Glycoprotein 130 Regulates Cardiac Myocyte Survival in Doxorubicin-Induced Apoptosis Through Phosphatidylinositol 3-Kinase/Akt Phosphorylation and Bcl-xL/Caspase-3 Interaction

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Background—We recently reported that the activation of glycoprotein (gp) 130 by leukemia inhibitory factor (LIF) upregulates Bcl-xL and exerts antiapoptotic effects in cardiac myocytes. In addition, LIF induces activation of phosphatidylinositol (PI) 3-kinase and Akt, which are known to be required for cell survival. However, their regulatory roles in cell death remain unknown.

Methods and Results—We investigated the fate of these proteins and the cytoprotective effects of LIF on doxorubicin (DOX)-induced apoptosis in cultured neonatal rat cardiac myocytes. Myocyte apoptosis increased significantly in DOX-treated cells but was significantly reduced by LIF pretreatment. The kinase activities of PI 3-kinase and Akt declined below basal levels but were partially recovered with LIF. Moreover, DOX-induced caspase-3 activation and decrease in Bcl-xL abundance are completely inhibited by LIF and caspase inhibitor. LIF phosphorylates Bad through PI 3-kinase and reduces the heterodimerization of Bad with Bcl-xL. Adenovirus transfer of the constitutively active form of Akt to cardiac myocytes restored cardiac myocyte survival after DOX treatment. Conversely, the dominant-negative form of Akt inhibited LIF-induced increase in cell viability and suppression of caspase-9 activation.

Conclusions—Activation of gp130 inhibits DOX-induced cell death in cardiac myocytes, resulting in the restoration of PI 3-kinase/Akt activities and in the inactivation of caspase-3, leading to facilitation of the protective function of Bcl-xL.

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Key Words: doxorubicin • kinases • caspases • glycoproteins

Interleukin-6–related cytokines, such as leukemia inhibitory factor (LIF) and cardiotrophin-1, use glycoprotein (gp) 130 as a common signal transducer and promote myocardial cell survival.1–3 We recently described that the activation of phosphatidylinositol (PI) 3-kinase through gp130 induced Akt phosphorylation in cardiac myocytes.4 Survival factors implicated in the activation of PI 3-kinase protect cells against undergoing apoptosis.5,6 Akt, a downstream target of PI 3-kinase, was found to be critical for the prevention of cell death.5,7

It has been reported that caspase-3, a cysteine protease, is crucial for the execution phase of myocardial apoptosis during ischemia/reperfusion.8 Interestingly, an important advance came with the discovery of Bad, a protein that promotes cell death by binding to and blocking the activity of Bcl-xL.9 The released Bcl-xL promotes cell survival by blocking the caspase protease cascade.10 Because Bcl-xL is upregulated through gp130 in cardiac myocytes,2 an investigation of the function of PI 3-kinase and its downstream target, Akt, would provide an intriguing explanation of the role of gp130 in myocardial cell survival.

Doxorubicin (DOX) is used against a wide variety of tumors; however, the long-term use of the drug is limited by the development of cardiomyopathy and congestive heart failure.11 Although a number of pharmacological mechanisms of DOX have been proposed to explain the alteration of myocardial cellular structure and function, much less is known about the signaling mechanisms used by survival factors that inhibit the commitment to myocardial cell death. Recently, stress-related pathways for cardiac myocyte survival and apoptosis have been found to play a critical role in the progression of heart failure.12,13
In the present study, we demonstrate that the treatment of cardiac myocytes with DOX induces apoptosis and that the activation of gp130 conveys survival signals through PI 3-kinase and its downstream target, Akt. Importantly, LIF suppressed DOX-induced apoptosis by blocking the activation of caspase-3 and preventing the decrease in levels of Bcl-xL.

**Methods**

**Reagents**
Recombinant mouse LIF, antibodies to PI 3-kinase, Akt, and antiphosphotyrosine (4G10), and Crossstain (substrate peptide for Akt) were purchased from Upstate Biotechnology Inc. DOX was kindly provided by Kyowa Hakko Kogyo Co Ltd (Tokyo, Japan). Antibody to caspase-3 was from Santa Cruz Biotechnology Inc. Antibodies to Bcl-xL, Bcl-2, and Bad were from Transduction Laboratories. Phospho-Akt and phospho-Bad antibodies and PD98059, a specific MEK-1 inhibitor, were purchased from New England Biolabs, Inc. All other chemicals were reagents of molecular biology grade and were obtained from standard commercial sources.

**Cell Culture and Viability Assay**
Primary cultures of neonatal rat cardiac myocytes were prepared from the ventricles of 1-day-old Sprague-Dawley rats (Nippon Dobutsu, Japan) as previously described.14 Cultured cardiac myocytes were serum-starved for 12 hours before the experiments. For the cell viability assay, cardiac myocytes were grown in 96-well plates and treated with 0.5 μmol/L DOX or were untreated in the absence or presence of 10^4 U/mL LIF. The cells were treated with 0.5 mg/mL MTT for 5 hours at 37°C and lysed in 100 μL dimethylformamide. Absorbance was determined at 570 nm in a microtiter plate reader.

**DNA Fragmentation Assay**
The detection of DNA ladder was performed by the method of Saeki et al15 with modification. In brief, 1×10^6 cells were lysed with 350 μL of lysis buffer containing 0.6% SDS and 0.1% EDTA (pH 8.0). After centrifugation, the supernatant was further incubated with 1 mg/mL heat-inactivated RNase A at 45°C for 90 minutes and with 200 μg/mL proteinase K for another 60 minutes. After ethanol precipitation, 2 μg of DNA sample was separated by electrophoresis on 1.8% agarose gel.

**Assays of Apoptosis**
In situ labeling of fragmented DNA was performed as previously described with MEBSTAIN Apoptosis Kit Direct (MBL).16 Cardiac myocytes were grown on slides and fixed with 4% paraformaldehyde in phosphate buffer and permeabilized with 0.2% Triton X-100 and 0.2% BSA. Cardiac myocytes were first incubated with α-actinin antibody (Sigma Chemical Co) at 37°C for 30 minutes and subsequently with anti-mouse IgG TRITC-labeled antibody (Sigma Chemical Co) for 1 hour at room temperature. Thereafter, 50 μL of lysis buffer containing 0.6% SDS and 0.1% EDTA (pH 8.0). After centrifugation, the supernatants were immunoprecipitated with 1 μg/mL of specific antiserum against Bcl-xL or Bad for 2 hours before addition of 30 μL protein A-Sepharose for 1 hour. Immunoprecipitates were washed, separated by 12.5% SDS-polyacrylamide gels, and transferred onto Immobilon-P membrane (Millipore Co). To detect the cleavage of Bcl-xL, the protein was prepared as described for measurement of caspase-3 activity. Immuno blot was incubated with primary and secondary antibodies for 1 hour and finally developed with an enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions.

**Phosphorylation of Akt and Bad**
For determination of phosphorylated forms of Akt and Bad, the cells were homogenized in 500 μL lysis buffer (mmol/L: Tris-HCl [pH 7.4] 20, NaCl 150, EDTA 1, PMSF 1, sodium orthovanadate 1, plus 1% Triton X-100, 1% Nonidet P-40, 10% glycerol, and 10 μg/mL aprotinin) and homogenized. After the centrifugation, the supernatants were immunoprecipitated with 1 μg/mL of specific antisera against Bcl-xL or Bad for 2 hours before addition of 30 μL protein A-Sepharose for 1 hour. Immunoprecipitates were washed, separated by 12.5% SDS-polyacrylamide gels, and transferred onto Immobilon-P membrane (Millipore Co). To detect the cleavage of Bcl-xL, the protein was prepared as described for measurement of caspase-3 activity. Immuno blots were incubated with primary and secondary antibodies for 1 hour and finally developed with an enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions.

**Adenovirus Vector Construction and Infection**
The recombinant replication-defective adenovirus expressing a constitutively active form of Akt (caAkt) and a dominant-negative form of Akt (dnAkt) were prepared as described previously.17Thirty-six hours after plating, cardiac myocytes were transfected with adenovirus vectors in M-199 with 10% FCS at a multiplicity of infection (MOI) of 100. 48 hours after infection, cardiac myocytes were prepared as described for measurement of caspase-3 activity. Immuno blots were incubated with primary and secondary antibodies for 1 hour and finally developed with an enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions.

**Statistics**
All data are expressed as mean±SEM. Statistical comparisons were performed with Student’s t test or ANOVA with Scheffe’s test when appropriate. Significance was accepted at P<0.05.

**Results**
LIF Suppressed DOX-Induced Cardiac Myocyte Apoptosis
To investigate whether LIF may suppress DOX-induced apoptosis in cardiac myocytes, a TUNEL assay was performed. Figure 1A shows photomicrographs of a typical TUNEL assay; the apoptotic nuclei were stained green. Compared with the control cells [LIF(−) DOX(−)], a significant increase in apoptotic cells was identified after 16 hours of 0.5 μmol/L DOX treatment [LIF(−) DOX(+)]. Quantification study revealed that 27.5±2.5% of the cells were TUNEL-positive after DOX versus 4.5±2.4% in the control
cells (Figure 1B, \( P < 0.05 \)). Treatment with \( 10^3 \) U/mL LIF 3 hours before DOX significantly reduced apoptotic cells \( [8.3 \pm 2.6\%, \; P < 0.05 \text{ versus } \text{LIF}(–) \text{ DOX}(+)\]).

Furthermore, treatment with DOX for 16 hours exhibited the typical ladder pattern of apoptosis, and LIF pretreatment suppressed the DNA fragmentation induced by DOX (Figure 1C). Consistent with the cytoprotective effects of LIF on TUNEL assay and the DNA ladder, LIF promoted the cell viability in DOX-treated cells, with an increase in MTT activity from 62% to 93% at 48 hours \( [P < 0.05 \text{ versus } \text{LIF}(–) \text{ DOX}(+)\]) (Figure 1D). Pretreatment with 20 \( \mu \text{mol/L} \) PD98059 further decreased MTT activity in DOX-treated cells, but the LIF-mediated increase in cell viability was still observed to the same extent.

**LIF Restores PI 3-Kinase/Akt Activities in DOX-Treated Cardiac Myocytes**

DOX treatment produced a decline in both kinase activities to below the basal levels, but these were restored to basal levels in the cells pretreated with LIF (Figure 2A and 2B). Extracellular signal-regulated kinase activation was not significantly affected by DOX treatment (Figure 2C). To determine whether the restoration of Akt phosphorylation by LIF is involved in the signaling of PI 3-kinase, the effect of its specific inhibitor, wortmannin, on Akt activation was examined. LIF-induced restoration of Akt kinase activity was completely inhibited by wortmannin but not by PD98059 (Figure 2D). Subsequent Western blot analysis with anti-Akt antibody was performed to verify the same amounts of Akt in all samples (Figure 2B and 2D, middle). These results correlated well with the data obtained with Akt kinase assays (Figure 2B and 2D, bottom).

**LIF Inhibits DOX-Induced Caspase-3 Activation in Cardiac Myocytes**

As shown in Figure 3A, cleavage of caspase-3 was observed in DOX-treated cells; however, preincubation of LIF completely eliminated the caspase-3 activation in DOX-treated cells. To further analyze whether the cytoprotective effect of LIF is mediated by decreased caspase-3 activity, we quantified the enzyme activity in DOX-treated cardiac myocytes. As shown in Figure 3B, DOX treatment induced a 4.5-fold (DOX 0.5 \( \mu \text{mol/L} \)) or 6.5-fold (DOX 1 \( \mu \text{mol/L} \)) increase in caspase-3 activity compared with the control cells. These augmented enzyme activities were significantly reduced in the cells pretreated with LIF or DEVD-CHO, an irreversible caspase inhibitor, in vitro.

**LIF and Caspase Inhibition Increase the Abundance of Bcl-xL but Not Bcl-2**

We next determined whether DOX would affect the protein level of Bcl-xL. Figure 4A shows that the Bcl-xL protein...
level was downregulated by 16 hours of DOX treatment in a dose-dependent manner. Incubation with 30 U of recombinant caspase-3 (BIOMOL Research Laboratories) completely inhibited Bcl-xL expression (lane 6). Pretreatment with LIF attenuated the DOX-induced decrease in Bcl-xL abundance in a dose-dependent manner (Figure 4B, lanes 3 to 5). To determine whether caspase activation was relevant to decreases in Bcl-xL abundance, DEVD-CHO was added before DOX treatment. Bcl-xL protein level markedly increased to the basal level with 100 μmol/L DEVD-CHO (lane 7). In contrast, the Bcl-2 level showed similar basal abundance in each sample (Figure 4A and 4B, bottom).

**LIF Phosphorylates Bad Through PI 3-Kinase in Cardiac Myocytes**

We subsequently examined the effect of LIF on Bad phosphorylation in cardiac myocytes. Figure 5A shows that LIF stimulation caused a substantial increase in Bad phosphorylation at 30 minutes, which continued up to 60 minutes. Treatment with DOX for 16 hours inhibited Bad phosphorylation in LIF-pretreated cells (Figure 5B). To test whether LIF induces Bad phosphorylation via a PI 3-kinase–dependent pathway, either wortmannin or PD98059 was preincubated for 30 minutes before LIF treatment. Wortmannin but not PD98059 blocked the LIF-induced Bad phosphorylation in DOX-treated cells (Figure 5B). Subsequent Western blot analysis with anti-Bad antibody was performed to verify the same amounts of Bad in all samples (Figures 5A and 5B, bottom).

**Phosphorylation of Bad Results in Sequestration From Bcl-xL Heterodimers Through PI 3-Kinase Activation**

We next examined whether the LIF-induced Bad phosphorylation actually results in sequestration from the heterodimers with Bcl-xL. Western blot analysis with Bcl-xL immunoprecipitates showed that DOX did not change the abundance of Bad protein level either bound with or free from Bcl-xL (Figure 6A and 6B, lanes 1 and 2). LIF treatment markedly reduced the amount of Bad that coimmunoprecipitated with Bcl-xL in DOX-treated cells (Figure 6A, lanes 2 and 3). In contrast, the amount of Bad remaining in the supernatant after depletion of the Bcl-xL complex, indicating the phosphorylated Bad species, showed a significant increase in the same cell preparation (Figure 6B, lanes 2 and 3). These effects of LIF were significantly inhibited by wortmannin pretreatment (Figure 6A and 6B, lanes 3 and 4).

**Figure 2.** LIF restores PI 3-kinase/Akt activities in DOX-treated cardiac myocytes. A, Top, Cardiac myocytes were treated with vehicle or 0.5 μmol/L DOX for 16 hours with or without 10^5 U/mL LIF. Position of PI 3-kinase product is indicated (PI 3-P). Bottom, Relative amounts of p85 subunit of PI3-kinase are shown by Western blot analysis. B, Top, Cardiac myocytes were treated as in A. Western blot analysis was performed with an antibody against phosphorylated Akt. Middle, Membranes were stripped and reprobed with anti-Akt antibody. Bottom, Cell lysates were immunoprecipitated with anti-Akt antibody, and Akt kinase activity was measured with Crossstide as a substrate. Data are expressed as mean±SEM from 7 independent experiments, each performed in triplicate. *P<0.05 vs DOX(−). C, ERK activation in DOX-treated cardiac myocytes. Myocytes were treated with 0.5 μmol/L DOX or 10^6 U/mL LIF for 60 minutes. Equal amounts of protein extracts were separated by 10% SDS-polyacrylamide gel, and Western blot analysis was performed with an anti-phosphospecific p44/p42 mitogen-activated protein kinase antibody (top). Blot was reprobed with anti-ERK1 and ERK2 antibodies (bottom). D, Cardiac myocytes were preincubated with either 100 nmol/L wortmannin or 20 μmol/L PD98059 for 30 minutes, followed by 16-hour DOX treatment with 10^5 U/mL LIF. All blots are representative of 4 independent experiments. Middle and bottom, Same as described in B. *P<0.05 vs DOX(−).

**Figure 3.** LIF inhibits DOX-induced caspase-3 activation in cardiac myocytes. A, Cardiac myocytes were treated with vehicle (lanes 1 and 3) or 0.5 μmol/L DOX (lanes 2 and 4) for 16 hours with or without 10^5 U/mL LIF. Active subunit p17 was assessed by immunoblot analysis with anti-caspase-3 antibody (1:1000 dilution). Positions of intact enzyme (p32) and cleaved product (p17) are indicated by arrowheads. B, Cardiac myocytes were incubated with DOX for 16 hours (solid bars) or preincubated with 10^6 U/mL LIF for 3 hours (open bars) or with 50 μmol/L DEVD-CHO for 1 hour (hatched bars) before DOX treatment at indicated concentrations. Data are presented as absolute fluorescence units per mg protein per hour. Data are expressed as mean±SEM from 5 independent experiments, each performed in triplicate. *P<0.01 vs DOX(−); †P<0.01, ‡P<0.05 vs DOX.
Akt Mediates Protective Effects of LIF in DOX-Induced Apoptosis

To determine the functional significance of Akt activation in LIF-mediated cytoprotective effect, adenovirus vectors expressing caAkt or dnAkt were transfected into cardiac myocytes. Transfection with caAkt or dnAkt did not show a significant effect in controls; however, caAkt restored MTT activity in DOX-treated cells (Figure 7A). Although transfection with Adeno-βgal showed no detectable effect on LIF-induced increase in MTT activity in DOX-treated cells, transfection with dnAkt eliminated the effect of LIF (Figure 7A).

Furthermore, as shown in Figure 7B, LIF prevented the DOX-induced increase in caspase-9 activity, and transfection of dnAkt eliminated the reduction of caspase-9 activity in LIF-treated cells.

Discussion

We demonstrated that DOX induced apoptosis in cardiac myocytes in conjunction with an increase in the number of TUNEL-positive cells, DNA strand breaks, and a decrease in MTT activity, and LIF acts as a survival factor to inhibit the effect of DOX. Although several reports have provided detailed discussion of DOX-induced cytotoxicity in cardiac myocytes, anthracycline-induced cardiac myocyte apoptosis is known to occur at concentrations below those that result in necrosis. This study contains 3 major findings.

First, LIF is a potent survival factor that significantly attenuates DOX-induced myocardial cell damage correlated with the restoration of PI 3-kinase/Akt activities. Second, LIF protects cells from DOX-induced apoptosis by inhibiting caspase-3 activation and maintaining the steady protein abundance of Bcl-xL. Third, LIF-induced PI 3-kinase activation promotes cell survival through Bad phosphorylation, leading to its sequestration away from Bcl-xL.

A large number of studies have shown that the PI 3-kinase/Akt signaling pathway delivers an important cell survival signal in several cell lines. Our data imply that the restoration of PI 3-kinase/Akt activities by LIF correlates well with cell survival. Although we previously showed that mitogen-activated protein kinase functions partially as a downstream target of PI 3-kinase in cardiac myocytes, extracellular signal–regulated kinases were not affected by DOX treatment and LIF-induced phosphorylation of Akt and Bad was not affected by PD98059 pretreatment. However, a substantial decrease in MTT activity was observed in PD98059-treated cardiac myocytes.

Several studies showed that overexpression of Bcl-xL and Bcl-2 inhibits the anti-cancer drug–induced caspase-3 activation and apoptosis. Recently, however, it has been reported that both antiapoptotic proteins are downstream death...
Akt regulates caspase-9 by protein phosphorylation in cardiac myocytes. Akt phosphorylation is responsible for transducing LIF-mediated survival signal in DOX-treated cardiac myocytes. Transfection of caAkt increased viability and inhibited caspase-9 activation in cardiac myocytes treated with 1.0 μmol/L DOX for 6 hours with or without 10^3 U/mL LIF pretreatment. Caspase-9 activity was measured as described in Methods. Data are expressed as folds of control from 4 independent experiments. *P<0.05 vs β-gal, †P<0.05 vs LIF(−).

Figure 7. Akt is necessary for LIF-mediated cytoprotection in DOX-treated cardiac myocytes. A, Cardiac myocytes were infected with adenovirus vectors expressing β-gal (open bars), caAkt (solid bars), or dnAkt (hashed bars). Cell viability was evaluated as described in Figure 1D. Results are mean±SEM from 4 independent experiments, each performed in triplicate. *P<0.05 vs β-gal, †P<0.05 vs LIF(−). B, Cardiac myocytes were incubated with 1.0 μmol/L DOX for 6 hours with or without 10^3 U/mL LIF pretreatment. Caspase-9 activity was measured as described in Methods. Data are expressed as folds of control from 4 independent experiments. *P<0.05 vs β-gal, †P<0.05 vs LIF(−).

Substrates of caspases. Cleavage of these cytoprotective proteins may convert them to lethal proteins to create a feedback loop with caspases. However, we failed to detect a cleaved 16-kDa fragment that was suggested to be a caspase-cleaved form of Bcl-xL, even in the recombinant active caspase-3–treated cells. Our data showed that caspase-3–dependent cleavage of the Bcl-2 family protein was observed only in Bcl-xL but not in Bcl-2. These results indicated that Bcl-2 was not a downstream death substrate of caspase-3 in cardiac myocytes.

It has been reported that stimulation of the PI 3-kinase signaling pathway with growth factors delivers a cell survival signal Akt phosphorylation. We examined whether Akt is responsible for transducing LIF-mediated survival signal in DOX-treated cardiac myocytes. Transfection of caAkt increased viability and inhibited caspase-9 activation in cardiac myocytes. Akt regulates caspase-9 by protein phosphorylation and is also known to phosphorylate Bad. Although expression of dnAkt eliminated the LIF-mediated increase in cell viability and decrease in caspase-9 activity in DOX-treated cells, phosphorylation of Bad was not affected (data not shown). Whether Akt or Raf-1 is essentially involved in the regulation of Bad is critical; therefore, further studies are necessary to elucidate how PI 3-kinase produces the phosphorylation of Bad in cardiac myocytes.

In summary, the present study demonstrates that LIF is a potent survival factor that activates PI 3-kinase/Akt signaling events in cardiac myocytes. LIF induced inhibition of caspase-3 activation and significantly abrogated the decreases in Bcl-xL protein levels in DOX-treated cells. Importantly, PI 3-kinase plays a critical role in a direct link between gp130 and Akt phosphorylation, resulting in the translocation of Bad from a membrane-associated Bcl-xL to the cytosol and in an increase in the steady-state levels of Bcl-xL to protect cells from apoptosis.

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