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Signal Transduction of the Protective Effect of Insulin Like Growth Factor-1 on Adriamycin-Induced Apoptosis in Cardiac Muscle Cells

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To determine whether Insulin-like growth factor (IGF-I) treatment represents a potential means of enhancing the survival of cardiac muscle cells from adriamycin (ADR)-induced cell death, the present study examined the ability of IGF-I to prevent cell death. The study was performed utilising the embryonic, rat, cardiac muscle cell line, H9C2. Incubating cardiac muscle cells in the presence of adriamycin increased cell death, as determined by MTT assay and annexin V-positive cell number. The addition of 100 ng/mL IGF-I, in the presence of adriamycin, decreased apoptosis. The effect of IGF-I on phosphorylation of PI, a substrate of phosphatidylinositol 3-kinase (PI 3-kinase) or protein kinase B (AKT), was also examined in H9C2 cardiac muscle cells. IGF-I increased the phosphorylation of ERK 1 and 2 and PKC ζ kinase. The use of inhibitors of PI 3-kinase (LY 294002), in the cell death assay, demonstrated partial abrogation of the protective effect of IGF-I. The MEK1 inhibitor-PD098059 and the PKC inhibitor-chelerythrine exhibited no effect on IGF-1-induced cell protection. In the regulatory subunit of PI3K-p85- dominant, negative plasmid-transfected cells, the IGF-1-induced protective effect was reversed. This data demonstrates that IGF-I protects cardiac muscle cells from ADR-induced cell death. Although IGF-I activates several signaling pathways that contribute to its protective effect in other cell types, only activation of PI 3-kinase contributes to this effect in H9C2 cardiac muscle cells.

Key words: Insulin-like growth factor (IGF-1), Phosphatidylinositol 3-kinase, Protein kinase B (AKT), PKC ζ kinase, H9C2 cardiac muscle cells

INTRODUCTION

Adriamycin (doxorubicin) is an anthracycline derivative and is one of the most frequently used antineoplastic agents in the treatment of leukemias and solid tumors (Ettinghausen *et al.*, 1986, Hitchcock-Bryan *et al.*, 1986). For the treatment of metastatic thyroid carcinoma, adriamycin is probably the best available agent (Martin *et al.*, 1991). It is also an important agent for the treatment of Hodgkins lymphomas (Liisa *et al.*, 1989). However, the severe cardiotoxicity of adriamycin is a major limiting factor for its effective use in the treatment of all malignancies (Doroshow *et al.*, 1991). The proposed mechanism for the cardiac toxic effect of adriamycin is the production of reactive oxygen species during its intracellular metabolism (Narayanan *et al.*, 1998; Nowak *et al.*, 1995). Significant efforts have been directed towards developing conjunctive therapy to decrease adriamycin-induced cardiotoxicity and enhance its anticancer therapeutic efficacy.

Pharmacological and clinical attempts to reduce the cardiotoxicity of adriamycin have been, thus far, only partially successful. Up to now, researchers have employed antioxidant agents such as superoxide dismutase (SOD), catalase and mannitol, which are known to scavenge superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals, respectively (Li *et al.*, 2000; Liu *et al.*, 2000).

Insulin-like growth factors (IGF-1 and IGF-2) are single-chain polypeptides that have structural homology with

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pro-insulin. IGF-1 has insulin-like short-term metabolic effects and growth factor-like long-term effects, on cell proliferation and differentiation of various cell types.

The importance of IGF-1, as an anti-apoptotic factor, is evidenced by observations in adriamycin-treated cardiac muscle cells. In this study, IGF-1 stimulated growth factor-related protein kinases, including phosphatidylinositol-3-OH-kinase (PI3K), protein kinase B (AKT), ERK and PKC. These results show that PI3K especially, has an essential role in the anti-apoptotic effects of IGF-1, in adriamycin-induced apoptosis of cardiac muscle cells.

MATERIALS AND METHODS

Materials

DMEM, fetal bovine serum, and trypsin were purchased from Life Technologies, Inc. The bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce. Adriamycin and IGF-1 were obtained from Sigma. The Annexin V-FITC apoptosis detection kit (6993KK) was obtained from PharMingen (San Diego, CA). All other chemicals were purchased from either Sigma or Aldrich. The purity of all reagents was at least analytical grade.

Detection of phosphatidyl serine with Annexin V-FITC

The annexin V-FITC cell membrane labeling assay was used to detect translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface, where it binds an annexin V-FITC conjugate and serves as an early marker of apoptosis. Non-adherent cells or cells grown on coverslips were incubated with annexin V-FITC for 15 min at room temperature in the dark and visualized by fluorescent microscopy with fluorescein filters as previously reported (Richard *et al.*, 1999).

Cytochrome c measurement

Cytosolic fractions were prepared from 1×10^7 H9C2 cardiac muscle cells by differential centrifugation in a buffer containing 250 mM sucrose as described previously (Chae *et al.*, 2000). Protein samples of 25 μg were loaded onto sodium dodecyl sulfate-15% polyacrylamide gel, subjected to electrophoresis and then electrophoretically transferred to nitrocellulose membranes. Western blots were probed with primary monoclonal anti-cytochrome c antibody (Pharmingen, San Diego, CA. USA) and secondary anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). These were then developed with an enhanced chemiluminescence kit (Kirkegaard & Perry Laboratories).

Assay of mitochondrial membrane potential ($\Delta\Psi m$)

To measure changes in $\Delta\Psi m$, the potential-sensitive fluorochrome DiO_6 (Molecular Probes, Eugene, OR) was used. Cells were incubated with various agents for 24 h, collected by centrifugation, and resuspended in PBS. DiO_6 (100 nM) was added and cells were incubated for 20 min at 37°C in the dark. Cells were analyzed by flow cytometry.

Fluorogenic substrate assay for caspase activity

Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl (pH 7.4), 10 μg/mL leupeptin, aprotinin (0.27 trypsin inhibitor/U/mL) and 100 μM PMSF. Caspase activity was determined by incubation of cell lysate (25 μg of total protein) with 50 μM of fluorogenic substrates such as AC-YVAD-AMC (caspase-1), AC-DEVD-AMC (caspase-3) or AC-LEHD-AFC (caspase-9) in a cell-free system buffer containing 10 mM Hepes (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol. The release of fluorescent 7-amino-4-methylcoumarin was measured for 1 h at 2 min intervals by spectrofluorometry.

Western blotting

Protein samples (100 μg) were mixed with an equal volume of 2×sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then separated by 10 or 12.5% SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by the semi-dry electrophoretic transfer method. The membranes were blocked in a 5% dry milk (1 h), rinsed and then incubated with primary antibodies (1:1000) in TBS overnight at 4°C. Primary antibody was removed, membranes were washed four times in TBS, then 0.1 μg/mL peroxidase-labeled mouse or rabbit secondary antibody was added for 1 h. Following four washings in TBS, the bands were visualized by ECL and exposed to X-ray film.

DCFDA assay

For measurement of intracellular H_2O_2, cells were loaded with 5 μM of 2, 7-dihydrodichlorofluorescin diacetate (DCF-DA, Molecular Probes) for 10 min. The esterified form of DCF-DA can permeate cell membranes and subsequently be deacetylated by intracellular esterase. The resulting compound, dichlorofluorescein, is reactive with H_2O_2 to give fluorescence. The cells were washed with PBS, an aliquot was taken for cell counting, and the rest was used to determine the relative amounts of H_2O_2 by measuring fluorescence with a spectrophotofluorometer with excitation and emission wavelengths of 495 nm and 520 nm, respectively. Cell numbers were determined in parallel, and fluorescence values were normalized to the number of cells in each sample.

PI3K assay

Cell lysates were immunoprecipitated with phosphotyrosine Ab-conjugated protein Gsepharose. The immunoprecipitates were assayed for PI3K activity as described previously (Linassier *et al.*, 1997). Briefly, beads were washed twice in lysis buffer and twice in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, and 0.1% β-mercaptoethanol at 25°C. Reactions were performed at 30°C for 15 min, in a total volume of 50 μl, in the presence of either 3 mM Mg^{2+} or 3 mM Mn^{2+}, 40 μM ATP and 200 μM lipid. In some experiments, assays were performed in the presence of 0.5% Nonidet P40 (NP40; BDH). Reactions were terminated with 1 M HCl/chloroform/methanol (1:1:1, by vol.), mixed and centrifuged to separate organic and aqueous phases. The lipid products in the lower layer were extracted and resolved by TLC.

PKC activity assay

The activity of PKC was determined by measuring the phosphorylation of a PKC peptide substrate, MBP. Cells were rinsed twice and treated in serum-free, alpha-MEM with 100 ng/mL IGF-1, for indicated times or, with the indicated concentrations of IGF-1 for 20 min. After aspiration of the medium, PKC activity was assayed by incubating the cells for 10 min in 100 μL of permeabilization kinase assay buffer per well as previously described (Vanio *et al.*, 1995). The buffer contained 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 1 mg/mL glucose, 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 mM β-glycerophophate, 2.5 mM $CaCl_2$, 5 mM EGTA, 100 μM [γ^{32}-P]-dATP (500 cpm/pmol), 50 μg/mL digitonin, and 50 μM acetylated-MBP. The reaction was terminated by addition of 10 μL of 25% TCA (w/v) and an aliquot was spotted onto a phosphocellulose disk, which was then washed with 1% (v/v) phosphoric acid and counted in a scintillation counter.

Transient transfection and LacZ cell death assay

Cells were plated 24 h before transfection at a density of 1×10^4 cells/well in a 6-well plate. Cells were co-transfected with pCMV-β-gal plasmid (1 μg) which expresses β-galactosidase, and plasmids (4 μg) containing DN-p85 or pcDNA3 control vector, utilizing the Lipofectamine transfection reagent. Transfections were performed in triplicate. Twenty-four hours after transfection, the cell medium was replaced with a fresh medium for 12 h, and then exposed to agents. Cells were washed after treatment and fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. These cells were washed twice more with PBS, resuspended for 4 to 24 h in staining solution containing PBS (pH 7.4), 1 mM $MgCl_2$, 10 mM $K_4Fe(CN)_6$, 10 mM $K_3He(CN)_6$ and 1 mM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; added just before use), and then washed twice with PBS. The β-galactosidase-positive cells (blue living cells) in the pcDNA- transfected well were counted. The number of blue living cells can be considered as 100%. The percentage of dead cells is calculated as a relative percentage compared with 100% of pcDNA-transfected cells.

Statistical analysis

Statistical differences were evaluated by analysis of variance (ANOVA) in dose-response experiments and two-tailed Students *t*-tests. In each case, the statistical test used is indicated and the number of experiments is stated individually in the legend of each figure.

RESULTS

Insulin-like growth factor induces cell resistance to adriamycin-induced apoptosis in cardiac muscle cells

To explore whether Insulin-like growth factor (IGF-1)

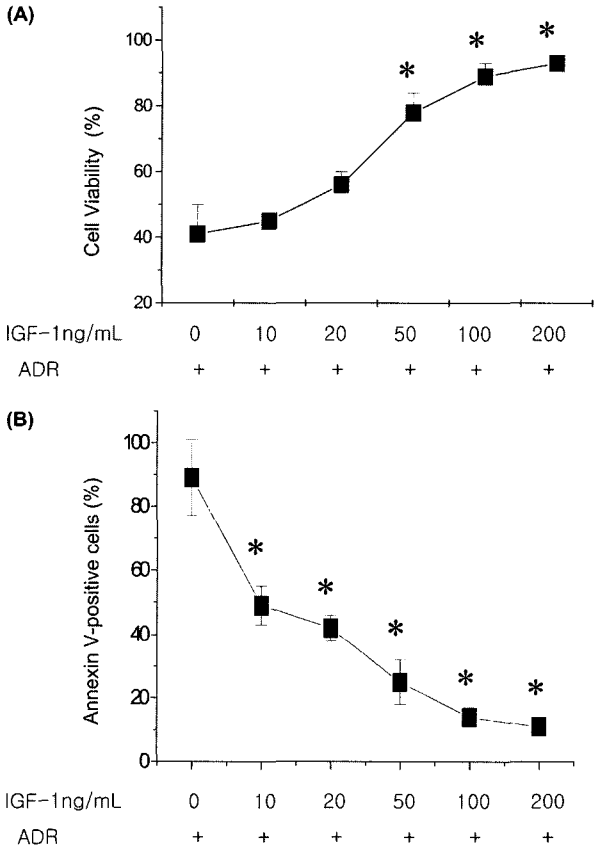

Fig. 1. IGF-1 reduces adriamycin-induced cell death in H9C2 cardiac muscle cells. Cells were treated with IGF-1 at indicated doses and then incubated with 2 μM adriamycin. (A) Cell viability was measured by MTT assay as described in Materials and Methods. (B) Apoptosis assay was performed using the Annexin V kit. The percentage of apoptotic cells (annexinV-positive cells/total cells ×100) was determined for a minimum of 20 microscopic fields (≥500 cells). Results are expressed as mean ± S.E. of three experiments. *P<0.05 versus adriamycin (2 μM).

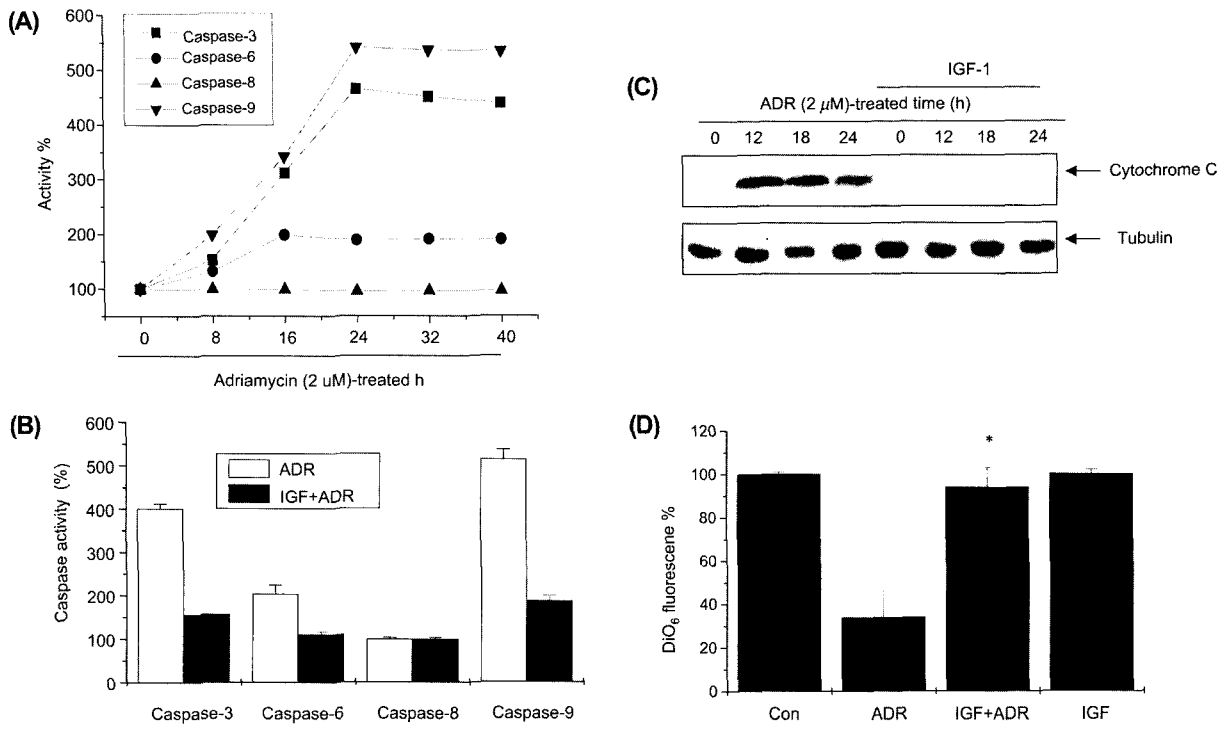

Fig. 2. IGF-1 inhibits adriamycin-induced activation of caspase-3, 6 and 9 in H9C2 cardiac muscle cells. (A) Cells were treated with adriamycin (2 μM) for various periods as indicated. The result is representative of the results of three independent experiments. (B) Cells were exposed to adriamycin for 24 h in the presence or absence of 100 ng/mL IGF-1 after which caspase-3, 6, 8 and 9 activities were measured as described in Materials and Methods. (C) Cells were treated with adriamycin (2 μM) in the presence or absence of 100 ng/mL IGF-1. Cytoplasmic fractions were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Cytochrome c was visualized by western blot analysis. (D) Cells were exposed to adriamycin (2 μM) for 24 h in the presence or absence of 100 ng/mL IGF-1. Mitochondrial membrane potential was assessed as described in Materials and Methods. (B) to (D) Results are expressed mean ± S.E. of three experiments. Results are expressed as mean ± S.E. of three experiments. *P<0.05 versus adriamycin (2 μM).

modifies adriamycin-induced responses in H9C2 cardiac muscle cells, the cells were exposed to IGF-1 (0, 10, 20, 50, 100 and 200 ng/mL), and subsequently stimulated with adriamycin (2 μM). Fig. 1A shows the effect of IGF-1 on cytotoxicity caused by adriamycin, in cardiac muscle cells at 24 h. At concentrations of more than 50 ng/mL of IGF-1, IGF-1 protected adriamycin-increased annexin V-positive cells: an early positive apoptosis marker. Maximum protection with IGF-1 against adriamycin-induced toxicity, was seen at concentrations of 100 or 200 ng/mL. At higher doses of IGF-1, there was no further improvement in the protective effect (data not shown).

Insulin-like growth factor regulates adriamycin-induced cytochrome c release, collapse of mitochondrial membrane potential (ΔΨm) and the subsequent caspase activation in cardiac muscle cells

Activation of the proteolytic caspase cascades is a pathway of apoptosis in diverse biological systems. Members of the caspase family are synthesized as proforms, which are proteolytically cleaved and activated during apoptosis (Takahashi *et al.*, 1999). In various cell types undergoing apoptosis, caspase-3, 6 and 9, constitute the major pool of activated caspases, regardless of the initial apoptotic stimuli (Perry *et al.*, 1997; Chae *et al.*, 2000). However, other caspases-caspase-1, 2 and 8, are also activated during the execution phase of apoptosis (Tatsta T *et al.*, 2000; Watt W *et al.*, 1999). Therefore, the fluorescent intensity of the caspase protease cleavage product, AMC, was monitored over various periods of incubation of adriamycin (0, 8, 16, 24, 32, and 40 h). Caspase-3 and 9 were markedly activated in adriamycin-treated H9C2 cardiac muscle cells. Additionally, caspase-6 was moderately activated in adriamycin-exposed cells, whereas caspase-8 was not affected by adriamycin (Fig. 2A). To further assess the influence of IGF-1 on adriamycin-induced caspase activation, caspase activity was measured in adriamycin-treated cells in the presence or absence of IGF-1. As shown in Fig. 2B, adriamycin-induced activation of caspase 3, 6 and 9, was significantly inhibited by IGF-1 (100 ng/mL).This data suggests that the regulation of caspase-3, 6 and 9-like cysteine protease activity is involved in the IGF-1-induced protective function on

adriamycin-induced apoptosis. Next to be examined was the effect of IGF-1 on cytochrome c release after 12, 18, or 24 h incubation with 2 μM adriamycin. IGF-1 blocked adriamycin-induced cytochrome c release into the cytoplasm (Fig. 2C).

In light of these changes in mitochondrial function, an assessment was made as to whether there was any decrease in mitochondrial membrane potential, using the potential-sensitive dye, DiO_6. The cardiac muscle cells exhibited loss of mitochondrial membrane potential ($\Delta\Psi m$) with 2 μM adriamycin. Loss of membrane potential persisted at 24 h (data not shown). In contrast, no change in $\Delta\Psi m$ was detectable after adriamycin treatment in the presence of IGF-1. Similar results were obtained with rhodamine 123 staining (data not shown). In this experiment, loss of $\Delta\Psi m$ lagged slightly behind changes in cytochrome c translocation. Fig. 2D, shows that IGF-1 had a regulatory effect on adriamycin-induced loss of $\Delta\Psi m$ in cardiac muscle cells. Therefore, the inhibition of apoptosis by IGF-1 is associated with a reduced release of cytochrome c into the cytoplasm and a reduced loss of $\Delta\Psi m$ in H9C2 cells.

Thus, IGF-1 protects adriamycin-induced cytochrome c release and the subsequent collapse of $\Delta\Psi m$ and caspase-3 and 9 activation in H9C2 cardiac muscle cells.

Insulin-like growth factor has no effect on adriamycin-induced ROS release in cardiac muscle cells

Reactive oxygen species (ROS) produced in cells were determined by measuring fluorescence after loading with DCFDA, a dye that is oxidized into a highly fluorescent form, in the presence of peroxides. DCFDA can be oxidized by any peroxidase and hydroperoxide, including H_2O_2. To specify the active oxygen species that were responsible for oxidation of DCF, an examination into whether IGF-1 prevents the oxidation of DCF was carried out. Cells were exposed to 2 uM adriamycin in the absence or presence of IGF-1 (100 ng/mL). The pretreatment with IGF-1 did not have any effect on DCF oxidation (Fig. 3).

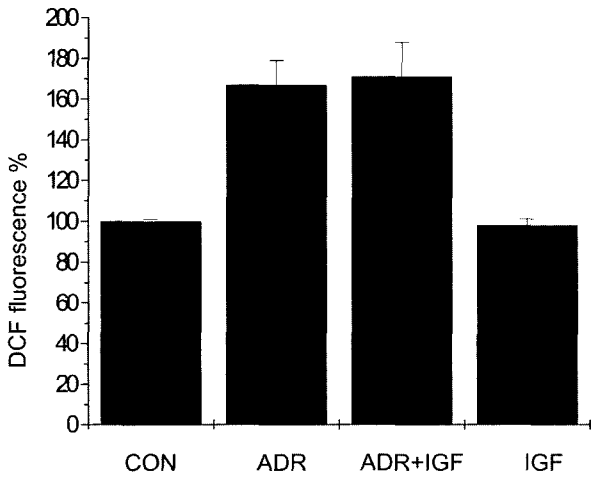

Fig. 3. IGF-1 has no effect on adriamycin-induced H2O2 release in rat ventricular cardiomyocytes. Cells were treated with adriamycin (2 μM) for 1 h in the presence or absence of 100 ng/mL IGF-1. The cells were then incubated with 100 μM DCF-DA or the equivalent amount of DMSO for 10 min at 37°C. These cells were washed with PBS, an aliquot was taken for cell counting and the rest was used to determine the relative amounts of H_2O_2 by measuring fluorescence with a spectrophotofluorometer (excitation, 495 nm; emission, 520 nm). Results are expressed mean ± S.E. of three experiments.

Insulin-like growth factor stimulates PI3 kinase in cardiac muscle cells

To determine the role of PI3K in the signaling pathways of IGF-1, the effect of IGF-1 on PI3K activity was examined using a lipid kinase assay, to monitor the conversion of PI into PI-P (Chesley A *et al.*, 2000). As shown in Fig. 4A, the incubation of IGF-1 (100 ng/mL) for 5 minutes increases

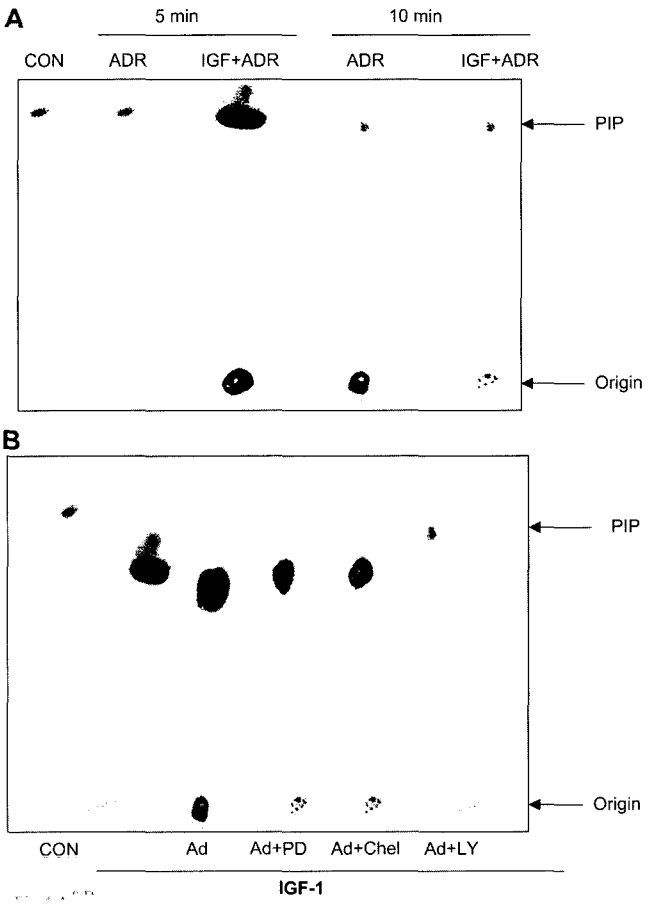

Fig. 4. IGF-1-induced PI3K activation is not associated with either PKC or ERK. (A) H9C2 cells were treated with adriamycin (2 μM) for 5 or 10 min in the presence or absence of 100 ng/mL IGF-1. (B) H9C2 cells were treated as indicated and lysates with equal amounts of protein were subjected to immunoprecipitation with anti-phosphotyrosine antibody. The immunocomplex was performed for PI3K activity assays as described under Materials and Methods.

PI-P production and the addition of a highly selective PI3K antagonist, LY294002 (10 μM), blocked this effect (data not shown). Based upon the reports, that a PKC-dependent pathway is implicated in the IGF-1-induced signaling pathway, the question of how PKC is involved in IGF-1-induced PI3K activation was examined. Treatment with a PKC inhibitor, chelerythrine, had no effect on IGF-1-induced PI3K activation. Similarly to the PKC inhibitor, the MEK inhibitor, PD098059 had no regulatory effect on IGF-1-induced PI3K activation (Fig. 4B). This shows that IGF-1 induces PI3K activation independent of PKC or ERK in cardiac muscle cells.

Insulin-like growth factor stimulates AKT and ERK activation in cardiac muscle cells

To evaluate whether AKT kinase, known as PI3K downstream kinase, is involved in the signaling pathway of IGF-1, the ability of IGF-1 to stimulate AKT activation in H9C2 cardiac muscle cells was examined. As shown in Fig. 5, the treatment of IGF-1 induced a rapid and transient AKT phosphorylation, which increased within 5 minutes and then decreased to baseline after 20 minutes. In addition, the examination as to whether IGF-1 is involved in the MAPK pathway required that we measured ERK 1 and 2 activations. IGF-1 treatment induced ERK 1 and 2 phosphorylations within 5 min and then decreased after 20 min (Fig. 6).

PI3K and ERK pathways are dissociated from IGF-1 signaling transduction

The direct effect of the MEK1 inhibitor, PD98059, on IGF-1-induced AKT phosphorylation, was tested in H9C2 cardiac muscle cells. Without the MEK 1 inhibitor, LY294002 (10 μM) completely inhibited AKT activation. In addition, the LY compound had no effect on IGF-1-induced ERK activation (Fig. 7). These results indicated that ERK and AKT

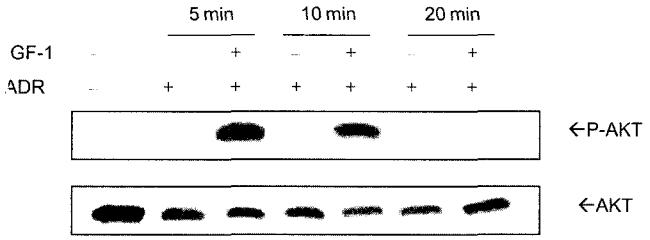

Fig. 5. The effect of IGF-I on Akt phosphorylation in H9C2 cardiac muscle cells. Cells were treated for various time intervals (0, 5, 10 or 20 min) with 2 μM adriamycin, in the presence or absence of 100 ng/mL IGF-I. Western blot analysis was performed using an antibody directed against phosphorylated Akt, at a dilution of 1:1,000. After detection of phospho-Akt, the blot was stripped and reprobed with a 1:1,000 dilution of an antibody directed against Akt. The results are representative of the results of three independent experiments performed using different cell lysates.

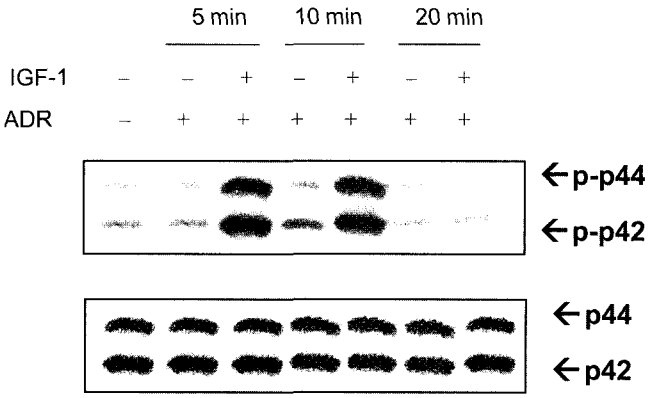

Fig. 6. The effect of IGF-I on ERK 1 and 2 phosphorylation in H9C2 cardiac muscle cells. Cells were treated for the indicated periods of time with 2 μM adriamycin, in the presence or absence of 100 ng/ml IGF-1. Phosphorylated and total ERK 1 and 2 levels were determined as described above. The results are representative of the results of three independent experiments performed using different cell lysates.

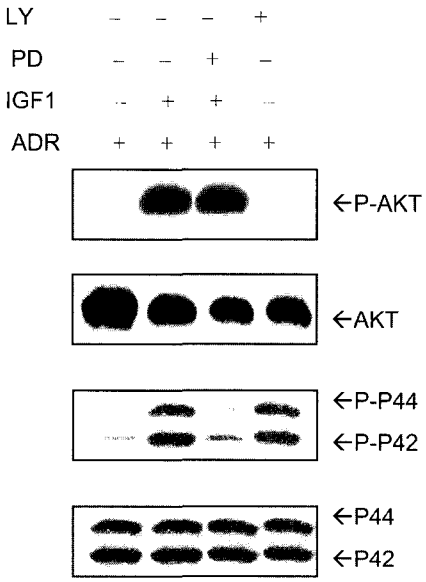

Fig. 7. ERK is not involved in IGF-1-induced AKT activation. Cells were treated as indicated for 10 min. Phosphorylated and total AKT levels were determined as described above. The results are representative of the results of three independent experiments performed using different cell lysates.

were dissociated in IGF-1-induced signaling pathways.

IGF-1 induces PKCζ activity in cardiac muscle cells

MBP can also be a substrate for in vitro assays of PKC activity. Among PKC isoenzymes, PKCζ plays a major role in MBP phosphorylation (Vanio *et al.*, 1995). This result shows that PKC ζ was increased in the presence of 100 ng/mL IGF-1. The treatment with IGF-1 induced a rapid and transient MBP phosphorylation, which increased

within 20 minutes and then decreased to baseline after 30 minutes (Fig. 8A). The next examination conducted was how PKC is involved in IGF-1-induced PI3K activation. Treatment with the MEK1 inhibitor, PD98059, had no effect on IGF-1-induced PKCζ activation. However, the PI3K specific inhibitor, LY294002, had a partial but significant regulatory effect on IGF-1-induced PKCζ activation (Fig. 8B). This shows that IGF-1 induces PKCζ activation independent of ERK and the PKCζ activity can be controlled by PI3K in H9C2 cardiac muscle cells. A similar result was obtained when worthmannin was used instead of the LY compound.

PI3K has an important role in the IGF-1-induced protective effect

To evaluate the biological significance of PI3K, ERK or PKC kinase activation by IGF-1, the effect of LY294002, PD098059 or chelerythrine chloride, on the IGF-1-induced protective effect in adriamycin-exposed cardiac muscles, was tested. Cells were pretreated for 30 min with each agents (10 μM LY294002, 10 μM PD98059 or 1 μM chelerythrine chloride), followed by 24 h of incubation with 2 μM adriamycin, in the presence or absence of 100 ng/

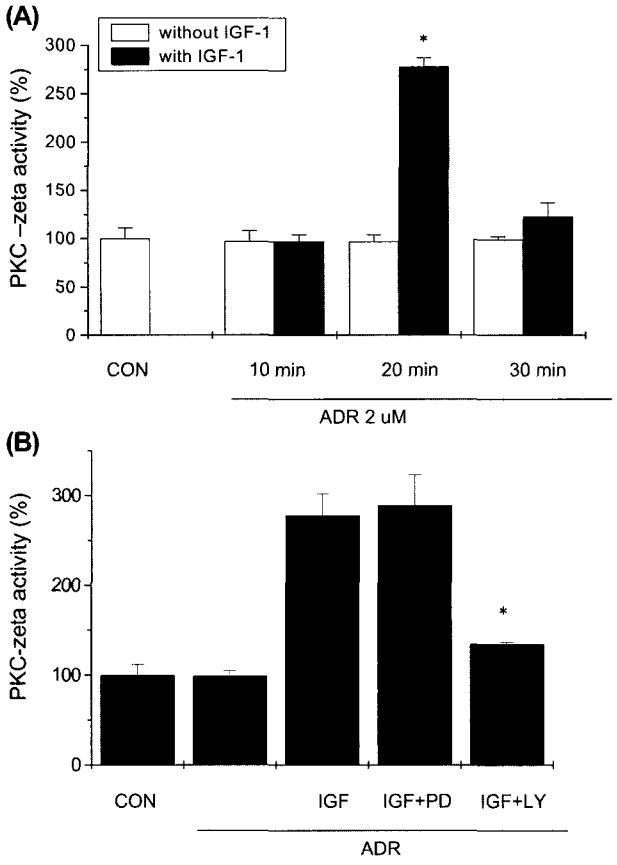

Fig. 8. PI3K but not ERK has no involvement in IGF-1-induced PKC ζ activation. (A) Cells were treated for the indicated periods of time with 100 ng/mL IGF-I in the presence of 2 μM adriamycin. *P<0.05 versus ADR-treated (without IGF-1). (B) Cells were treated with the indicated agents. Phosphorylated MBP was measured as described in Materials and Methods. Results are expressed as mean ± S.E. of three experiments. *P<0.05 versus IGF-1+ ADR.

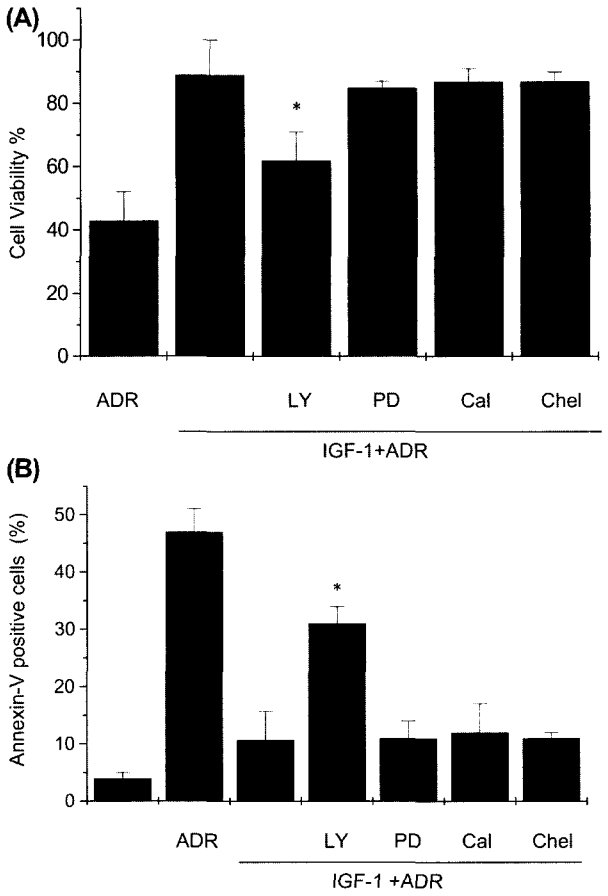

Fig. 9. PI3K has a critical role in the IGF-1-induced protective effect in cardiac muscle cells. A. Cells were treated with 10 μM LY 294002, 10 μM PD98059, 1 μM calphostin C or 1 μM chelerythrine. Thirty minutes later, 100 ng/mL IGF-1 and 2 μM adriamycin were added prior to incubation for 24 h. MTT assay was performed as described in Materials and Methods. *P<0.05 versus IGF-1+ADR. B. Cells were treated as described in panel A. Apoptosis assay was performed using the Annexin V kit. The percentage of apoptotic cells (annexinV-positive cells/total cells ×100) was determined for a minimum of 20 microscopic fields (≥500 cells). Results are expressed as mean ± S.E. of three experiments. *P<0.05 versus IGF-1+ADR. C. DN-p85 transfection abolished IGF-1-induced anti-cell death activity. H9C2 cells were transfected with pCMV-β-gal (1 μg/mL) and either DN-p85 (4 μg/mL) or empty vecor (4 μg/mL). After 24 h of transfection, the cell medium was replaced with the fresh medium and the cells were exposed to 2 μM adriamycin, in the presence or absence of 100 ng/ml IGF-1. After treatment, cells were washed and fixed to determine β-galactosidase activity, as described under Materials and Methods. The surviving cells were counted as the number of blue cells expressing β-galactosidase activity. The numbers of β-galactosidase-positive cells under the empty vector-transfection were considered as 100%. Results are expressed as mean ± S.E. of three experiments. *P<0.05 versus IGF-1 + ADR (β-gal-transfected cells).

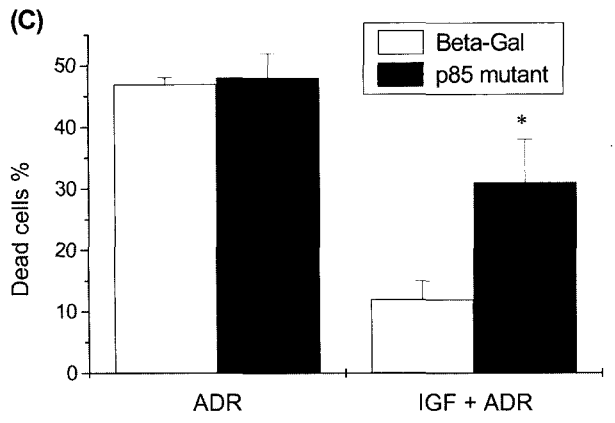

Fig. 9. Continued.

ml IGF-1. Each agent was present throughout the incubation period. LY294002 regulated IGF-1-increased cell viability (Fig. 9A). Figure 9B shows a similar protective effect. This protective effect was significantly inhibited by LY294002. However, the other inhibitors had no effect on the IGF-1-induced protective effect. Although IGF-I activates several signaling pathways, that contribute to its protective effect in other cell types, only activation of PI 3-kinase contributes to this effect in H9C2 cardiac muscle cells. A transient transfection death assay was therefore conducted to investigate PI3K-involved regulation of apoptosis. H9C2 cardiac muscle cells were transfected with pcDNA3 empty vector or p85 (a regulatory subunit of PI3K) dominant negative plasmid in combination with pCMV-β-gal. Twenty-four hours after transfection, transfected cells were changed to fresh media and treated with 2 μM adriamycn, in the presence or absence of IGF-1. The transfection results revealed that the DN-p85 transfection, but not the control pcDNA3, abolished IGF-1-induced cell survival activity when exposed to 2 μM adriamycin (Fig. 9C).

DISCUSSION

In the present study, it has been shown that IGF-1 effectively protects cardiac muscle cells against adriamycin-induced apoptosis. Several recent studies have indicated that apoptosis occurs in the human heart with end stage cardiac failure or acute myocardial infarction, suggesting the involvement of apoptosis in the cardiovascular diseases (Formigli *et al.*, 1998). From this present study, it is also speculated that apoptosis may be involved in the genesis of adriamycin-induced cardiomyopathy, although the final conclusion should only be rendered after in vivo or human study.

Our experiments show that caspase-3, 6 and 9 processing and the release of cytochrome c, precede apoptosis by adriamycin, thus indicating a temporal and perhaps casual relationship. Furthermore, the release of cytochrome c into the cytoplasm is blocked by IGF-1, suggesting that IGF-1 treatment propagates an inhibitory signal that determines mitochondrial cytochrome c release. Cytochrome c is an essential component of the mitochondrial respiratory chain and is also released from the mitochondria in response to apoptotic stimuli. These stimuli include UV irradiation, etoposide, staurosporine, actinomycin D, H_2O_2 and Ara-C. Our results provide evidence that IGF-1 significantly attenuated adriamycin-mediated apoptosis via cytochrome c release and caspase-3, 6 and 9 activation, in H9C2 cardiac muscle cells.

Several oxidative stress-inducible genes become activated during adaptation. Other studies reported an induction of the expression of Bcl-2 after preconditioning (Andoh *et al.*, 2000). Bcl-2 may be regarded as an important cellular component, which not only guards against apoptotic cell death but also impinges on multiple cellular events.

It has been reported that prevention of apoptosis with IGF-1, is associated with an increased abundance of Bcl_2 and a decrease of Bax (Wang *et al.*, 1998). As the anti-apoptotic functions of Bcl-2 can be antagonized by other pro-apoptotic proteins, such as Bax, the IGF-1-induced decrease of Bax and the stimulation of Bcl-2 expression, may contribute to the anti-apoptotic actions of IGF-1. Consistently with these reports, several lines of evidence suggest that mitochondrial membrane potential may be the effector of both cytochrome c release and Bcl_2/Bax induction (Maulik *et al.*, 1999;Andoh *et al.*, 2000). An observation of a loss of $\Delta\psi_m$, beginning 18 h after the onset of adriamycin treatment and continuing for at least 48 h (data not shown) was made. Although loss of $\Delta\psi_m$ is not necessary for cytochrome c release in some systems (Kluck *et al.*, 1997), it is often sufficient for cytochrome c release to occur (Yang *et al.*, 1997). Therefore, it is possible that loss of $\Delta\psi_m$ contributes to cytochrome c release in cardiac muscle cells. Loss of $\Delta\psi_m$ is indicative of the opening of large, nonselective channels in the inner membrane, known as the PT pores. It has been previously proposed that opening of the PT pores would allow the entry of solutes and water into the mitochondrial matrix, thereby causing swelling of the matrix (Martin *et al.*, 1999). Due to the greater surface area of the inner membrane, this swelling would cause rupture of the outer mitochondrial membrane, liberating proteins such as cytochrome c that reside in the intermembrane space. Additional experiments are needed to determine whether loss of $\Delta\psi_m$ causes the initial release of cytochrome c from cardiac muscle cells or merely amplifies the release, once initiated by other mechanisms. The results of this study show that IGF-1 prevents both cytochrome c release and loss of $\Delta\psi_m$. The data presented here strongly indicates that apoptosis occurs after adriamycin treatment and IGF-1 protects against the anti-cancer agent-induced apoptosis in cardiac muscle

cells.

Adriamycin stimulates reactive oxygen species release, resulting in irreversible cardiomyopathy. Most of the supportive evidence has been obtained from *in vitro* studies including reports that adriamycin increases lipid peroxydation and free radical production in heart tissue, and that free radical scavengers such as *N*-acetylcysteine, vitamin E, superoxide dismutase, and catalase decrease the severity of adriamycon-induced oxidative damage (Geetha *et al.*, 1989; Kang *et al.*, 1996). In this study, adriamycin induced ROS release. However, IGF-1 had no regulatory effect on the release of ROS (Fig. 3). Although the role of free radical/oxidative stress in adriamycin-induced injury is supported by the recent demonstration of apoptotic cell death during exposure to the anthracycline agent, our data shows that ROS is not involved in the IGF-1-induced protective mechanism.

In this study, the role of the PI3-kinase pathway was investigated using specific inhibitors and dominant negative p85; PI3K regulatory subunit constructs. The present study demonstrated in a model of adriamycin-induced apoptosis that there is a signal transduction pathway responsible for the anti-apoptotic properties of IGF-I in cardiac muscle cells, the PI3K and AKT pathway. For the PI3K assay, anti-pTyr not anti-subunit of PI3K was used, since it has been suggested that most of the insulin-activated portion of PI3K can be precipitated by anti-pTyr (Okamoto *et al.*, 1993). This experiment reveals that in cardiac muscle cells, LY294002 sufficiently blocks PI3K-mediated AKT phosphorylation without affecting MEK1-dependent ERK1 and ERK2 phosphorylation, indicating an independent activation of these two signal transduction pathways. In addition, it was observed that PD98059 completely blocked ERK 1 and 2 phosphorylations without affecting either PI3K or AKT activation. In this study, the down-regulation of PI3K has a regulatory effect on IGF-1-induced PKC ζ activation, suggesting PI3K could be an upstream pathway of PKC ζ. However, the specific inhibitor of PKC, chelerythrine, did not have any effect on IGF-1-induced protection in this system. Although PKC can be a downstream kinase of PI3K, the kinase is not related to the IGF-1-protective effect in H9C2 cardiac muscle cells. It is suggested therefore, that IGF-I activates at least PI3K and subsequent AKT pathways leading to enhanced survival. The signal transduction pathway may represent a cellular strategy to amplify survival signals in cells. This study shows that IGF-I protects cardiac muscle cells from ADR-induced apoptosis and that the activation of PI3K contributes to the protective effect in H9C2 cardiac muscle cells.

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