In Cardiomyocyte Hypoxia, Insulin-Like Growth Factor-I–Induced Antiapoptotic Signaling Requires Phosphatidylinositol-3-OH-Kinase–Dependent and Mitogen-Activated Protein Kinase–Dependent Activation of the Transcription Factor cAMP Response Element–Binding Protein

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Background—A variety of pathologic stimuli lead to apoptosis of cardiomyocytes. Survival factors like insulin-like growth factor-I (IGF-I) exert anti-apoptotic effects in the heart. Yet the underlying signaling pathways are poorly understood.

Methods and Results—In a model of hypoxia-induced apoptosis of cultured neonatal cardiomyocytes, IGF-I prevented cell death in a dose-dependent manner. Antiapoptotic signals induced by IGF-I are mediated by more than one signaling pathway, because pharmacological inhibition of the phosphatidylinositol-3-OH-kinase (PI3K) or the mitogen-activated protein kinase kinase (MEK1) signaling pathway both antagonize the protective effect of IGF-I in an additive manner. IGF-I-stimulation was followed by a PI3K-dependent phosphorylation of AKT and BAD and an MEK1-dependent phosphorylation of extracellular signal–regulated kinase (ERK) 1 and ERK2. IGF-I also induced phosphorylation of cAMP response element–binding protein (CREB) in a PI3K- and MEK1-dependent manner. Ectopic overexpression of a dominant-negative mutant of CREB abolished the antiapoptotic effect of IGF-I. Protein levels of the antiapoptotic factor bcl-2 increased after longer periods of IGF-I-stimulation, which could be reversed by pharmacological inhibition of PI3K as well as MEK1 and also by overexpression of dominant-negative CREB.

Conclusions—In summary, our data demonstrate that in cardiomyocytes, the antiapoptotic effect of IGF-I requires both PI3K- and MEK1-dependent pathways leading to the activation of the transcription factor CREB, which then induces the expression of the antiapoptotic factor bcl-2. (Circulation. 2001;104:2088-2094.)

Key Words: apoptosis ■ hypoxia ■ signal transduction ■ growth factors

Several lines of evidence suggest that progressive loss of cardiomyocytes due to apoptosis significantly contributes to the development of heart failure (for a recent review, see Hauensetter and Izumo1). A variety of stimuli that are known to participate in the pathogenesis of heart failure have been shown to induce cardiomyocyte apoptosis, including hypoxia, ischemia and reperfusion, and oxidative stress.

Insulin-like growth factor I (IGF-I) regulates several pleiotropic cellular responses, including proliferation, hypertrophic growth, and protection from apoptosis. Binding of IGF-I to its specific cell-surface receptor results in activation of an intracytoplasmatic tyrosin kinase, which phosphorylates insulin receptor substrate-1. Tyrosyl-phosphorylated insulin receptor substrate-1 then interacts with numerous SH2 domain-containing proteins, including phosphatidylinositol-3-OH-kinase (PI3K) and the guanine nucleotide exchange factor Grb2/SOS. Whereas PI3K initiates phospholipid turnover and activation of AKT, Grb2/SOS activation results in initiation of the mitogen-activated protein kinase (MAPK) cascade by sequential phosphorylation and activation of the proto-oncogenes Ras and Raf and of mitogen-activated protein kinase kinase (MEK) 1 and MEK2. The activation of AKT has been shown to be of major importance for the antiapoptotic properties of IGF-I (reviewed by Datta et al8). AKT exerts its antiapoptotic properties by either activating antiapoptotic targets or inactivating proapoptotic factors. Among the known targets of AKT are the bcl-2-family member BAD, procaspase-9, the transcription factors nuclear factor-κB...
cAMP response element–binding protein (CREB),
Forkhead family of transcription factors,
glycogen synthase kinase (GSK)-3β.
The MAPK family is also important for IGF-I–dependent signal transduction. Particularly, extracellular signal–regulated kinase (ERK) 1 and ERK2 have been shown to be involved in hypertrophic growth of cardiomyocytes as well as protection from apoptosis.

Recent studies provided data supporting the notion that PI3K and AKT are involved in IGF-I–dependent survival pathways in cardiomyocytes in vitro and in vivo. However, these studies leave room for speculations as to whether other signaling pathways may contribute to IGF-I–dependent antiapoptotic effects as well. Furthermore, nothing is known about the identity and role of factors acting downstream of PI3K and AKT and MAPK in survival pathways in cardiomyocytes. In the present study, we demonstrate that IGF-I exerts its antiapoptotic properties in cardiac myocytes at least via two distinct signaling pathways, the PI3K and the MEK1 pathways. We identify transcriptional-dependent and -independent mechanisms responsible for the antiapoptotic properties of IGF-I.

Methods

Materials

Horse serum and cell culture medium were obtained from Gibco, recombinant IGF-I and wortmannin were from Sigma, and PD098059 and SB203580 were from Calbiochem. Phospho-specific antibodies were purchased from New England Biolabs, monoclonal Bcl-2 antibody and monoclonal BAD antibody were from Transduction Laboratories, polyclonal anti-ERK antibody from Santa Cruz, α-sarcomeric actin-antibody was from Sigma, and horseradish peroxidase–conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG antibodies were from Amersham.

Cell Culture

Monolayer cultures of neonatal rat cardiac cells were prepared as described previously, except for the omission of insulin in the culture medium. After 24 hours of culture, horse serum was reduced to 0.1% (vol/vol), and cells were exposed to hypoxia 24 hours later. Myocyte cultures contained ~95% myocytes as assessed by staining with an α-sarcomeric actin antibody. Hypoxic conditions could be created by placing cells in a hypoxia chamber (Labotect, Model 2015) with a water-saturated atmosphere composed of 5% CO2 and 95% N2. After 8 hours, the Po2 in the medium reached a steady-state level of 15 mm Hg. Inhibitors were added to the culture medium 15 minutes before IGF-I.

Recombinant Adenoviral Vectors and Infection

Construction of adenovirus type 5 constructs was performed as described previously. The recombinant adenoviruses contained expression cassettes either for a dominant-negative CREB mutant (Ad-dnCREB) or the lacZ-cDNA (Ad-lacZ) under control of the cytomegalovirus early gene promoter. The dominant-negative CREB mutant cDNA has an alanine residue substituting the serine at position 133. Cardiomyocyte cultures were infected 24 hours after seeding in serum-free medium with a multiplicity of infection of 10 for 3 hours. Exposure to hypoxia and IGF-I was performed after an additional 24 hours.

Cell Viability Assessment by Trypan Blue Exclusion Staining

The amount of dead cells was assessed by staining trypsinized cells after treatment with trypan blue, and the fraction of blue cells was quantified by counting under a microscope. The analyzer was blinded toward the treatment of cells. To include cells that already detached from the plate during the process of cell death, cells from the supernatant of each dish were also included. In each dish a total number of at least 500 cells were counted.

Analysis of DNA Fragmentation

Analysis of nucleosomal fragmentation of genomic DNA was performed as described elsewhere. TUNEL staining (Clontech) and measurement of histone-bound DNA fragments by cell death ELISA (Boehringer) were performed using commercially available kits as described elsewhere.

Western Blot and Immunoprecipitation Experiments

Western blot analysis, immunoprecipitation, preparation of subcellular fractions, and stimulation with 0.1 mmol/L H2O2 plus 0.1 mmol/L FeSO4 have also been described previously. For densitometric analysis of scanned blots, the NIH Imager software was used.

Statistical Analysis

The results are expressed as mean±SEM of at least 3 independent experiments, unless stated otherwise. Statistical significance was determined using an unpaired t test. For multiple comparisons, data were subjected to ANOVA followed by a Bonferroni adjustment. A value of P<0.05 was considered significant.

Results

IGF-I Prevents Hypoxia-Induced Apoptosis in Cardiomyocytes Via PI3K- and MEK1-Dependent Signaling Pathways

Exposure of cultured neonatal cardiomyocytes to hypoxic culture conditions led to time-dependent increase of dead cells, as assessed by trypan-blue exclusion staining (41±3.2% after 24 hours to 56±1.5% after 48 hours of hypoxia, Figure 1A). Supplementation of culture medium with IGF-I during hypoxia dose-dependently decreased the amount of dead cells (Figure 1A). To assess whether apoptosis contributed to hypoxia-induced cell death, internucleosomal fragmentation of DNA was analyzed. Figure 1B indicates that after 24 hours of hypoxic culture conditions, characteristic DNA laddering is present, which was attenuated in the presence of IGF-I. These results could be confirmed by in situ nick-end labeling (TUNEL assay) combined with α-sarcomeric actin staining for cardiac myocytes. After 24 hours of hypoxia, 36.46±4.189% of cardiac myocyte nuclei were TUNEL-positive, and IGF-I-supplementation reduced the amount significantly (21.05±2.361; P<0.05 versus hypoxia) (Figure 1C). To ascertain whether different signaling pathways contribute to IGF-I–mediated inhibition of cardiac myocyte apoptosis, we pharmacologically inhibited key enzymes of IGF-I–dependent signaling cascades. The amount of apoptosis was measured by an apoptosis-specific cell death ELISA (Figure 1D). Inhibition of PI3K (using wortmannin) reduced the protective effect of IGF-I by 60.9% (P<0.01); inhibition of MEK1 (using PD098059) showed a reduction by 48.3% (P<0.05). Parallel inhibition of both pathways antagonized the effect of IGF-I by 81.6% (P<0.01). Inhibition of p38 MAPK (using SB 203580) had no effect on IGF-I–mediated survival. These results suggest that for hypoxic...
cardiomyocytes, IGF-I serves as a survival factor through the activation of both PI3K and MEK1.

**IGF-I Mediates Phosphorylation of AKT and ERK1 and ERK2 Using Distinct Signaling Pathways**

Recently, IGF-I–dependent activation of downstream targets of PI3K as well as MEK1 have been shown in cardiac myocytes.17,18,22 We performed Western blot analysis using phospho-specific antibodies against AKT and ERK1 and ERK2 to prove IGF-I–dependent activation of these factors in hypoxic cardiac myocytes. As can be seen in Figures 2A and 2B, IGF-I induces phosphorylation of AKT and ERK1 and ERK2 in cells cultured under hypoxic conditions for 24 hours, whereas hypoxia alone does not induce phosphorylation of AKT and ERK1 and ERK2. Pharmacological inhibition of MEK1 but not PI3K or p38 MAPK prevents IGF-I–induced phosphorylation of ERK1 and ERK2 (Figure 2A), whereas phosphorylation of AKT can be blocked using a PI3K inhibitor (Figure 2B). The time course of ERK1 and ERK2 phosphorylation in response to IGF-I differed from that of AKT in that it showed an early peak after 30 to 60 minutes, declined thereafter, and showed a more moderate second elevation still detectable after 24 hours (data not shown).

To rule out a crosstalk between the two pathways activated by IGF-I, we investigated the effect of the PI3K inhibitor wortmannin on ERK1 and ERK2 phosphorylation. Although 10 nmol/L wortmannin was sufficient to inhibit IGF-I–mediated ERK1 and ERK2 phosphorylation (Figure 2C), whereas phosphorylation of AKT can be blocked using a PI3K inhibitor (Figure 2B). The time course of ERK1 and ERK2 phosphorylation in response to IGF-I differed from that of AKT in that it showed an early peak after 30 to 60 minutes, declined thereafter, and showed a more moderate second elevation still detectable after 24 hours (data not shown).
conclude, therefore, that IGF-I–induced ERK1 and ERK2 phosphorylation does not depend on PI3K.

Both IGF-I and Hypoxia Mediate Phosphorylation of BAD

For investigation of downstream signaling molecules in the PI3K and MEK1 pathways, we first immunoprecipitated total BAD followed by the analysis of the phosphorylation status of BAD serine residues S112 and S136 using phospho-specific antibodies. In normoxia, IGF-I caused rapid and transient phosphorylation at both serine residues (Figure 3A). However, phosphorylation at S112 occurred to a lower extent, because we had to double the amount of protein for the immunoprecipitation to receive a signal that was comparable to that of phosphorylated S136. The effect of IGF-I on the phosphorylation of BAD was dependent on PI3K-mediated signaling, because wortmannin could abrogate the IGF-I–dependent phosphorylation at both residues (Figure 3A). Importantly, longer periods of hypoxic culture conditions alone were sufficient to induce increasing phosphorylation of BAD on both serine residues. However, this occurred independent of PI3K or MEK1, and the amount of phosphorylation was comparable between serine residues S112 and S136 (Figure 3B).

Apoptosis of cardiomyocytes involves translocation of BAD to the mitochondrial membrane. For investigation of downstream signaling molecules in the PI3K and MEK1 pathways, we first immunoprecipitated total BAD followed by the analysis of the phosphorylation status of BAD serine residues S112 and S136 using phospho-specific antibodies. In normoxia, IGF-I caused rapid and transient phosphorylation at both serine residues (Figure 3A). However, phosphorylation at S112 occurred to a lower extent, because we had to double the amount of protein for the immunoprecipitation to receive a signal that was comparable to that of phosphorylated S136. The effect of IGF-I on the phosphorylation of BAD was dependent on PI3K-mediated signaling, because wortmannin could abrogate the IGF-I–dependent phosphorylation at both residues (Figure 3A). Importantly, longer periods of hypoxic culture conditions alone were sufficient to induce increasing phosphorylation of BAD on both serine residues. However, this occurred independent of PI3K or MEK1, and the amount of phosphorylation was comparable between serine residues S112 and S136 (Figure 3B).

Apoptosis of cardiomyocytes involves translocation of BAD to the mitochondrial membrane. We therefore analyzed subcellular localization of BAD in cardiomyocytes cultured under hypoxic conditions. Western blots of mitochondrial and cytosolic fractions showed modestly increased amounts of BAD bound to the mitochondrial membrane during the first 12 hours of hypoxia (2.3-fold increase compared with control after 12 hours). Thereafter, the amount of mitochondria-bound BAD decreased to basal levels (Figure 3B). However, control experiments using H2O2 to induce cardiomyocyte apoptosis resulted in a much more pronounced translocation of BAD. These data suggest that although via distinct pathways, both IGF-I and hypoxia mediate antiapoptotic effects, which include the phosphorylation and thus sequestration of BAD in the cytosol.

Antiapoptotic Effect of IGF-I Requires the Activation of CREB

The transcription factor CREB has been shown to be involved in antiapoptotic signaling. Interestingly, in PC12 cells, IGF-I stimulation results in CREB activation by PI3K- and MAPK-dependent phosphorylation at serine residue 133. We therefore examined CREB phosphorylation at serine 133 in IGF-I-stimulated hypoxic cardiac myocytes using a phospho-specific antibody. As shown in Figure 4A, IGF-I leads to significant phosphorylation of CREB (4.7-fold over control). Inhibition of PI3K or MEK1 resulted in a decrease of IGF-I–mediated CREB phosphorylation (59.5% or 54%, respectively), whereas combination of both inhibitors blocked 91.5% of IGF-I–induced CREB phosphorylation. These data indicate an additive effect of PI3K- and MEK1-dependent pathways in IGF-I–induced phosphorylation of CREB in cardiomyocytes.

To determine the functional significance of IGF-I–induced activation of CREB in cultured cardiomyocytes, cells were infected with an adenovirus harboring a dominant-negative mutant of CREB (Ad-dnCREB) or with a control virus (Ad-lacZ) and treated with hypoxia and IGF-I. Western blot analysis using phospho-specific CREB antibody revealed that overexpression of dnCREB abolishes the IGF-I–induced phosphorylation at serine 133, whereas the control virus had no such effect (data not shown). In hypoxic cardiac myocyte cultures, overexpression of dnCREB significantly attenuated the protective effect of IGF-I as assessed by cell death ELISA (hypoxia+IGF-I, 6.62±0.11-fold over control, versus hypoxia+IGF-I+Ad–dnCREB, 10.38±0.55-fold over control, P<0.01), whereas it had only little effect on basal survival of normoxic cardiac myocytes (normoxia+Ad–dnCREB, 2.3±0.32-fold over control; P=NS) (Figure 4B).
IGF-I Treatment Induces Expression of bcl-2 via CREB-Dependent Transcription

In PC12 cells, CREB transcriptional activity induces the expression of antiapoptotic bcl-2. Therefore, we analyzed bcl-2 protein levels of IGF-I-treated hypoxic cardiomyocyte cultures. Bcl-2 protein levels were increased after 18 hours of IGF-I treatment and declined slowly after 36 hours (24 hours, 1.82±0.19-fold over control, *P<0.05 vs control; 48 hours, 1.44±0.03-fold over control, *P=0.05) (Figure 5). Pharmacological inhibition of PI3K or MEK1 alone reduced IGF-I-induced bcl-2 expression (≈50% compared with 24-hour IGF-I). Combination of both inhibitors or overexpression of dnCREB completely abolished IGF-I-induced bcl-2 expression. These data indicate that IGF-I induces bcl-2 expression via PI3K- and MEK1-dependent activation of the nuclear transcription factor CREB.

Discussion

The present study demonstrates in a model of hypoxia-induced apoptosis that there are at least two signal transduction pathways responsible for the antiapoptotic properties of IGF-I in cardiomyocytes, the PI3K and AKT pathway as well as the MEK1 and ERK1 and ERK2-pathway. We identified the transcription factor CREB as an antiapoptotic target regulating bcl-2 expression in a PI3K- and MEK1-dependent manner. Besides this transcription-dependent protective event, we demonstrate phosphorylation of BAD as an additional survival pathway activated by IGF-I but also by hypoxia itself. A simplified signaling scheme summarizes the signaling events induced by IGF-I and hypoxia (Figure 6).

Several studies describe phosphorylation and inactivation of the proapoptotic bcl-2 family member BAD as one mechanism for AKT-mediated survival. BAD exerts its apoptotic function by forming heterodimers with mitochondria-bound bcl-2 and bcl-XL, neutralizing their pro-
tective effect, and promoting cell death.\textsuperscript{77} After phosphoryla-
tion of two serine residues embedded within the 14-3-3
consensus–binding site, BAD is sequestered in the cytosol
bound to 14-3-3.\textsuperscript{28} Although phosphorylation of serine resi-
due S136 has been shown to be mediated by AKT,\textsuperscript{9} different
mechanisms have been proposed for phosphorylation at
serine residue S112, including mitochondria-anchored protein
kinase A\textsuperscript{9,29} and MEK1-dependent p90 ribosomal S6 kinases
(RSKs).\textsuperscript{30} Our results demonstrate that IGF-I induces phos-
phorylation of BAD mainly at position S136 and to a lower
extent at position S112 in a PI3K-dependent manner. Inter-
estingly, hypoxic culture conditions lead to an extensive
phosphorylation of BAD at both positions, and neither the
inhibition of PI3K nor that of MEK1 are able to block this
effect. This indicates that IGF-I and hypoxia activate distinct
upstream signaling pathways leading to BAD phosphoryla-
tion. In the case of hypoxia, the phosphorylation of BAD
could reflect a protective mechanism of those cells that
survive hypoxia. This idea is supported by the finding that at
early time points only, when BAD is not fully phosphorylat-
ed, there is a slight increase of BAD bound to the mitochon-
drial membrane. However, at later time points, hypoxia-
duced phosphorylation of BAD prevents additional
translocation of BAD to the mitochondrial membrane. An-
other possibility is that the phosphorylation of BAD has no
effect on the execution of the apoptotic cell death program,
because it has been shown in a hemopoietic cell line stably
induced phosphorylation of BAD prevents additional
survival signals in cells.  

Cellular survival promoted by the PI3K and AKT signaling
pathway as well as the MAPK signaling pathway has also
been described to occur by transcription-dependent mecha-
nisms. The transcription factor CREB has been shown to be
a target of several signaling molecules, including AKT,\textsuperscript{12,23}
p90RSK,\textsuperscript{32} and MAPKs.\textsuperscript{25,30,33} Besides being a critical
regulator of immediate early gene transcription, CREB in its
activated form mediates survival of different cell types by
enhancing transcription of antiapoptotic bcl-2 family mem-
bers.\textsuperscript{23,24,34} A functional relevance for CREB-dependent tran-
scription in the heart derives from studies in human failing
and nonfailing heart samples, where binding of phosphory-
lated CREB to CRE sites has been demonstrated.\textsuperscript{35} Trans-
genic mice overexpressing dominant-negative CREB specif-
ically in the heart develop a dilated cardiomyopathy with
impaired cardiac function; however, no significant increase
in the amount of apoptotic nuclei could be demonstrated.\textsuperscript{36}
Our results indicate the importance of CREB as a transducer of
IGF-I–mediated survival signals in cardiomyocytes. CREB-
mediated survival signals emerge from different signal trans-
duction pathways and result in enhanced transcription of
bcl-2 and possibly other antiapoptotic proteins, such as
bcl-X\textsubscript{L}, which has been demonstrated to be expressed after
IGF-I stimulation in PC12-cells.\textsuperscript{37}

Other survival factors, such as leukemia inhibitory factor
and cardiotrophin-1, protect cardiomyocytes from apoptosis
by signaling through the glycoprotein 130 receptor. Activa-
tion of both the PI3K and AKT pathway\textsuperscript{38} and the MEK1
pathway\textsuperscript{39} have been demonstrated in response to glycopro-
tein 130 stimulation; however, these studies focus on only
one signal transduction pathway mediating the survival ef-
facts. Lately, some reports have demonstrated parallel or
subsequent activation of independent survival pathways as a
 cellular strategy to escape deleterious influence.\textsuperscript{30} Examples
from cell types such as 3T3 fibroblasts and leiomyosarcoma
cells\textsuperscript{40} and PC12 cells\textsuperscript{5} demonstrate additive effects of PI3K
and MAPK signal transduction on survival. An involvement
of PI3K in MEK1-dependent signaling pathways has been
proposed by some groups\textsuperscript{41} but denied by others.\textsuperscript{42,43} One
study suggests a conditional inhibition of the MAPK cascade
by wortmannin, depending on the signal strength.\textsuperscript{44} Our
experiments revealed that in cardiomyocytes, wortmannin in
a dose of 20 nmol/L sufficiently blocks PI3K-mediated AKT
phosphorylation without affecting MEK1-dependent ERK1
and ERK2 phosphorylation, indicating an independent acti-
vation of these two signal transduction pathways. In addition,
we observed an additive effect of wortmannin and PD98059
regarding the inhibition of CREB phosphorylation and ex-
pression of bcl-2. We conclude, therefore, that IGF-I activates
at least two independent pathways leading to enhanced
survival. Parallel activation of these independent signal trans-
duction pathways may represent a cellular strategy to amplify
survival signals in cells.

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