Reperfusion-Activated Akt Kinase Prevents Apoptosis in Transgenic Mouse Hearts Overexpressing Insulin-Like Growth Factor-1

Kazuhiro Yamashita, Jan Kajstura, Daryl J. Discher, Bernard J. Wasserlauf, Nanette H. Bishopric, Piero Anversa, Keith A. Webster

Abstract—Hearts of wild-type and insulin-like growth factor-1 overexpressing (Igf-1+/−) transgenic mice were subjected to Langendorff perfusions and progressive periods of ischemia followed by reperfusion. Apoptosis was measured by DNA nucleosomal cleavage and a hairpin probe labeling assay to detect single-base overhang. Transgenic hearts subjected to 20 minutes of ischemia and 4 hours of reperfusion (I/R) sustained a rate of apoptosis of 1.8±0.3% compared with 4.6±1.1% for wild-type controls (n=4; P<0.03). Phosphorylation of the protein kinase Akt/protein kinase B was elevated 6.2-fold in transgenic hearts at baseline and increased another 4.4-fold within 10 minutes of reperfusion, remaining elevated for up to 2 hours. I/R activated Akt in wild-type hearts but to a lesser extent (1.6±0.3-fold). Pretreatment of transgenic hearts with wortmannin immediately before and during ischemia eliminated reperfusion-mediated activation of Akt and neutralized the resistance to apoptosis. The stress-activated kinase p38 was also activated during ischemia and reperfusion in both wild-type and transgenic hearts. Pretreatment with SB203580 (10 μmol/L) blocked both p38 activation and phosphorylation of Akt and differentially modulated apoptosis in wild-type and transgenic hearts. These results demonstrate that Akt phosphorylation during I/R is modulated by IGF-1 and prevents apoptosis in hearts that overexpress the IGF-1 transgene. (Circ Res. 2001;88:609-614.)

Key Words: ischemia ■ hypoxia ■ phosphoinositol-3′-kinase ■ p38 mitogen-activated protein kinase ■ SB203580

Protective and antiapoptotic properties of insulin-like growth factor-1 (IGF-1) have been demonstrated in different models of myocardial ischemia and infarction1–4 as well as in isolated cardiac myocytes subjected to ischemic or oxidative stress.5,6 IGF-1 stimulates the phosphoinositol-3′ (PI3)-kinase pathway, producing phosphoinositides that promote activation of the kinase Akt.7,8 Activated Akt kinase plays a central role in suppressing apoptosis by modulating the activities of Bcl-2 family proteins,9 caspase 9,10 and Fas ligand.11 Transfer of mutationally activated PI3-kinase and Akt genes has been shown to prevent apoptosis of cardiac myocytes in vitro,6 and activated Akt delivered by an adenovirus vector reduced apoptosis in the intact heart subjected to ischemia and reperfusion (I/R).4 We previously reported that Igf-1+/− transgenic mice overexpressing IGF-1 had reduced rates of necrosis and apoptosis after myocardial infarction caused by coronary artery ligation.1 The same hearts were also resistant to necrosis but not apoptosis caused by nonocclusive coronary artery constriction.12 Other work has shown that the endogenous PI3-kinase pathway is activated in the postinfarcted myocardium, where it may contribute to cell survival and hypertrophy.13–15 Regulation of the endogenous PI3-kinase pathway during I/R has not been described. We report here that Igf-1+/− transgenic mouse hearts are resistant to I/R-mediated apoptosis through a novel pathway involving enhanced basal activation of Akt and superinduction by reperfusion. In these hearts, elevated basal IGF-1 supports an amplified response of the PI3-kinase pathway that shifts the balance between antiapoptotic Akt and proapoptotic p38 after reperfusion.

Materials and Methods

IGF-1 Transgenic Mice

The generation of transgenic mice expressing the human insulin-like growth factor-1 gene under the direction of the rat α-myosin heavy chain gene promoter has been described previously.16 In all studies, mice of both sexes heterozygous17 for the IGF-1 transgene between 8 to 12 weeks of age were compared with age-matched controls.

Langendorff Perfusions

Our methods for isolating and perfusing mouse hearts by the Langendorff method have been described previously.17 Briefly,
hearts were retrograde-perfused with a phosphate-free Krebs-Heineleit buffer equilibrated at 37°C with 5% CO₂/95% O₂, pH 7.4, and the Langendorff apparatus was housed in a temperature-controlled (37°C) and humidified incubator. Before treatments, hearts were perfused at constant pressure (80 mm Hg) with a flow rate of 2 to 4 mL/min for a 30-minute stabilization period. Global ischemia was applied by eliminating flow for the period indicated; hearts ceased to beat after 3 to 5 minutes of ischemia, and all hearts resumed contractions during reperfusion. Where indicated, drugs including wortmannin (Sigma Chemical Co) and SB203580 (Boehringer) were added to the perfusion buffer 15 minutes before ischemia and remained in the perfusion for 15 minutes after reperfusion. At the end of the perfusion period, hearts were either frozen rapidly on dry ice and stored at −95°C or immersed in 1.5% paraformaldehyde.

Analysis of DNA Fragmentation (Laddering) and In Situ Ligation of Hairpin With Single-Base 3’ Overhang

Our procedures for analyzing DNA fragmentation and in situ ligation with hairpin probes have been described previously.12,17–19 For fragmentation assays, DNA samples (5 to 8 µg DNA) were subjected to electrophoresis in 2% agarose gels and imaged by ethidium bromide staining and digital photography. In some cases, the band density of the ladders was measured using an NIH Image program with Adobe Photoshop. For in situ ligations, hearts were fixed in 1.5% paraformaldehyde and tissue sections were labeled in a buffer containing T4 ligase and 35 ng/µL hairpin probe with single base 3’ overhang (Synthetic Genetics Corp) followed by FITC-extravidin staining (Sigma). Myocyte cytoplasm was detected by α-sarcomeric actin labeling; nuclei were stained by propidium iodide. Sections were analyzed with a confocal microscope (MRC-1000, BioRad Laboratories). Apoptotic index was obtained by counting an average of 15 000 myocyte nuclei in each heart.

Western Blot and Kinase Analysis

Our procedures for Western blots have been described in detail elsewhere.17 Blots were probed with specific antibodies against Akt, phospho-Akt, p38, and phospho-p38 (New England Biotechnology) and visualized using enhanced chemiluminescence (Pierce). Kinase assays were used as described previously, with anti-p38 antibody to immunoprecipitate p38 from solubilized heart tissues and myelin basic protein as substrate.20

Results

DNA Fragmentation During I/R

Igf-1+/− and wild-type hearts were perfused for 30 minutes and subjected to increasing periods of ischemia followed by 4 hours of reperfusion. DNA was extracted from the left ventricles and analyzed by gel electrophoresis as described in Materials and Methods. Representative results are shown in Figure 1A. DNA nucleosomal cleavage was observed after 20 minutes of ischemia and 4 hours of reperfusion and increased with the duration of ischemia up to 50 minutes (Figure 1, top, lanes 1 through 4). DNA cleavage in the Igf-1+/− hearts only appeared after 40 and 50 minutes of ischemia (n=4). To confirm this finding, 4 hearts from each strain were subjected to 20 minutes of ischemia and 4 hours of reperfusion, and apoptotic DNA fragmentation was measured in situ by detection of single-base overhang using a hairpin probe assay, as described in Materials and Methods. Figure 1 (bottom) shows examples of myocyte fields from wild-type and Igf-1+/− hearts, both selected for high density of apoptotic nuclei. In these fields, 60% of the wild-type nuclei and 34% of the Igf-1+/− nuclei were identified as apoptotic. Analyses of more

Figure 1. Apoptosis of wild-type and Igf-1−/− hearts induced by I/R. Excised hearts were perfused by the Langendorff system, as described in Materials and Methods. Top, Global ischemia was sustained for intervals between 20 and 50 minutes, followed by 4 hours of reperfusion. Arrows indicate DNA fragments at ~300, 500, 700, 900, and 1000 nucleotide base pairs. Bottom, Hairpin probe labeling of apoptotic myocytes from wild-type (A through C) and Igf-1+/− (D through F) hearts subjected to 20 minutes of ischemia and 4 hours of reperfusion. Blue fluorescence (A and D) indicates total nuclei stained with propidium iodide, green fluorescence (B and E) is the hairpin-FITC-extravidin stain, and red stain (C and F) is α-sarcomeric actin.

than 15 000 nuclei in each of 4 hearts from both strains revealed a reduction in apoptosis; 1.8±0.3% versus 4.6±1.1% (P<0.03) for transgenic and wild-type hearts, respectively, subjected to 20 minutes of ischemia and 4 hours of reperfusion.

Activation of Akt and Reversal of Protection by Wortmannin

Western blots of wild-type and Igf-1−/− hearts probed with antibodies against phospho-Akt and total Akt are shown in Figure 2A, and quantitation of the results is shown in Figure 2B. There was no difference in the total Akt protein between wild-type and Igf-1−/− samples (n=3). Wild-type hearts were weakly positive for phospho-Akt at baseline and showed a small but significant 1.6±0.3-fold increase immediately after reperfusion that remained elevated for 1 to 2 hours. In the Igf-1−/− hearts, phospho-Akt was 6.2±1.1-fold higher than the wild-type at baseline (n=4), and this was induced an additional 4.4±0.9-fold (n=3) within 10 minutes of reperfusion. Overall, there was >10-fold more phospho-Akt in transgenic hearts than in wild-type hearts 10 minutes after reperfusion, and the activation of Akt was sustained for at least 2 hours. Ischemia up to 50 minutes by itself was not
FIGURE 2. Expression of Akt and effects of wortmannin. A and B, Wild-type and Igf-1−/− hearts were subjected to I/R, and extracted proteins were analyzed by Western blot with anti-Akt antibodies as indicated. Western blots were quantitated by densitometric analyses, as described in Materials and Methods, and quantitation includes results from 3 separate experiments (±SEM). Phospho-Akt was significantly increased in the transgenic hearts compared with wild-type hearts at all time points (P<0.01). C, Replicate hearts were perfused as in Figure 1 or with 200 nmol/L wortmannin (left) or 20 nmol/L wortmannin (right) for 15 minutes before and immediately after ischemia or for 4 hours without ischemia as indicated. After 4 hours of reperfusion, DNA was analyzed by gel electrophoresis as in Figure 1 (top).

sufficient to activate Akt, indicating a critical role for reperfusion in the induction of this kinase; indeed, Akt kinase activation was also inhibited when 10 mmol/L N-acetyl cysteine was included during I/R (n=2, data not shown).

To determine whether Akt activation contributes to the cardioprotection observed in IGF-1−/− overexpressing hearts, wild-type and Igf-1−/− hearts were perfused with 200 nmol/L wortmannin before and immediately after ischemia. Results from replicate hearts are shown in Figure 2C (left panel). Wortmannin had only minimal effect on I/R-induced DNA cleavage in wild-type hearts. In contrast, wortmannin treatment resulted in a marked increase in DNA cleavage in IGF-1 transgenic hearts, equivalent to that seen in wild-type hearts. In experiments not shown here, we found that 200 nmol/L wortmannin also inhibited extracellular signal–regulated kinase (ERK) activity in reperfused hearts; therefore, transgenic mouse hearts were treated with 20 nmol/L wortmannin before I/R, a concentration that does not effect ERK. Representative results are shown in the right panel of Figure 2C. The lower wortmannin concentration also stimulated I/R-mediated apoptosis, supporting the predominant role of PI3-kinase in preventing apoptosis in the transgenic hearts. Wortmannin treatment alone did not cause increased DNA fragmentation. Densitometric quantitation of the bottom 3 bands in the ladders, as described in Materials and Methods, indicated that the increased fragmentation in transgenic hearts by wortmannin treatment was very significant (P<0.001, n=4), whereas wortmannin treatment had no significant effect on fragmentation in the wild-type hearts. These results indicate that superinduction of endogenous Akt is a key determinant of the cardioprotection observed in IGF-1−/− overexpressing mice.

Activation of p38 and Differential Responses to SB203580

The stress-activated protein kinase (SAPK) p38 has been assigned a proapoptotic role in various models of I/R in vitro21,22 and in vivo,23,24 an activity that is probably associated with the p38α isoform.23 SAPK/p38 has also been implicated as an upstream kinase for mitogen-activated protein kinase–activated protein kinase-2, which can activate Akt under some conditions.25,26 Therefore, we analyzed the expression of p38 in wild-type and Igf-1−/− mouse hearts subjected to I/R. The kinetics and magnitude of p38 phosphorylation in response to I/R were similar (Figures 3A and 3B). Phosphorylation was activated during the ischemic phase in both sets of hearts and was sustained through 1 hour of reperfusion, and there was a second later phase of activation starting at 2 to 4 hours of reperfusion. These 2 phases probably correspond to stimuli initiated by ischemia and reperfusion, respectively.27,28 Quantitation of the results indicated a possible slight lag in the responses of phospho-p38 in the transgenic compared with the wild-type hearts (Figure 3B).

To determine the contribution of p38 activation to apoptosis, hearts were perfused with the p38 inhibitor SB203580 during ischemia, as described in Materials and Methods. These results are shown in Figure 3C. In wild-type hearts, pretreatment with SB203580 decreased but did not eliminate I/R-induced DNA cleavage, as would be predicted from previous studies.18,22,29 Unexpectedly, SB203580 treatment increased DNA fragmentation in the Igf-1−/− hearts. Densitometric quantitation of the ladders, as described for Figure 2, indicated that the effects of SB203580 were significant (P<0.001 for both wild-type and transgenic hearts, n=4). There was no significant difference between wild-type and SB203580–treated transgenic hearts subjected to I/R.

SB203580 has recently been shown to inhibit both p38 and Akt kinase within a narrow concentration range.30 As shown in Figure 3D, perfusion with wortmannin blocked Akt activation selectively, but 10 μmol/L SB203580, which is a concentration frequently used for this kind of analysis,22,24,29 prevented the induction of both p38 and Akt by I/R. These results present an interesting anomaly; the net effect of simultaneously inhibiting both p38 and Akt is protective in wild-type cells but promotes apoptosis in the transgenic hearts. This suggests that p38 activation during I/R is dominant in wild-type hearts, whereas in Igf-1−/− hearts under the same conditions, antiapoptotic Akt is dominant.

Discussion

Our results support previous studies that have described cardioprotective and antiapoptotic roles of Akt in different conditions. This provides a possible explanation for the apparent differential role of Akt in transgenic and wild-type cells. Indeed, our results indicate that Akt is sufficient to promote cardioprotection and antiapoptosis, whereas additional autocrine and paracrine factors, such as IGF-1, may further enhance these effects. However, our results also suggest that Akt activation is not the only mechanism by which IGF-1 exerts its cardioprotective effects. Indeed, p38 activation has been implicated in various models of I/R, and our results indicate that p38 activation is associated with increased DNA fragmentation in wild-type hearts, whereas in transgenic hearts, p38 activation is associated with decreased DNA fragmentation. This suggests that the balance between Akt and p38 activation is critical for the cardioprotection observed in IGF-1−/− overexpressing mice.
models of ischemia and hypoxia in vitro and in vivo. In addition, we show that the endogenous PI3-kinase pathway is activated by reperfusion in a manner that reflects the availability of IGF-1. Igf-1/2 transgenic myocytes from 75-day-old mice secrete 4-fold more IGF-1 and maintain almost twice the serum levels of IGF-1 as their littermates. In these hearts, we found that the basal level of phosphorylated Akt was increased 6-fold above the equivalent wild-type level, presumably because of the continuous overproduction of IGF-1 in these hearts. The additional stimulation of phospho-Akt by I/R, most pronounced in the Igf-1/2 hearts but also observed in wild-type hearts, places Akt kinase among the lengthening list of kinases that respond to the reperfusion stimulus.

Both the induction of phospho-Akt by I/R and the resistance of the Igf-1/2 hearts to apoptosis were blocked by wortmannin. This confirms the role of PI3-kinase in both responses. The relatively greater induction of Akt in the Igf-1/2 hearts presumably reflects the primed state of PI3-kinase in these hearts. The stimulus for I/R-mediated activation of Akt is not clear. A previous study using isolated neonatal cardiac myocytes demonstrated that Akt was induced when the culture medium was switched from a low-pH, high-lactate medium containing sodium dithionite and deoxyglucose to normal buffered medium. Akt is also activated by preconditioning in the rat heart. Therefore, activation of Akt in response to reperfusion/reoxygenation occurs in vitro and in vivo. The stimulus may be directly linked to the redox changes that accompany I/R, as seems to be the case for c-Jun N-terminal kinase and ERK activation. In results not shown here, we found that I/R in the presence of 10 mmol/L N-acetyl cysteine prevented Akt activation, supporting a role for reactive oxygen. The minimal impact of wortmannin on apoptosis in the wild-type hearts reflects the low basal level of phospho-Akt and the weak response of Akt to I/R in these hearts. This in turn presumably reflects the low basal activity of the PI3-kinase pathway in wild-type hearts.

The differential responses to SB203580 underscore the importance of the balance between the PI3-kinase and p38 pathways in determining cell fate in the ischemic reperfused heart. Wild-type hearts have low basal phospho-Akt that is only weakly activated by I/R. Therefore, endogenous Akt offers only minimal protection in these hearts, and the principle action of SB203580 is to inhibit p38 and block apoptosis. This result is consistent with several previous studies that have described partial protection of normal myocardium by treatments with SB 203580. In contrast, the PI3-kinase-Akt kinase pathway in Igf-1/2 hearts is extremely robust, generating 10-fold more phospho-Akt than wild-type hearts during I/R and suppressing apoptosis even though p38 is activated in parallel. Under these conditions, SB 203580 has both proapoptotic and antiapoptotic effects because of the dual block of Akt and p38, and the outcome is the net effect of these. Because inhibition of p38 only partially prevents apoptosis, the contribution of phospho-Akt seems to be dominant in the transgenic hearts, and the net effect of SB203580 treatment is to stimulate apoptosis.
The results confirm the cardioprotective role of IGF-1 and the PI3-kinase-Akt pathway. By extrapolation, augmentation of this pathway by either gene or protein transfer may also provide a promising clinical strategy. Indeed, elevated IGF-1 correlates with improved recovery of postischemic hearts.1,13–15 Our studies have shown that IGF-1−/− transgenic mouse hearts are resistant to apoptosis or necrosis in 3 different models of ischemia, confirming the disease-resistant phenotype of these hearts. However, the IGF-1−/− mice also have side effects that include an age-dependent hypertrophy and hyperplasia of the heart and various other organs that appears within the first year of life and may compromise the protective effects of IGF-1 in older mice.16 There may also be increased cancer risk for these mice. Clearly, these side effects are not compatible with chronic global IGF-1 therapy as a treatment strategy for ischemic heart disease.

A key feature of the studies reported here is that protection from I/R in the IGF−1−/− mice may actually be enhanced by the stimulus of reperfusion itself. Although increased circulating IGF-1 supports a global increase of PI3-kinase and phospho-Akt in the IGF−1−/− hearts, superinduction of Akt is confined to the ischemic-reperfused myocardium, providing both a spatial and temporal containment of the therapy. If the basal IGF-1 transgene expression could be silenced in the healthy (nondiseased) heart while maintaining or preferably amplifying activation during I/R, it may be possible to eliminate the side effects while maintaining protection. Incorporation of hypoxia-responsive, oxidative stress, and silencer elements in the transgene promoter that specifically activate gene expression during ischemia or reperfusion may help to accomplish this and perhaps create a feasible, safe method for delivering IGF-1 genes to diseased hearts.37–39

Acknowledgments

This work was supported by grants HL44578 (to K.A.W.) and AG15756 (to P.A.) from the National Institutes of Health and an Established Investigator Award from the American Heart Association (to N.H.B.).

References

17. Webster KA, Discher DJ, Kaiser S, Hernandez OM, Sato B, Bishopric NH. Hypoxia-activated apoptosis of cardiomyocytes requires reoxygona
20. Laderoute KR, Webster KA. Hypoxia/reoxygona


Reperfusion-Activated Akt Kinase Prevents Apoptosis in Transgenic Mouse Hearts Overexpressing Insulin-Like Growth Factor-I
Kazuhito Yamashita, Jan Kajstura, Daryl J. Discher, Bernard J. Wasserlauf, Nanette H. Bishopric, Piero Anversa and Keith A. Webster

Circ Res. 2001;88:609-614
doi: 10.1161/01.RES.88.6.609

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/6/609

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/