Expression of Constitutively Active Phosphatidylinositol 3-Kinase Inhibits Activation of Caspase 3 and Apoptosis of Cardiac Muscle Cells

Received for publication, May 15, 2000, and in revised form, August 29, 2000
Published, JBC Papers in Press, September 27, 2000, DOI 10.1074/jbc.M004108200

Weihua Wu‡, Wen-Lieng Lee‡, Yvonne Y. Wu‡, Daniel Chen‡, Tsun-Jui Liu‡, Andy Jang§, Prem M. Sharma¶, and Ping H. Wang‡‡

From the ‡Department of Medicine and Biological Chemistry, Division of Endocrinology, Diabetes, and Metabolism, University of California, Irvine, California 92697, the §Department of Medicine, Division of Hematology and Oncology, University of California, Irvine, California 92697, and the ¶Department of Medicine, Division of Endocrinology, University of California, San Diego, California 92162

Apoptosis of cardiac muscle cells contributes to the development of cardiomyopathy. Recent studies showed that insulin-like growth factor I (IGF-I) inhibits apoptosis of cardiac muscle cells and improves myocardial function in experimental heart failure. This study was carried out to elucidate the role of phosphatidylinositol 3-kinase (PI 3-kinase) in the anti-apoptotic actions of IGF-I in cardiomyocytes and to explore whether expression of constitutively active PI 3-kinase can inhibit apoptosis in cardiomyocytes. Apoptosis of primary cardiomyocytes was induced by doxorubicin treatment and serum withdrawal. Transduction of cardiomyocytes with constitutively active PI 3-kinase specifically lead to serum phosphorylation of Akt, whereas phosphorylation of IGF-I receptor, IRS1/2 and p44/42 mitogen-activated protein kinase were not increased. In the cardiomyocytes transduced with constitutively active PI 3-kinase, activation of the pro-apoptotic caspase 3 was attenuated and fragmentation of DNA was reduced. Preincubating cells with PI 3-kinase inhibitor LY294002 was associated with loss of anti-apoptotic actions of IGF-I and PI 3-kinase. Neither IGF-I nor constitutively active PI 3-kinase lead to serum phosphorylation of Bad, suggesting that the anti-apoptotic effects of PI 3-kinase are not mediated through Bad phosphorylation in cardiac muscle cells. To determine whether activation of caspase 3 is sufficient to induce apoptosis in cardiomyocytes, an engineered TAT-caspase 3 protein was introduced to cardiomyocytes. Significant reduction of cell viability occurred in the cardiomyocytes transduced with active caspase 3, indicating that activation of caspase 3 is sufficient to cause cardiomyocyte death. These findings indicate the existence of an IGF-I receptor-PI 3-kinase-caspase 3 pathway in cardiomyocytes that plays an important role in the anti-apoptotic actions of IGF-I in heart. Moreover, these data suggest that modulation of PI 3-kinase activities may represent a potential therapeutic strategy to counteract the occurrence of apoptosis in cardiomyopathy.

Apoptosis of cardiac muscle cells have been observed during fetal development of cardiac tissue, in various models of myocardial injuries, and during the development of heart failure (1–9). Because myocytes rarely proliferate in adult cardiac muscles, loss of cardiac muscle cells leads to a permanent loss of cardiac functional units. Thus myocardial apoptosis may contribute to or aggravate the development of myocardial dysfunction in various cardiac diseases. The occurrence of cardiac apoptosis can be suppressed with insulin-like growth factor I (IGF-I) (10–15). IGF-I is a growth factor that activates multiple intracellular signaling pathways. How these pathways modulate apoptosis signaling in cardiomyocytes is not yet clear. Recent studies have shown that in various types of cells the anti-apoptotic effects of IGF-I disappeared when activation of PI 3-kinase was inhibited. Thus it appears that the anti-apoptotic effects of IGF-I require activation of PI 3-kinase. PI 3-kinase is composed of an 85-kDa (p85) regulatory subunit and a 110-kDa catalytic subunit (p110). Activation of PI 3-kinase leads to activation of downstream signaling molecules such as Akt/PKB. How PI 3-kinase/Akt signaling modulates apoptosis signaling is not completely understood. Datta et al. (16) have observed that activation of Akt lead to serine phosphorylation of Bad, a pro-apoptotic protein of Bcl-2 family, and in turn suppressed apoptosis of transformed cells. However, whether activation of PI 3-kinase can lead to phosphorylation of Bad in cardiac muscle cells is not known. Our laboratory (13, 14) has observed that doxorubicin increased the activity of caspase 3 and induced apoptosis of cardiac muscle cells, whereas IGF-I suppressed activation of caspase 3 and apoptosis of cardiomyocytes. Caspase 3 is a key component of apoptotic signaling that mediates both mitochondria-dependent and -independent apoptotic signaling. Although the anti-apoptotic effects of IGF-I may be in part mediated by PI 3-kinase, it is unclear whether activation of PI 3-kinase pathway alone suppresses activation of caspase 3 in cardiac muscle or in other tissues. The aim of this study was to investigate whether activation of PI 3-kinase can lead to suppression of caspase 3 activation and in turn inhibit the occurrence of apoptosis in cardiac muscle cells. The results show that transduction of a constitutively active PI 3-kinase into cardiomyocytes suppressed activation of caspase 3 and attenuated apoptosis of cardiomyocytes. Moreover, trans-

* This work was supported by grants from the NIHLB, National Institutes of Health, American Heart Association, and American Diabetes Association (to P. H. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medicine, Med Sci I, C240, University of California, Irvine, CA 92697. Tel.: 949-824-9811; Fax: 949-824-2200; E-mail: phwang@uci.edu.

§ To whom correspondence should be addressed: Dept. of Medicine, Division of Endocrinology, Diabetes, and Metabolism, University of California, Irvine, CA 92697, the §Department of Medicine, Division of Hematology and Oncology, University of California, Irvine, California 92697, and the ¶Department of Medicine, Division of Endocrinology, University of California, San Diego, California 92162

The abbreviations used are: IGF-I, insulin-like growth factor I; PI 3-kinase, phosphatidylinositol 3-kinase; Akt/PKB, protein kinase B; MEK, MAP kinase kinase; PBS, phosphate-buffered saline; MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IRS, insulin receptor substrate.
duction of active caspase 3 into cardiomyocytes was associated with decreased cardiomyocyte viability. Since activation of PI 3-kinase was not associated with serine phosphorylation of Bad, we concluded that in cardiac muscle cells, PI 3-kinase/Akt signaling inhibited caspase cascades through a mechanism independent of Bad phosphorylation. These results thus provide a novel link between PI 3-kinase pathway and suppression of caspase 3 activation in a tissue where apoptosis plays a significant pathophysiological role.

**EXPERIMENTAL PROCEDURES**

**Materials—** Fetal bovine serum (FBS) and cell culture medium were purchased from Irvine Scientific (Santa Ana, CA). Immobilin-P membranes were from Millipore Co. (Bedford, MA). Anti-Akt, anti-phospho-Akt (Ser^{473}), anti-phospho-p44/42 MAP kinase (Thr^{389/422}Tyr^{397/426}), anti-Bad, anti-phospho-Bad (Ser^{361}), antibodies, phospho-MBP-Bad and MBP-Bad fusion proteins were from New England Biolabs (Beverly, MA). Anti-phospho-Tyr (pY99) antibody was from Santa Cruz Biologals.

**Cell Monolayers—** Cardiomyocytes were maintained in a serum free DMEM supplemented with 1% FBS and 20 ng/ml human IGF-I. To test whether IGF-I suppression of cardiomyocyte apoptosis is inhibited by LY294002 treatment, cardiomyocytes were plated in 96- or 48-well plates. Then the cells under-

**DNA Fragmentation—** Apoptosis is accompanied by fragmentation of DNA. To determine the occurrence of DNA fragmentation, total DNA was extracted from control and treated cardiomyocytes using Gentra DNA Isolation Kit (Minneapolis, MN) according to the manufacturer’s instructions. In brief, both attached cells and detached cells floating in the medium were collected by scraping and centrifuging (2000 × g for 10 min). After incubating with 300 µl of Lysis Buffer at 65 °C for 30 min, the lysates were digested by 1.5 µl of RNase A (4 mg/ml) at 37 °C for 1 h. The proteins were excluded by adding 100 µl of Protein Precipitation Solution and cleared by microfuge. Then the DNA in supernatant was precipitated by adding 300 µl of isopropanol ethanol at −20 °C for 1 h. The DNA pellets were dissolved in TE buffer (pH 7.4) and equal amounts of DNAs were resolved with 1.5% agarose gel and stained with 0.5 µg/ml ethidium bromide.

**Immunoblotting and Immunoprecipitation—** Cell monolayers were incubated with defined medium in the presence or absence of doxorubicin, IGF-I, and LY294002. The cells were harvested and solubilized with a lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mM EDTA, pH 8.0, 3 µg/ml aprotinin, 3 µg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoro-ride, 20 mM NaF, 10 mM NaPP, and 2 mM Na$_2$VO$_4$). Equal amounts of proteins were separated by SDS-PAGE. The resolved proteins were then electrophoretically transferred to a polyvinylidene difluoride filter. The filters were then incubated with blocking solution (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 5% nonfat milk or 3% bovine serum albumin, and 0.1% Tween 20) at room temperature for 1 h and then with primary antibodies for 1.5 h, washed three times (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) incubated with 1:10,000 diluted 400 µg/ml peroxi-

**Caspase 3 Activity Assay—** The activities of caspase 3 were determined from the CPP32 assay kit (CLONTECH, Palo Alto, CA) as described previously (13, 14). In brief, the cells were solubilized, and 100 µg of lysate proteins were reacted with 50 µl DEVD-AFC at 37 °C for 45 min. The samples were analyzed with a Cytofluor 2300 Fluorescence Measurement System (Millipore) at excitation of 425 nm and emission of 530 nm.

**Measurements of Annexin V Staining with Epifluorescence Microscope—** Exposure of phosphatidylserine on outer plasma membrane is a key sign of apoptosis, thus staining of phosphatidylserine with annexin V can be used as a marker to define the occurrence of apoptosis (19). Annexin V staining was performed with Vybrant Apoptosis Assay Kit (Molecular Probe, Eugene, OR) according to the manufacturer’s instructions. For this purpose, cardiomyocytes were grown in 60-mm Petri dishes with PBS, the cells were washed twice with annexin-binding buffer containing 0.5% fluorescein isothiocyanate-annexin V for 30 min at room temperature. After briefly rinsed with PBS, the cardiomyocytes were analyzed under an Zeiss Axiphot epifluorescence microscope at the wavelengths of 480 nm (excitation) and 530 nm (emission). The images were recorded with a Senays digital camera and analyzed with PathVision imaging software. For each dish, 10 random microscopic fields were recorded and the number of annexin V-positive cells were counted. After the results of annexin V staining were recorded, the cardiomyocytes were immediately fixed with methanol/aceton (50:50) and stained with propidium iodide to count the number of cells on each dish. Since propidium iodide stains all cells after fixation, the average number of cardiomyocytes from 10 random microscope fields was then used as the denominator to calculate the percent of annexin V-positive cardiomyocytes in each dish.

**Cell Viability—** Cell viability was performed by staining the cells with calcine AM (Molecular Probe, Eugene, OR) as described previously (20). Calcine AM is hydrolyzed to calcine and retains in live cells, and thus serves as an indicator for cell viability. To measure cell viability, cardiomyocytes were plated in 96- or 48-well plates. Then the cells under-

**TAT Fusion Proteins—** The construction of TAT fusion protein expression vectors that were used in this study has been described previously (21–23). In brief, TAT fusion proteins were prepared by soni-

**RESULTS**

**IGF-I Suppression of Cardiomyocytes Apoptosis Is Inhibited by LY294002—** To test whether IGF-I suppression of cardiomyocytes apoptosis requires activation of PI 3-kinase, we used an in vitro model of cardiomyocytes apoptosis induced with doxorubicin (13, 14). Primary neonatal cardiomyocytes was incubated with 0.4 µg doxorubicin in serum-free medium and IGF-I was added prior to incubation with doxorubicin when indi-
cated. After 6 h of doxorubicin incubation DNA was extracted from cardiomyocytes and resolved with 1.5% agarose gel to determine the occurrence of DNA fragmentation. Fig. 1 shows that IGF-I attenuated doxorubicin-induced fragmentation of DNA. When PI 3-kinase inhibitor LY294002 (20 μM) was added to the cultured medium before adding IGF-I, IGF-I could no longer retard the occurrence of DNA fragmentation. Moreover, LY294002 aggravated doxorubicin-induced fragmentation of DNA. These results strongly suggest that the survival effects of IGF-I in cardiomyocytes require activation of PI 3-kinase pathway, and raised the possibility that activation of PI 3-kinase plays an important role in maintaining the viability of cardiomyocytes.

Expression of Active PI 3-Kinase in Cardiomyocytes Activated Akt—To test the hypothesis that PI 3-kinase activation leads to inhibition of cardiomyocytes apoptosis, a constitutively active PI 3-kinase was transferred to primary cardiomyocytes via adenoviral vector (Ad5-p110*). With adenoviral vector, PI 3-kinase was transferred to primary cardiomyocytes via infection with recombinant adenovirus, the cells were serum-deprived overnight. The control cells and the cells transduced with Ad5-lacZ were stimulated with IGF-I (10^{-8} M) for 5 min when indicated. Cell lysates (50 μg of protein) were immunoblotted with anti-phospho-Akt, anti-Akt, anti-phosphotyrosine (phospho-Tyr), or anti-p44/42 MAP kinase antibodies. Autophosphorylation of IGF-I receptor (IGF-IR) β-subunit and phosphorylation of IRS1/2 were detected by anti-phospho-Tyr antibodies.

Activation of PI 3-Kinase in Cardiomyocytes Inhibited Activation of Caspase 3 and Did Not Phosphorylate Bad—To further establish the specificity of Akt activation in the cardiomyocytes infected with Ad5-p110*, these cells were incubated with LY294002 for 1 h. The data showed that phosphorylation of Akt in Ad5-p110*-transduced cells was suppressed to a level nearly undetectable (Fig. 3A). To define the effects of PI 3-kinase activation on caspase 3, we have investigated activation of caspase 3 in the cells transduced with Ad5-p110* (Fig. 3, B and C). Caspase 3 is activated by cleavage of proenzyme into active subunits, therefore we first investigated processing of caspase 3 by assessing the presence of active 20-kDa subunits with Western blot (Fig. 3B). The active fragments were nearly undetectable in the normal control cells and in the cells transduced with Ad5-lacZ, whereas doxorubicin treatment significantly increased the abundance of the active caspase 3 fragment. In contrast, the induction of active caspase 3 fragment after doxorubicin treatment was less in the cells transduced with Ad5-p110*. When Ad5-p110*-infected cardiomyocytes were preincubated with LY294002 to block activation of PI 3-kinase, abundant active caspase 3 fragments were detected upon doxorubicin treatment. Similar changes of caspase 3 enzyme activities were also observed. In the cells transduced with Ad5-lacZ, doxorubicin treatment increased the activities of caspase 3 and IGF-I treatment suppressed it as expected (Fig. 3C). In the cells transduced with Ad5-p110*, doxorubicin-activated caspase 3 activities was attenuated to a level similar to IGF-I treatment. Conversely, addition of LY294002 to Ad5-p110*-transduced cells lead to a loss of inhibition on caspase 3 activities. These data indicate that PI 3-kinase signaling inhibits activation of caspase proenzyme and hence suppresses the activities of caspase 3 in doxorubicin-treated cardiomyocytes.

Phosphorylation of Bad at serine 136 is one of the potential mechanisms through which Akt inhibits caspases activation (16). To study whether IGF-I and PI 3-kinase stimulation can lead to phosphorylation of Bad in cardiomyocytes, cardiomyocytes were either stimulated with IGF-I or transduced with Ad5-p110* and the cell lysates were immunoprecipitated with anti-Bad antibody and then immunoblotted with anti-pBad (Ser^{136}) antibody. The results are shown in Fig. 4. IGF-I stimulation in control cells or Ad5-lacZ-cells was not associated with serine phosphorylation of Bad. Moreover, overexpression of Ad5-p110* failed to induce Bad phosphorylation. These findings suggest that inhibition of caspase 3 by IGF-I and by overexpression of PI 3-kinase in cardiomyocytes does not involve serine 136 phosphorylation of Bad.

Activation of PI 3-Kinase Signaling Attenuated Cell Death—The next series of experiments are designed to determine whether activation of PI 3-kinase signaling can inhibit cell death. For this purpose, we first analyzed the occurrence of DNA fragmentation, a hallmark of apoptosis. To detect DNA fragmentation, DNA was extracted and resolved on 1.5% agarose gel electrophoresis. Control, DMEM containing 10% FBS. Dose, 0.4 μM doxorubicin. LY, 20 μM LY294002.
Expression of Constitutively Active Phosphatidylinositol 3-Kinase

FIG. 3. A, LY294002 blocked phosphorylation of Akt in cardiomyocytes transduced with Ad5-p110*. The cells were transduced with Ad5-p110* for 36 h and serum-fasted overnight prior to treatment with LY294002. Equal amounts of cell lysates were resolved with SDS-PAGE and immunoblotted with anti-phospho-Akt antibodies. B, transduction of Ad5-p110* suppressed induction of active caspase 3 fragment. The cells were transduced with Ad5-lacZ or Ad5-p110* for 36 h, and treated with doxorubicin in serum-free medium for 6 h to induce apoptosis. Cell lysates (100 μg of protein) were resolved with SDS-PAGE and blotted with anti-active caspase 3 antibodies. C, activation of PI 3-kinase inhibited the enzymatic activities of caspase 3. The cells was transduced with Ad5-lacZ or Ad5-p110* (5 plaque forming units/cell) for 36 h and treated with IGF-I, LY294002, or doxorubicin as indicated. Cell lysates (100 μg of protein) were assayed for caspase 3 activities as described under “Experimental Procedures.” The data represent mean ± S.E. from four independent experiments. FBS, DMEM containing 10% fetal bovine serum. S, serum-free DMEM; I, 10−8 M IGF-I; D, 0.4 μM doxorubicin. LY, LY294002.

FIG. 4. IGF-I stimulation and PI 3-kinase activation did not induce serine 136 phosphorylation of Bad in cardiomyocytes. The cells were transduced with Ad5-lacZ or Ad5-p110* (5 plaque forming units/cell) for 36 h and serum-fasted overnight. Ad5-lacZ-cells were stimulated with vehicles or 102 M IGF-I for 15 min. Cell lysates were first immunoprecipitated with anti-Bad antibodies (1:500) and then immunoblotted with anti-phospho-Bad (Ser136) antibodies. Bad from cardiomyocytes migrated around 26–27 kDa. Recombinant MBP-Bad fusion proteins (nonphosphorylated and phosphorylated) that migrated around 67 kDa served as positive control.

Fig. 5. Activation of PI 3-kinase suppressed DNA fragmentation in cardiomyocytes. The cells were transduced with Ad5-lacZ or Ad5-p110*, serum-deprived overnight, and incubated with doxorubicin, IGF-I, and/or LY294002 as indicated. Equal amounts of DNA were resolved with 1.5% agarose gel. FBS, DMEM containing 10% fetal bovine serum; S, serum-free DMEM; I, 10−8 M IGF-I; D, 0.4 μM doxorubicin; LY, 20 μM LY294002.

rose gel. As shown in Fig. 5, doxorubicin treatment lead to fragmentation of DNA in the control cells with Ad5-lacZ, but DNA fragmentation was suppressed in the cells stimulated with IGF-I and in the cells transduced with Ad5-p110*. Addition of PI 3-kinase inhibitor LY294002 reversed the inhibitory effects of PI 3-kinase on DNA fragmentation.

In addition to DNA fragmentation, the effects of Ad5-p110* on cardiomyocyte apoptosis were confirmed with annexin V staining. Annexin V has high affinity for phosphatidylserine. In apoptotic cells the membrane phospholipid phosphatidylserine is translocated from the inner to the outer plasma membrane, thus annexin V can bind to the exposed phosphatidylserine in apoptotic cells (19). In the cells transduced with Ad5-lacZ, doxorubicin treatment increased the number of annexin V-stained cardiomyocytes and IGF-I reduced the number of annexin V-positive cells as we have anticipated (Fig. 6). Moreover, the effects of IGF-I was reversed with preincubation of LY294002. These data confirmed the results of the DNA fragmentation study (Fig. 1) that IGF-I suppression of cardiomyocyte apoptosis requires activation of PI 3-kinase pathway. In the cells transduced with Ad5-p110* and treated with doxorubicin, the number of annexin V-positive cells were decreased and preincubation with LY294002 blocked the protective effects of Ad5-p110*. To further define the effects of active PI 3-kinase on cardiomyocyte survival, the cells were transduced with either Ad5-lacZ or Ad5-p110* and then the cells were incubated with doxorubicin to induce apoptosis (Fig. 7). As expected, in the control cells doxorubicin treatment was associated with a significant reduction in the number of viable cells, and IGF-I reduced the cytotoxic effects of doxorubicin. In the cells transduced with Ad5-p110*, the cytotoxic effects of doxorubicin was attenuated to a level similar to the IGF-I-treated cells (p = 0.43). These experiments indicate that activation of PI 3-kinase increased cardiac muscle resistance to the cytotoxicity of doxorubicin.

Activation of Caspase 3 Decreased Cardiomyocytes Survival—The above studies defined the effects of PI 3-kinase signaling on caspase 3 and apoptosis of cardiomyocytes. However, whether activation of caspase 3 alone can lead to cardiomyocyte death is not known. To this end, a TAT-caspase 3 fusion protein (TAT-wild type Casp 3) was transduced into cardiomyocytes to induce activation of caspase 3. TAT-wild type Casp 3, substi-
The cells were transduced with Ad5-lacZ or Ad5-p110* serum-deprived overnight and incubated with doxorubicin, IGF-I, and/or LY294002 as indicated. Apoptosis of cardiomyocytes was determined by annexin V staining. This bar graph depicts the proportions of cardiomyocytes stained positive for annexin V in each experimental group. *, p < 0.01 versus control (10% FBS). #, p < 0.01 versus doxorubicin treatment (Ad5-lacZ, S+D). S, serum-free DMEM; I, 10−4 M IGF-I; D, 0.4 μM doxorubicin. LY, 20 μM LY294002.

**Fig. 6. Annexin V staining confirmed the anti-apoptotic effects of IGF-I and p110* in cardiomyocytes.** The cells were transduced with Ad5-lacZ or Ad5-p110*, serum-deprived overnight, and incubated with doxorubicin, IGF-I, and/or LY294002 as indicated. Apoptosis of cardiomyocytes was determined by annexin V staining. This bar graph depicts the proportions of cardiomyocytes stained positive for annexin V in each experimental group. *, p < 0.01 versus control (10% FBS). #, p < 0.01 versus doxorubicin treatment (Ad5-lacZ, S+D). S, serum-free DMEM; I, 10−4 M IGF-I; D, 0.4 μM doxorubicin. LY, 20 μM LY294002.

**Fig. 7. Activation of PI 3-kinase enhanced cardiomyocyte survival.** The cells were transduced with Ad5-lacZ (control cells) or Ad5-p110* for 24 h. When indicated the cells were treated with IGF-I and/or 50 nM TAT-HIV-Pro. Various concentrations of TAT-wild type Casp 3/TAT-mutant Casp 3 and/or 50 nM TAT-HIV-Pro were added to DMEM containing 1% FBS for 16 h in cardiomyocytes. TAT-HIV-protease was co-transduced to activate the recombinant wild type caspase 3 that replaced the natural cysteine cleavage sites with HIV-protease cleavage sites. The cells were stained with 1 μM calcein AM and the intensity of fluorescence was used to quantitate the amount of live cells. The data represent mean ± S.E. from six independent experiments. Control, DMEM containing 1% FBS. Casp3, TAT-wild type Casp 3; mCasp3, TAT-mutant Casp 3; HIV-Pro, TAT-HIV-protease.

**Fig. 8. Activation of caspase 3 leads to cardiomyocytes death.** Various concentrations of TAT-wild type Casp 3/TAT-mutant Casp 3 and/or 50 nM TAT-HIV-Pro were added to DMEM containing 1% FBS for 16 h in cardiomyocytes. TAT-HIV-protease was co-transduced to activate the recombinant wild type caspase 3 that replaced the natural cysteine cleavage sites with HIV-protease cleavage sites. The cells were stained with 1 μM calcein AM and the intensity of fluorescence was used to quantitate the amount of live cells. The data represent mean ± S.E. from six independent experiments. Control, DMEM containing 1% FBS. Casp3, TAT-wild type Casp 3; mCasp3, TAT-mutant Casp 3; HIV-Pro, TAT-HIV-protease.

**DISCUSSION**

The findings of this study delineated the relationship of PI 3-kinase, caspase 3 activation, and apoptosis in cardiac muscle cells. Apoptosis of cardiac muscle cells was recently recognized as a new paradigm that occurs in human and experimental models of heart failure. This emerging concept of cardiomyocyte apoptosis has important implications on cardiac function because loss of cardiomyocytes could be a fundamental part of the myocardial process that initiates or aggravates heart failure. For example, administration of doxorubicin is associated with heart failure and reduced number of cardiac muscle cells per functional units (24), and in vitro incubation of cardiomyocytes with doxorubicin lead to activation of caspase 3 and occurrence of apoptosis (13, 14). IGF-I can attenuate apoptosis of cardiac muscle cells induced by doxorubicin and apoptosis of cardiac muscle cells in various experimental models of cardiomyopathy (10, 11, 13–15, 25, 26). Although considerable progress has been made in understanding the signaling pathways of IGF-I, relatively little is known about how IGF-I signaling modulates apoptotic signaling. The results of this study indicate that activation of caspase 3 was sufficient to cause cardiomyocyte death and that activation of PI 3-kinase signaling can suppress activation of caspase 3 and hence inhibit the occurrence of apoptosis.

There is ample evidence suggesting that activation of PI 3-kinase may lead to suppression of caspase 3 and DNA fragmentation in a variety of cells. Most of the evidence derived from studies using pharmacological inhibitors of PI 3-kinase (LY294002 and wortmannin), inhibition of PI 3-kinase with these compounds lead to cell apoptosis or loss of anti-apoptotic effects of growth factors (25–30). Several other studies used dominant negative constructs of PI 3-kinase to inhibit PI 3-kinase signaling and further confirmed that PI 3-kinase is essential in maintaining cell survival (10, 28). However, from these studies it is not clear whether activation of PI 3-kinase alone is sufficient to protect the cells from apoptosis induction and whether PI 3-kinase activation suppresses caspase activation. A downstream target of PI 3-kinase that may mediate the anti-apoptotic actions of PI 3-kinase is Akt. Akt pathways may modulate sequential steps of apoptosis signaling. Activation of Akt may lead to phosphorylation of Bad (16), induction of Bcl-2 family of proteins (31), inhibition of cytochrome c release from...
mitochondria (32), and phosphorylation and inactivation of caspase 9 (33). Some of these observations appear to be dependent upon the experimental system used. For example, Akt phosphorylation of caspase 9 was reported in human neoplastic cells, but murine caspase 9 lacks the phosphorylation motif and cannot be phosphorylated by Akt (33). Moreover, we were unable to find phosphorylation of Bad by IGF-I or by activation of PI 3-kinase in cardiomyocytes. Thus it appears survival signaling may employ different mechanisms to antagonize apoptosis in different cells. These signaling steps can be variably dependent on the type of tissue, the type of species, the state of cell transformation or differentiation, and the nature of apoptosis inducers. The lack of Bad phosphorylation in IGF-1-stimulated and in Ad5-p110* -tranduced cardiomyocytes suggests that PI 3-kinase/Akt signaling may utilize survival pathways independent of Bad.

When cardiomyocytes were incubated with the PI 3-kinase inhibitor LY294002, the protective effects of IGF-I and Ad5-p110* were reversed and caspase 3 activities increased to a level higher than the caspase 3 activities induced by doxorubicin alone. LY294002 is a very effective inhibitor of PI 3-kinase signaling as phosphorylation of Akt in LY294002-treated cells was suppressed to a level lower than the basal phosphorylation of Akt in control cardiomyocytes. Adding LY294002 alone to culture medium for a prolonged period of time lead to induction of moderate cardiomyocyte apoptosis (data not shown). It is possible that LY294002 completely inhibited the basal activities of PI 3-kinase and thus lead to a greater induction of apoptosis in the cardiomyocytes treated with both doxorubicin and LY294002. Together with our finding that activation of PI 3-kinase inhibited induction of apoptosis in cardiomyocytes, these observations indicate PI 3-kinase is essential for cardiomyocyte viability.

Caspases are stored as proenzymes and can be cleaved into active fragments upon apoptosis induction. The process of cleavage is mediated by proteolysis and generates two subunits (10 and 20 kDa), these two subunits form heterodimers that eventually produce the enzymatic activities of caspases (34). Caspase 3 plays a pivotal role in execution of apoptosis; ES cells deficient in caspase 3 were resistant to apoptosis induction (35). In human cardiomyopathy, apoptosis of cardiac muscle cells is associated with release of cytochrome c and activation of caspase 3 (36). Activation of myocardial caspase 3 was also observed in experimental models of post-transplant cardiac rejection (37), which are accompanied by inflammatory reactions and myocardial apoptosis. These studies suggest that caspase 3 represents a potential therapeutic target for suppression of myocardial apoptosis.

Caspase 3 activation may lead to propagation of apoptosis signaling and structural disorganization of cells. Intracellular targets of caspases cascades include ICAD, gelsolin, PK2, PKC, lamin, NuMa, actin, catenin, FAK, PARP, and several transcription factors (38). The exact roles of these molecules in apoptosis are not entirely clear at present. However, studies thus far suggest that these molecules represent different components of apoptosis machinery that ultimately terminate survival signaling, dissolve cell integrity, promote cell packaging, and facilitate phagocytosis of cell remnants. Using peptide inhibitor of caspases, Bialik et al. (39) have shown that cardiomyocytes apoptosis induced by serum and glucose withdrawal can be attenuated. Our data show that activation of caspase 3 alone was sufficient to cause cell death in cardiac muscle, suggesting preservation of myocardial viability can be achieved with inhibition of caspase 3. Since PI 3-kinase has additional beneficial effects on cell growth and metabolism, gene transfer of active PI 3-kinase to cardiomyocytes may have potential therapeutic implications on the preservation of cardiac muscle in cardiac diseases.

Parrizas et al. (40) have reported that inhibition of PI 3-kinase or MEK signaling with chemical inhibitors lead to attenuation of the anti-apoptotic actions of IGF-I in cardiomyocytes. In IGF-I signaling pathways, p85 PI 3-kinase subunit interacts with phosphorylated IRS 1/IRS 2 and then activates p110 catalytic subunit. There are conflicting data on whether activation of PI 3-kinase lead to activation of Ras and MAP kinase signaling. Activation of PI 3-kinase resulted in activation of Raf-1/MEK pathways in some experimental systems, whereas other studies failed to demonstrate activation of MAP kinase signaling by PI 3-kinase (41). Activation of MEK/ERK signaling was not observed in the cardiomyocytes transduced with active PI 3-kinase. These findings support the notion that in cardiac muscle cells MEK/ERK signaling is not downstream from PI 3-kinase, and suggest that the anti-apoptotic effects of PI 3-kinase activation in cardiomyocytes does not involve activation of MEK/ERK pathway.

Acknowledgements—We thank Dr. Steven Dowdy for providing TAT fusion protein vectors and Dr. Robert J. Smith for providing anti-IGF-1 receptor antibodies. We are grateful to Jeffrey Hsu for excellent technical assistance in preparing primary cultures of cardiomyocytes.

REFERENCES

1. MacLellan, W. R. & Schneider, M. D. (1997) Circ. Res. 81, 137–144
2. Williams, R. S. (1999) J. Biol. Chem. 274, 2247–2255
3. Delpy, E., Hatem, S. N., Andrieu, N., de Vaumas, C., Henaff, M., Rucker-Burl, C., Jaffrezou, J.-P., Laurent, G., Levade, T. & Mercadier, J. J. (1999) Cardiovasc. Res. 43, 398–407
4. Saraste, A., Pulkit, K., Kallajoji, M., Heikkila, P., Laine, P., Mattila, S., Nieminen, M. S., Parvinen, M. & Voipio-Pulkki, L.-M. (1999) Eur. J. Clin. Invest. 29, 380–386
5. Von Harsdorf, R., Li, P. F. & Dietz, R. (1999) Circulation 99, 2934–2941
6. Yue, T.-L., Wang, C., Homanic, A. M., Kiddy, K., Kellar, P., DeWolff, W. E., J., Hart, T. K., Thomas, H. C., Storer, B., Gu, J.-L., Wang, X. & Feuerstein, G. Z. (1998) J. Mol. Cell. Cardiol. 30, 495–507
7. Zhu, W., You, Y., Akawa, R., Harada, K., Kudoh, S., Uozumi, H., Hayashi, D., Gu, Y., Yamanishi, T., Nagai, R., Yahashi, M., Nomoto, K. & Kouda, E. (1999) Proc. Natl. Acad. Sci. USA 96, 8031–8035
8. Fonseca, R., Anderson, M., Kettermann, A., Blakesley, V., Sapag-Hagar, M., Sugden, P., LeRoith, D. & Lavandero, S. (1997) J. Biol. Chem. 272, 19115–19124
9. Li, Q., Li, B., Wang, X., Lei, A., Jana, K. P., Liu, Y., Kajtuzeta, J., Baserve, R. & Averges, P. (1997) J. Clin. Invest. 100, 1991–1999
10. Wang, L., Ma, W., Markovich, R., Chen, J.-W. & Wang, P. H. (1998) Circ. Res. 83, 516–522
11. Wang, L., Ma, W., Markovich, R., Lee, W.-L. & Wang, P. H. (1998) Endocrinology 139, 1354–1360
12. Lee, W.-L., Chen, J.-W., Ting, C.-T., Ishiwata, T., Lin, S.-J., Korc, M. & Wang, P. H. (1999) Endocrinology 140, 4851–4859
13. Datta, S. R., Dead, H., Tak, T., Masters, F., Hu, Gotob, Y. & Greenberg, M. E. (1997) Cell 91, 231–241
14. Flink, I. L., Edwards, J. G., Bahl, J. J., Liew, C., Sole, M. & Morkin, E. (1992) J. Biol. Chem. 267, 9911–9916
15. Hu, Q., Klippel, A., Muslin, A. J., Fintz, W. J. & Williams, L. T. (1995) Science 268, 100–103
16. Vermes, I., Hansen, C., Steffens-Nakken, H. & Reutelingsperger, C. (1995) J. Immunol. Methods 184, 39–51
17. De Clerck, L. S., Britsch, C. H., Mertens, A. M., Moens, M. M. & Stevens, W. J. (1994) J. Immunol. Methods 172, 115–124
18. Nagahara, H., Vocero-Akkhali, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissay, N. A., Becker-Hapak, M., Ehshvsky, S. A. & Dowdy, S. (1998) Nature Med. 4, 1449–1452
19. Schwartz, S. R., Ho, A., Vocero-Akkhali, A. & Dowdy, S. F. (1999) Science 283, 1569–1572
20. Vocero-Akkhali, A. M., Heyden, N. V., Lissay, N. A., Ratner, L. & Dowdy, S. F. (1999) Nature Med. 5, 29–33
21. Olsen, R. D. & Mulsolin, P. S. (1999) FASEB J. 13, 3076–3086
22. Tse, T.-S. & Chen, M. K. (1999) J. Biol. Chem. 274, 4228–4235
23. Matsui, T., Li, L., Monte, F. D., Fukui, Y., Franke, T. F., Hajar, R. J. & Rosenweig, A. (1999) Circulation 100, 2373–2379
24. Parrizas, M., Saltiel, A. & LeRoith, D. (1997) J. Biol. Chem. 272, 154–161
25. Crowley, R. J. & Freeman, R. S. (1998) J. Neurosci. 18, 2693–2693
Expression of Constitutively Active Phosphatidylinositol 3-Kinase

29. Kulik, G. & Weber, M. (1998) Mol. Cell. Biol. 18, 6711–6718
30. Ryu, B. R., Ko, H. W., Jou, I., Nah, J. S. & Gwag, B. J. (1999) J. Neurobiol. 39, 536–546
31. Matsuzaki, H., Tamatani, M., Mitsuda, N., Namikawa, K., Kiyama, H., Miyake, S. & Tohyama, M. (1999) J. Neurochem. 73, 2037–2046
32. Kennedy, S. G., Kandel, E. S., Cross, T. K. & Hay, N. (1999) Mol. Cell. Biol. 19, 5800–5810
33. Fujita, E., Jinbo, A., Matuzaki, H., Konishi, H., Kikkawa, U. & Momoi, T. (1999) Biochem. Biophys. Res. Commun. 264, 550–555
34. Fernandes-Alnemri, T., Litwack, G. & Alnemri, E. S. (1994) J. Biol. Chem. 269, 30761–30764
35. Colussi, P. A. & Kumar, S. (1999) Immunol. Cell Biol. 77, 58–63
36. Narula, J., Pandey, P., Arbustini, E., Haider, N., Narula, N., Kolodgie, F. D., Dal Bello, B., Semigran, M. J., Bielsa-Masdeu, A., and Dec, G. W., Israels, S., Ballester, M., Virmani, R., Saxena, S. & Kharbanda, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8144–8149
37. Koglin, J., Granville, D. J., Glysing-Jensen, T., Mudgett, J. S., Carthy, C. M., McManus, B. M. & Russell, M. E. (1999) Circulation 99, 836–842
38. Rosen, A. & Casciola-Rosen, L. (1997) J. Cell. Biochem. 64, 50–54
39. Bialik, S., Cryns, V. L., Drincic, A., Miyata, S., Wollowick, A. L., Srinivasan, A. & Kitsis, R. N. (1999) Circ. Res. 85, 403–414
40. Parrizas, M., Saltiel, A. R. & LeRoith, D. (1997) J. Biol. Chem. 272, 154–161
41. Egawa, K., Sharma, P. M., Nakashima, N., Huang, Y., Huver, E., Boss, G. R. & Olefsky, J. M. (1999) J. Biol. Chem. 274, 14306–14314
