with wortmannin (+WT), an inhibitor of the upstream Akt activator, PI3-K, but not the MAPK inhibitor PD98059 (+PD). The lower panel shows total Akt to demonstrate similar protein loadings. Similarly, phosphorylation of ERK1/2 was observed after 10 min treatment with IGF-1 (100 ng/ml) (Fig. 2B; lane 2) which was blocked by PD98059 (Fig. 2B; lane 3) but not wortmannin (Fig. 2B; lane 4). The lower panel shows total ERK2 to demonstrate similar protein loadings.

To assess whether the activation of the PI3-K/Akt or ERK1/2 mediated IGF-1 protection against osmotic-induced cell death, cells were treated with sorbitol plus IGF-1 (100 ng/ml) in the presence/absence of wortmannin and PD98059 and cell survival determined using MTT metabolism. Figure 2C shows that IGF-1 significantly increased cell survival in fibroblasts compared with sorbitol alone and this was completely abolished in the presence of wortmannin but not PD98059 suggesting a role of the PI3-K/Akt pathway in protection. Further studies were undertaken to investigate the mechanism whereby IGF-1 prevented osmotic-induced cell death.

### *Effect of IGF on Osmotic Stress-Induced Perturbation in Mitochondrial Membrane Potential and DNA Fragmentation*

To determine the effects of osmotic stress and IGF-1 on $\Delta\psi_m$, fibroblasts were treated with sorbitol for 3 h prior to the addition of the fluorophore $DiOC_6(3)$. Figure 3A shows that osmotic stress induced a significant increase in the number of cells with depolarized mitochondria compared with cells with fluorophore alone. Treatment with IGF-1 significantly attenuated the osmotic stress-induced reduction in fluorescence and thus the collapse of $\Delta\psi_m$. However, blocking the PI3-K/Akt pathway with wortmannin abolished this effect of IGF-1. Furthermore, to show that IGF-1 protected against apoptosis we have used DNA fragmentation a characteristic feature of apoptotic cell death. Fibroblasts treated with sorbitol for 5 h had a significantly increased number of cells with fragmented DNA compared with control cells (Fig. 3B). However, treatment with IGF-1 significantly reduced the proportion of fragmented DNA in a wortmannin-dependent manner.

### *Effect of IGF-1 on Osmotic-Induction of Caspase-3 Cleavage*

Recent reports have demonstrated that sorbitol induces caspase-3 cleavage and thus may play a key role in osmotic-induced apoptosis (9, 27). We therefore examined the effect of IGF-1 on osmotic-stress-dependent caspase-3 activation by determining cleavage of procaspase-3 and its downstream target PKC$\delta$. Figure 4A shows that under control conditions no cleavage products were observed suggesting that caspase-3 had not been activated. However, after 4 h of sorbitol treatment, 20 kDa and 12 kDa cleavage products were detected (Fig. 4A, lane 2) indicating activation of caspase-3. Treatment with sorbitol in the presence of IGF-1 significantly reduced the appearance of the cleavage product (Fig. 4A, lane 3). Furthermore, blocking the protective PI3-K/Akt pathway with wortmannin prevented the IGF-1-induced delay in the appearance of the cleavage products. To ascertain whether the reduction in osmotic stress-induced procaspase-3 cleavage by IGF-1 equated with a decrease in enzyme activation, caspase-3 activity was determined by investigating the effects on proteolytic degradation of PKC$\delta$,

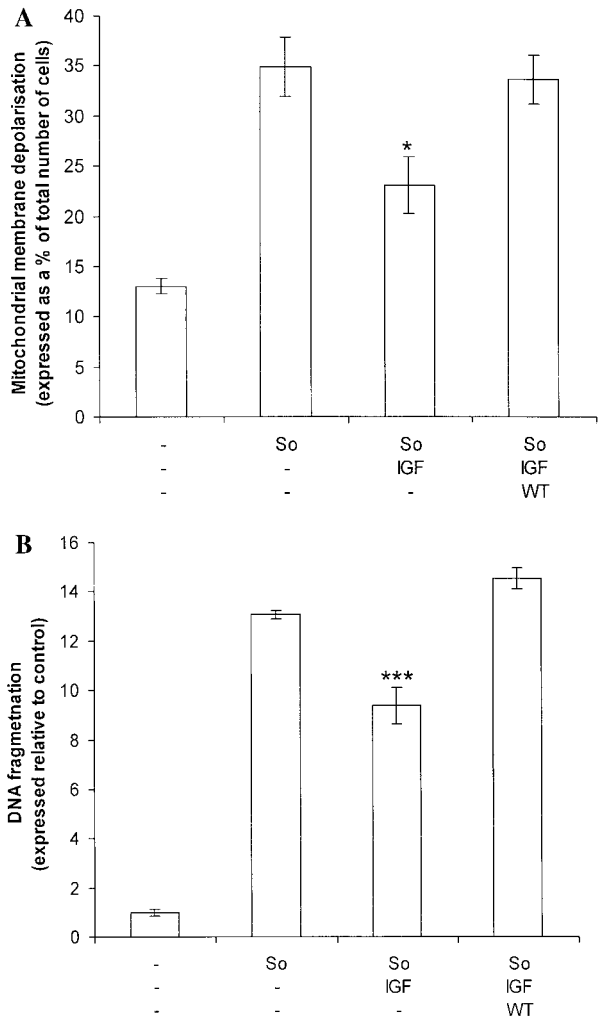


**FIG. 3.** IGF-1 reduces osmotic stress-induced decrease in mitochondrial $\Delta\psi_m$ and DNA fragmentation. Cardiac fibroblasts were treated with IGF-1 (100 ng/ml) in the presence/absence of wortmannin (100 nM) during treatment with sorbitol (500 mM). In (A), cells were treated for 3 h, trypsininsed and treated for 20 min with $DiOC_6(3)$. $DiOC_6(3)$ fluorescence (FL1-H) was measured by flow cytometry and the number of cells displaying $DiOC_6(3)$ fluorescence determined for each treatment and expressed as a percentage of the total number of cells. In (B), cells were treated for 5 h, trypsininsed and fixed overnight in 70% ethanol. Cells were then treated for 30 min with prodidium iodide (PI) and PI fluorescence (FL2-H) measured by flow cytometry and the number of cells displaying a loss of PI fluorescence determined for each treatment and expressed as a percentage of controls. Results are expressed as mean ± S.E.M ($n$ = 6), $*P < 0.05$; $***P < 0.001$ compared with sorbitol alone.

a downstream target of active caspase-3. Figure 4B shows that, consistent with the activation of caspase-3, sorbitol alone also induced the proteolytic degradation of PKCδ resulting in the appearance of a 40 kDa fragment (Fig. 4B, lane 2) whilst pretreatment with IGF-1 reduced but did not abolish the proteolytic cleavage of PKCδ (Fig. 4B, lane 3). However, wortmannin prevented the IGF-1-induced delay in the appearance of the 40 kDa fragment (Fig. 4B, lane 4).

## DISCUSSION

The protective effect of IGF-1 against apoptotic cell death in the central nervous system has been well established. Similarly, there has been a limited but increasing, interest in the cytoprotective role of IGF-1 within the heart. However, the vast majority of these studies have focussed on the ability of IGF-1 to protect cardiomyocytes against apoptotic cell death whilst generally ignoring the other cells that make up the heart, namely the cardiac fibroblasts. In this study we demonstrate for the first time that IGF-1 is capable of promoting cytoprotection against osmotic stress-induced apoptosis in cardiac fibroblasts. Thus, IGF-1 is capable of protecting not only the cardiomyocytes within the heart (3, 15, 16, 28) but also the surrounding cardiac fibroblasts against apoptotic stimuli. However, the challenge is to elucidate the signaling mechanism(s) required for this protection and how this impinges on the apoptotic process.

The various cellular effects of IGF-1 are initiated by the binding of the ligand to its membrane bound receptor and result in the subsequent activation a plethora of intracellular signaling pathways (29, 30). Of these pathways, two major parallel cytoprotective pathways, incorporating PI3-K/Akt and MEK/ERK, are activated by IGF-1 in a number of cell types (31–34) and, consistent with these findings, we demonstrate here that both pathways are activated upon IGF-1 treatment in cardiac fibroblasts. Previous studies have demonstrated the requirement of either the PI3-K/Akt pathway (4, 33, 35) or MEK/ERK pathway alone (32) or a

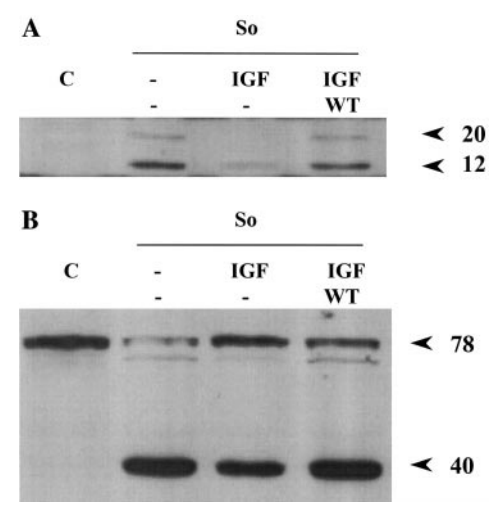


**FIG. 4.** IGF-1 reduces osmotic stress-induced (A) cleavage of caspase-3 and (B) PKCδ proteolytic degradation. Cardiac fibroblasts were treated with IGF-1 (100 ng/ml) during treatment with 500 mM sorbitol for 4 h. Cells were lysed in 2× SDS–PAGE sample buffer, resolved by 12.5% SDS–PAGE and analyzed by Western blotting for (A) caspase-3 or (B) PKCδ. Results are representative of three independent experiments.

combination of both pathways (20, 34) in protecting against a variety of different apoptotic stimuli. In this study we have used the specific inhibitors of inhibitors of PI3-K (wortmannin) and MEK (PD98059) to determine the role of these two pathways in survival and demonstrate that IGF-1 mediates protection by via the PI3-K/Akt pathway, but not MEK/ERK pathway. Thus these results demonstrate that the activation of the PI3-K/Akt pathway is both necessary and sufficient to promote IGF-1 mediated protection against osmotic stress-induced apoptosis in cardiac fibroblasts but is independent of MEK/ERK pathway. Therefore, it appears that the requirement of PI3-K/Akt and ERK/MEK acting alone or in combination is dependent not only on the apoptotic stimulus against which IGF-1 protects but also the particular cell type used.

Although the PI3-K/Akt pathway is required for IGF-1 mediated protection against osmotic stress-induced apoptosis, an understanding of how this pathway impinges on the cell death process is necessary but this requires a knowledge of the apoptotic process itself. Apoptotic signaling varies from cell to cell and, depending on the specific trigger, any one of a number of different signaling pathways can be activated. In general, a key event in the apoptotic pathway is the mitochondria permeability transition (MPT), which may lead to the release of cytochrome c and subsequent activation of caspase-3 (12). Indeed, previous studies using osmotic stress have proposed that apoptosis is induced via the release of cytochrome c from the mitochondria and subsequent activation of caspase-3 (9, 11, 27). Consistent with these studies we demonstrate here that sorbitol-induced osmotic stress activates caspase-3 in cardiac fibroblasts. In addition, we also demonstrate that osmotic stress-induced apoptosis is associated with a reduction in mitochondrial membrane potential characteristic of the MPT. Thus, the potential sequence of events leading to osmotic stress-induced apoptosis appears to be via the perturbation of mitochondrial function leading to the mitochondrial permeability transition and subsequent release of cytochrome c.

Once released cytochrome c then induces the cleavage and activation of caspase-3 and the further dismantling of the cell. Therefore, there are a number of different levels at which IGF-1 may impinge on the osmotic stress-induced apoptotic process to promote survival. Caspase-3 is an obvious target as, once activated, it coordinates the downstream proteolytic effects seen during apoptosis. Indeed, we (this report) and others (1, 2, 28) have demonstrated that IGF-1 prevents the cleavage and activation of this protease. However, although preventing or delaying caspase-3 activation may be the ultimate target of IGF-1, it does not appear to be the direct site of action for the cytoprotective signaling pathways as our results demonstrate that IGF-1 is capable of delaying the reduction in mitochondrial membrane potential.

It has been indicated that members of the Bcl2 family may affect the mitochondrial transmembrane potential. These proteins possess either pro-apoptotic (e.g., Bad and Bax) or anti-apoptotic properties (e.g., Bcl-2 and Bcl-$x_L$) and it appears that relative level of these proteins is essential for determining the fate of the cell. Indeed, IGF-1 has previously been shown to modulate the expression of both pro- and anti-apoptotic Bcl-2 proteins by either increasing Bcl-2 and Bcl-$x_L$ levels (33) or alternatively attenuating the expression of pro-apoptotic Bcl-2 proteins including Bax (28). However, we have investigated the expression of a number of these Bcl-2 proteins, including Bcl-2, Bax, Bad and Bcl-$x_L$, during osmotic treatment in the presence/absence of IGF-1 and found that levels of these proteins are unaffected (results not shown).

Therefore, it appears that IGF-1 mediated protection against osmotic stress-induced apoptosis in cardiac fibroblasts does not require modulation of Bcl-2 protein levels. However, the activity of the pro-apoptotic Bcl-2 proteins may also be regulated by phosphorylation and thus the balance between phosphorylated and unphosphorlyated states of pro-apoptotic Bcl-2 proteins may play a crucial role in determining the fate of the cell. Indeed, a number of survival factors have been shown to induce the phosphorylation of pro-apoptotic Bcl-2 family proteins via the PI3-K/Akt pathway and thus induce protection (4, 36, 37). Although this may account for the protective effect of IGF-1 in other cell types, further studies are required to ascertain the role of the pro-apoptotic Bcl-2 family of proteins in osmotic stress-induced apoptosis in cardiac fibroblasts.

In summary, our data demonstrate for the first time that IGF-1 protects cardiac fibroblasts against osmotic stress via the activation of the PI3-K/Akt but not MEK/ERK pathway. This pathway appears to impinge on the apoptotic pathway by impeding the osmotic stress-induced reduction in mitochondrial function and subsequent activation of caspase-3 and DNA fragmentation. Although the precise mechanism remains to be elucidated, we speculate that IGF-1 induced activation of the PI3-K/Akt may modulate the function of the pro-apoptotic proteins.

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