Cell Death and Differentiation (2008) 15, 521–529

www.nature.com/cdd

Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II

S Miyamoto[1], AN Murphy[1] and JH Brown*,[1]

Akt activation supports survival of cardiomyocytes against ischemia/reperfusion, which induces cell death through opening of the mitochondrial permeability transition pore (PT-pore). Mitochondrial depolarization induced by treatment of cardiomyocytes with H_20_2 is prevented by activation of Akt with leukemia inhibitory factor (LIF). This protective effect is observed even when cardiomyocytes treated with LIF are permeabilized and mitochondrial depolarization is elicited by elevating Ca^{2+}. Cell fractionation studies demonstrate that LIF treatment increases both total and phosphorylated Akt in the mitochondrial fraction. Furthermore, the association of Akt with HK-II is increased by LIF. HK-II contains consensus sequences for phosphorylation by Akt and LIF treatment induces PI3K- and Akt-dependent HK-II phosphorylation. Addition of recombinant kinase-active Akt to isolated adult mouse heart mitochondria stimulates phosphorylation of HK-II and concomitantly inhibits the ability of Ca^{2+} to induce cytochrome *c* release. This protection is prevented when HK-II is dissociated from mitochondria by incubation with glucose 6-phosphate or HK-II-dissociating peptide. Finally LIF increases HK-II association with mitochondria and dissociation of HK-II from mitochondria attenuates the protective effect of LIF on H_20_2-induced mitochondrial depolarization in cardiomyocytes. We conclude that Akt has a direct effect at the level of the mitochondrion, which is mediated via phosphorylation of HK-II and results in protection of mitochondria against oxidant or Ca^{2+}-stimulated PT-pore opening.
Cell Death and Differentiation (2008) **15,** 521–529; doi:10.1038/sj.cdd.4402285; published online 7 December 2007

Cardiomyocyte cell death has been suggested to contribute to cardiac pathophysiology including ischemia/reperfusion injury and development of heart failure.[1,2] The protein kinase Akt and its downstream signaling cascade, support cell survival in many systems including the heart.[3–6] Receptor stimuli that have been reported to elicit Akt-mediated cell protection in the heart include ligands for receptor tyrosine kinases (insulin-like growth factor-1:IGF-1),[3,4] glycoprotein 130 (cardiotrophin-1; CT-1, leukemia inhibitory factor; LIF)[7,8] and G-protein-coupled receptors (sphingosine 1-phosphate; S1P).[9] Sustained expression of constitutively active Akt (myr-Akt) prevents cardiomyocyte death induced by ischemia/reperfusion both *in vitro*[3,4] and *in vivo*,[4,5] as does expression of nuclear-targeted Akt, suggesting that transcriptional events mediate the cardioprotective effects of Akt.[10] There is also evidence that more acute protective effects are mediated through Akt signaling. For example, Akt activation by insulin or S1P decreases ischemia/reperfusion damage in isolated perfused heart or *in vivo*.[9,11] The acute cardioprotective effects of Akt activation, observed within hours, are unlikely to be mediated through transcriptional events. Thus post translational effects, as might result from Akt-mediated phosphorylation, should be considered. Akt has been reported to confer mitochondrial protection through phosphorylation of the apoptotic protein Bad.[12] GSK3β, another substrate for Akt, has also recently been reported to contribute to mitochondrial protection mediated by Akt.[13] Several lines of evidence demonstrate that cardiomyocyte injury induced by ischemia/reperfusion results from increased cytosolic Ca^{2+} and generation of reactive oxygen species, which subsequently leads to mitochondrial depolarization and initiates a cell death cascade.[2,14,15] Ischemic injury and oxidative stress disrupt mitochondrial integrity through opening of a mega channel referred to as the permeability transition (PT) pore. The PT-pore has been suggested to be composed of VDAC, ANT, cyclophilin D (Cyp D) and HK-II,[16,17] although the molecular constituents of the pore continue to be disputed.[18–21]

Hexokinases I and II (HK-I and HK-II) have mitochondrial-binding motifs and these enzymes have been demonstrated to localize at mitochondria as well as in cytosol.[13,22] A role for hexokinase in mitochondrial protection was recently reported.[22–26] Interestingly the ability of Akt to protect against cytochrome *c* release and apoptosis in fibroblasts was shown to be decreased by HK-II dissociation from mitochondria.[27] The question of whether hexokinase is a mediator of cell survival in the heart has not been examined, although HK-II is abundantly expressed in cardiomyocytes. It is also not known whether PT-pore opening and mitochondrial integrity are

[1]Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636, USA
*Corresponding author: JH Brown, Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636, USA.
Fax: +858 534 4337; E-mail: jhbrown@ucsd.edu
Keywords: Akt; Hexokinase-II; mitochondria; permeability transition pore
Abbreviations: PT-pore, permeability transition pore; HK-II, hexokinase-II; PI3K, phosphoinositide 3-kinase; PDK-1, phosphoinositide-dependent kinase-1; Rho-GDI, Rho-guanine nucleotide dissociation inhibitor; LIF, leukemia inhibitory factor; LY, LY 294002; PAS antibody, phospho-Akt substrate antibody; Cs-A, cyclosporine-A; NRVMs, neonatal rat ventricular myocytes; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel
Received 23.4.07; revised 02.10.07; accepted 16.10.07; Edited by G Kroemer; published online 07.12.07

regulated through the interaction of Akt and HK-II. More specifically the possibility that the effect of HK-II might be regulated by its phosphorylation by Akt, has not been considered. The current study presents evidence that agonist-induced Akt activation in cardiomyocytes promotes its interaction with mitochondria and that phosphorylation of HK-II plays a key role in enabling Akt to protect mitochondria against Ca^{2+}-induced mitochondrial depolarization and cytochrome *c* release.

Results

H_2O_2 treatment is widely used to mimic the reactive oxygen species generation that occurs with oxidative stress. Treatment with H_2O_2 has been shown to induce PT-pore-dependent cell death in cardiomyocytes.[28] A sequelae and indicator of PT-pore opening is depolarization of the mitochondrial membrane. Mitochondrial membrane potential can be assessed in cardiomyocytes loaded with TMRE, using confocal microscopy and fluorescence imaging, as validated in our previous work.[6] As shown in Figure 1a treatment with 100 μM H_2O_2 lead to a dramatic decrease in TMRE fluorescence within 60 min, indicative of mitochondrial depolarization. This response was inhibited by pretreatment with EGTA or cyclosporine A (Figure 1b), consistent with involvement of elevated Ca^{2+} and PT-pore opening in H_2O_2-induced mitochondrial depolarization. The decrease in TMRE fluorescence induced by H_2O_2 was also prevented by LIF, a highly efficacious activator of Akt (Figure 1a and b). To demonstrate whether the protective effect of LIF is mediated through Akt activation we used triciribine (Akt inhibitor V), reported to block Akt activation without inhibiting PI3K or PDK-1.[29] The ability of inhibitor V to block Akt activation was first confirmed by measuring LIF-induced phosphorylation of the T308 and S473 sites on Akt, as shown in Figure 1c. Inhibitor V and the PI3K inhibitor LY 294002 (LY) were determined to block the protective effects of LIF on H_20_2-induced mitochondrial depolarization (Figure 1b). A protective effect of LIF on H_20_2-induced DNA fragmentation (assessed at 18 h) was also observed and blocked by PI3K inhibition (data not shown).

The data presented in Figure 1 provide evidence that LIF inhibits H_2O_2-induced mitochondrial depolarization through Akt activation. To more directly evaluate the role of Akt activation on mitochondrial membrane potential we used a permeabilized cell system, in which mitochondrial membrane depolarization could be induced by manipulating Ca^{2+} levels. Cardiomyocytes were first loaded with TMRE then treated with LIF or vehicle for 15 min, after which time the plasma membrane was permeabilized by addition of saponin (50 μg/ml) for 60 s. As we have previously reported, mitochondrial membrane potential is not perturbed by this saponin permeabilization protocol.[6] The extramitochondrial Ca^{2+} concentration was then raised to induce mitochondrial membrane depolarization (Figure 2). In control cells, raising Ca^{2+} to 2 μM (from the physiological resting level of 100 nM) decreased TMRE fluorescence and further elevating Ca^{2+} to 10 μM fully depolarized the mitochondria (Figure 2a). When the same experiment was performed in cells pretreated with LIF, mitochondrial depolarization induced by 2 μM Ca^{2+} was largely prevented and the Ca^{2+} curve was right shifted

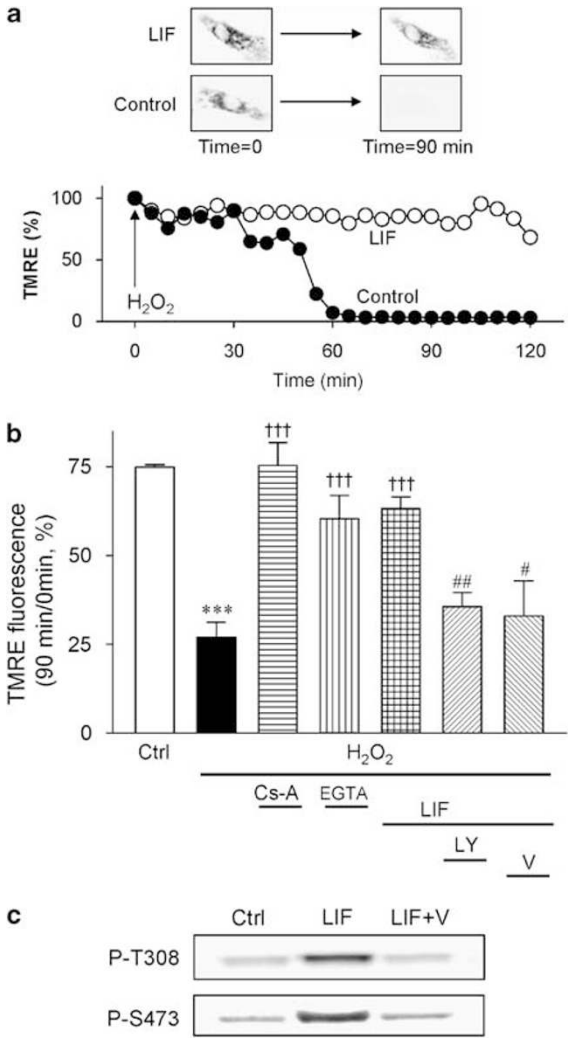

Figure 1 LIF treatment prevents H_2O_2-induced mitochondrial depolarization through a PI3K/Akt pathway. (**a** and **b**) Cardiomyocytes were loaded with 50 nM TMRE for 20 min and fluorescence was subsequently visualized by confocal microscopy. EGTA (2 mM) or cyclosporine A (1 μM) were added 30 min before H_2O_2 treatment. LIF (1 nM) was added 15 min before H_2O_2. LY294002 (LY, 10 μM) or the Akt inhibitor triciribine (inhibitor V, 3 μM) were added 30 min before LIF treatment. Values represent averages ± S.E. ($n = 50$–60 cells from four different experiments) $^{***}P < 0.001$ *versus* Ctrl; $^{\dagger\dagger\dagger}P < 0.001$ *versus* H_2O_2; $^{\#}$ and $^{\#\#}P < 0.05$, $P < 0.01$ *versus* H_2O_2 + LIF. (**c**) Akt inhibitor V (3 μM) was shown to block phosphorylation of Akt at the threonine 308 and serine 473 in response to 1 nM LIF

(Figure 2b–d). The effect of LIF was inhibited by LY or inhibitor V (Figure 2d) indicating that PI3K and Akt activation are required for LIF to protect against Ca^{2+}-induced mitochondrial depolarization.

The finding that the protective effect of LIF treatment is still evident in permeabilized cells implies that Akt activation induced a stable change at the level of the mitochondrion. We hypothesized that Akt, once activated, might localize to mitochondria and tested this by fractionating control and LIF-treated cardiomyocytes into cytosolic and mitochondrial fractions. Immunoblotting for VDAC and Rho-GDI, used as mitochondrial and cytosolic markers respectively, showed the cross contamination in these two fractions to be minimal

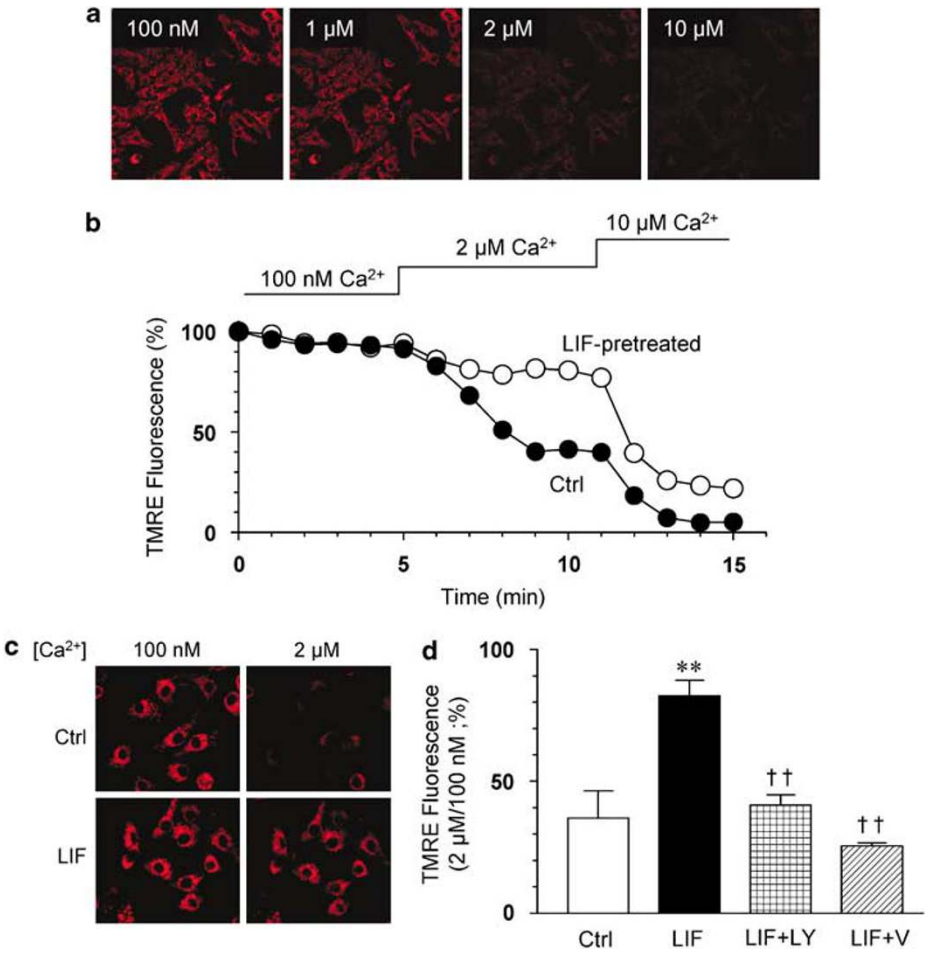

Figure 2 LIF pretreatment inhibits Ca^{2+}-induced mitochondrial depolarization in permeabilized cardiomyocytes. Cells were loaded with 50 nM TMRE for 20 min and treated with or without LIF (1 nM) for 15 min before saponin permeabilization. The plasma membrane was permeabilized by addition of saponin (50 μg/ml, 60 s). TMRE fluorescence was measured by confocal microscopy and mitochondrial depolarization was induced by raising the Ca^{2+} concentration. (**a**) Effects of raising Ca^{2+} concentration on TMRE fluorescence in permeabilized myocytes. (**b–d**) LIF pretreatment inhibits 2 μM Ca^{2+}-induced mitochondrial depolarization. In (**d**), LY294002 (LY; 10 μM) or inhibitor V (3 μM) were added 30 min before LIF treatment. $^{**}P<0.01$ *versus* Ctrl; $^{\dagger\dagger}P<0.01$ *versus* LIF. Values represent averages ± S.E. ($n=45$–60 cells from three different experiments)

(Figure 3a). Some Akt was associated with mitochondria prior to stimulation, as also demonstrated in neuroblastoma cells and HEK293 cells.[30] Most notably, however, the total Akt found in the mitochondrial fraction increased following LIF treatment (Figure 3a and b), suggesting that Akt is redistributed upon its activation. This was supported by the concomitant increase in the amount of activated (Ser 473 phosphorylated) Akt in the mitochondrial fraction following LIF treatment. The increases in total and phosphorylated-Akt in the mitochondrial fraction were blocked by inhibitor V or LY (Figure 3a and b). These results suggest that LIF-mediated Akt activation leads to its redistribution to mitochondria and to increases in mitochondrial Akt activity.

We next determined whether interactions of Akt with HK-II were elicited by LIF treatment (Figure 4). Cell lysates were immunoprecipitated with an Akt antibody and immunoblotted for hexokinase-II (HK-II). LIF treatment increased the association of Akt with HK-II and this association was prevented by PI3K inhibition (Figure 4a). Additional studies were carried out using an HK-II antibody for the immunoprecipitation. As shown in Figure 4b, both total and phosphorylated Akt association with HK-II increased following LIF treatment. These experiments provide further support for the conclusion that

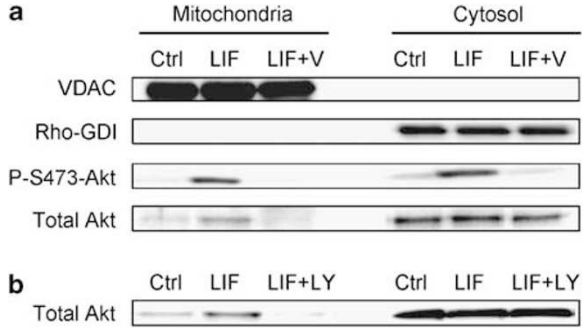

Figure 3 Akt redistributes to mitochondria upon its activation. Cells were treated with LIF (1 nM) plus or minus inhibitor V (3 μM) (**a**) or LY (10 μM) (**b**), fractionated into mitochondrial and cytosolic fractions and blotted with VDAC, Rho-GDI, phosphorylated Akt (S473) and total Akt antibodies. Rho-GDI and VDAC antibodies were used as cytosolic and mitochondrial markers

LIF induces a PI3K and Akt activation-dependent association between HK-II and Akt.

Searching the NCBI database revealed that HK-II contains an Akt phosphorylation site (consensus sequence; RXRXXS/T). This sequence, containing threonine at position 473 (Thr^{473}), is conserved in rat, mouse and human (Figure 5a). The ability of LIF to enhance the association between activated Akt and HK-II, and the presence of an Akt phosphorylation consensus sequence on HK-II, suggested that LIF might induce Akt-mediated HK-II phosphorylation. To determine whether HK-II is phosphorylated in response to Akt activation cells were treated with LIF and whole cell lysates or mitochondrial fractions prepared. HK-II was immunoprecipitated and the immunoprecipitates were subjected to Western blotting with a phospho-Akt substrate antibody (PAS antibody). This antibody recognizes the phosphorylated Akt consensus sequence and has been widely utilized to detect proteins phosphorylated by Akt.[30,31] PAS antibody staining revealed increases in HK-II phosphorylation in both the whole cell lysate and mitochondrial fraction following LIF treatment (Figure 5b). These increases in HK-II phosphorylation were also shown to be blocked by PI3K (LY) or Akt (V) inhibition (Figure 5b).

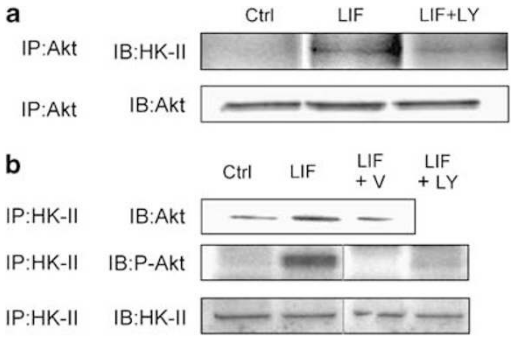

Figure 4 Interaction of Akt with HK-II is increased by LIF treatment. Cardiomyocytes were treated with LIF (1 nM) for 10 min. Inhibitor V (3 μM) or LY (10 μM) were added 30 min before LIF treatment. Whole cell lysates were immunoprecipitated with an Akt antibody (**a**) or a HK-II antibody (**b**), and subjected to Western blotting with antibodies to HK-II, Akt or phosphorylated Akt (S473)

A series of studies was then carried out using cardiac mitochondria from adult mouse heart. Mitochondria were isolated as described in Materials and Methods and HK-II was immunoprecipitated from the isolated mitochondria. The HK-II immunoprecipitate was then subjected to *in vitro* phosphorylation using recombinant kinase-active Akt (ΔPH, S473D) or wild type Akt. The kinase reactions were carried out in the presence or absence of Akt and ATP, and HK-II phosphorylation assessed by Western blotting with PAS antibody. As shown in Figure 6a, recombinant kinase-active Akt catalyzed a marked ATP-dependent increase in phosphorylated HK-II. Wild-type Akt was, as expected, inactive in this cell free assay. Similarly HK-II phosphorylation was detected when Akt treatment was carried out under the conditions used to examine Ca^{2+}-induced cytochrome *c* release (Figure 6b and c). Cytochrome *c* release from isolated mitochondria, assessed as the increase in cytochrome *c* in the supernatant and decrease in the mitochondrial pellet, was induced by raising the extramitochondrial Ca^{2+} concentration to 30 μM (Figure 6c). Calcium-induced cytochrome *c* release was inhibited not only by cyclosporine-A (Cs-A), a PT-pore inhibitor, but also by pretreatment of the isolated mitochondria with recombinant kinase-active Akt and ATP (Figure 6c). Since Cs-A sensitive Ca^{2+}-induced cytochrome *c* release from isolated mitochondria is established to result from PT-pore opening, these data provide additional support for the conclusion that Akt exerts a direct protective effect at the level of the mitochondrial PT pore through HK-II phosphorylation.

To address the question of whether mitochondrial HK-II is required for Akt-mediated inhibition of cytochrome *c* release, mitochondria were treated with either glucose 6-phosphate (G6P) or an HK-II-dissociating peptide. G6P has been well documented to release HK-II from mitochondria.[32] The HK-II-dissociating peptide was designed based on the mitochondrial-binding motif in the hydrophobic N terminus of HK-II, which is critical for mitochondrial binding. This peptide has been

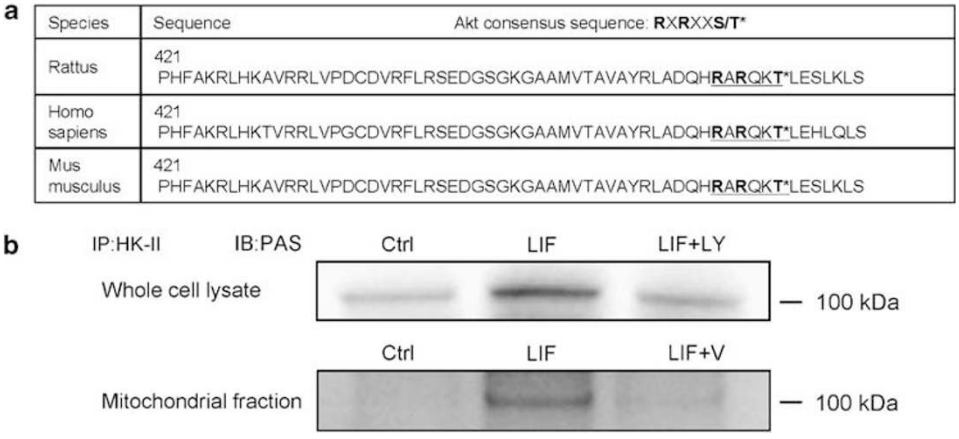

Figure 5 HK-II contains a consensus sequence for Akt-mediated phosphorylation and is phosphorylated by Akt. (**a**) The Akt phosphorylation consensus sequence is preserved in rat, mouse and human. (**b**) Cardiomyocytes were treated for 10 min with LIF (1 nM) plus or minus inhibitor V (3 μM) or LY (10 μM), added 30 min prior to LIF. Whole cell lysates or mitochondrial fractions were prepared. HK-II was immunoprecipitated and immunoprecipitates were subjected to Western blot with a Phospho-Akt Substrate (PAS) antibody, which recognizes phosphorylated Akt consensus sequence

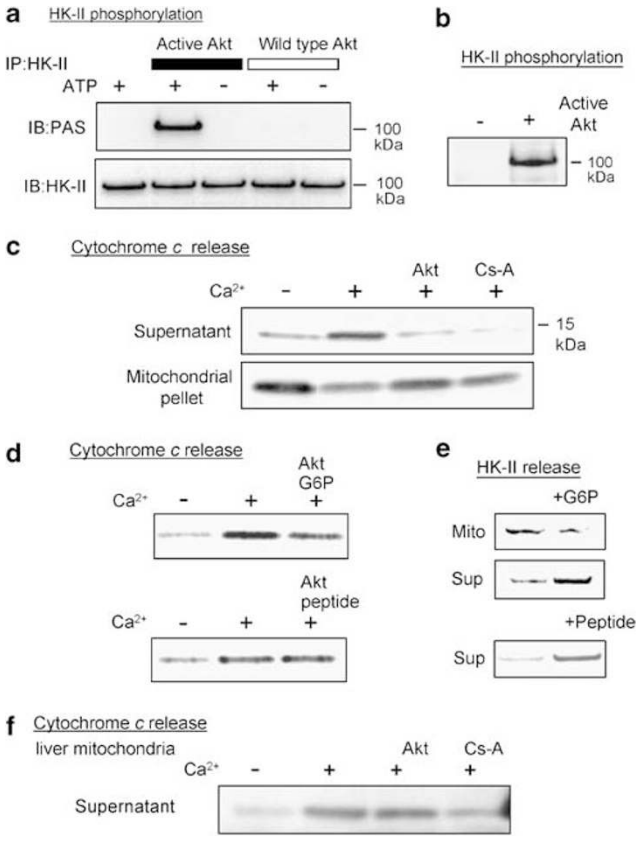

Figure 6 Recombinant Akt phosphorylates HK-II and inhibits Ca^{2+}-induced cytochrome *c* release in isolated mitochondria from adult mouse heart. (**a**) Mitochondria were isolated from adult mouse heart and HK-II was immunoprecipitated. Recombinant kinase active Akt (ΔPH, S473D) or wild type Akt were added to immunoprecipitated HK-II in the presence or absence of ATP (see Materials and Methods). After 15 min, samples were centrifuged, resuspended in 2 × LDS running buffer and boiled for 5 min. SDS gels were probed with PAS antibody or with an antibody to HK-II. (**b**) Mitochondria were isolated from adult mouse heart and resuspended in intracellular buffer (see Materials and Methods). Recombinant kinase active Akt was treated for 10 min and mitochondria lysed in RIPA buffer. HK-II was immunoprecipitated and its phosphorylation was assessed with PAS antibody. (**c**) Mitochondria were resuspended in intracellular buffer and recombinant kinase active Akt or Cyclosporine-A (Cs-A) were added for 10 min. Mitochondria were resuspended in buffer containing 30 μM Ca^{2+} to induce cytochrome *c* release. Mitochondrial and supernatant fractions were then collected and subjected to Western blotting with cytochrome *c* antibody. (**d** and **e**) Mitochondria were treated with glucose 6-phosphote (G6P, 2 mM) or HK-II-dissociating peptide (Genemed Synthesis Inc. 50 μM) for 20 min, collected, treated with recombinant active Akt and cytochrome *c* release was induced by Ca^{2+}. (**f**) Mitochondria were isolated from liver and cytochrome *c* release was induced as described in (**c**)

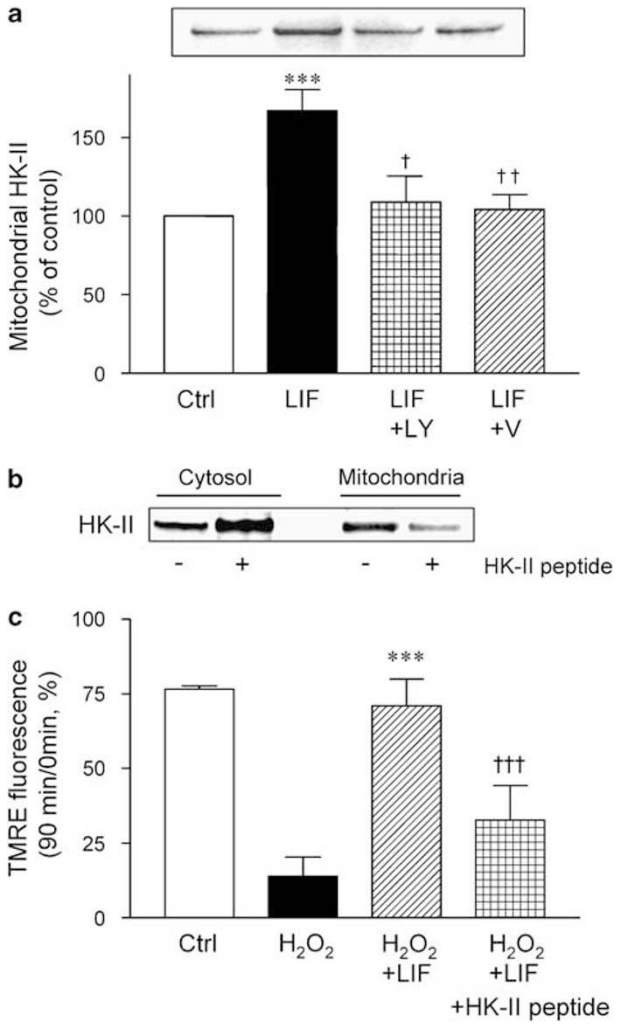

Figure 7 HK-II association with mitochondria is increased by LIF/Akt and required for LIF-induced mitochondrial protection. (**a**) Cardiomyocytes were treated with LIF (1 nM) plus or minus inhibitor V (3 μM) or LY (10 μM). HK-II in the mitochondrial fraction was detected by Western blotting. Values represent averages ± S.E. ($n = 3$–6). ***$P < 0.001$ *versus* Ctrl; † and $^{\dagger\dagger}P < 0.05$, $P < 0.01$ *versus* LIF. (**b**) Cardiomyocytes were treated with a cell-permeable HK-II-dissociating peptide (Genemed Synthesis Inc.) (25 μM), fractionated into cytosol and mitochondria fractions and subjected to Western blotting for HK-II. (**c**) Cardiomyocytes were loaded with TMRE and mitochondrial depolarization was induced by addition of 100 μM H_2O_2 (see Figure 1 legend). Some cells were treated with 25 μM HK-II-dissociating peptide for 30 min prior to addition of LIF (1 nM). Values represent averages ± S.E. ($n = 40$–50 cells from three different experiments). ***$P < 0.001$ *versus* H_2O_2; $^{\dagger\dagger\dagger}P < 0.001$ *versus* H_2O_2 + LIF

previously shown to force dissociation of HK-II from mitochondria in a presumably competitive manner.[23,27] As shown in Figure 6d and e, both interventions caused HK-II dissociation from isolated mitochondria and the effect of subsequent addition of recombinant kinase active Akt on Ca^{2+}-induced cytochrome *c* release was markedly diminished, supporting a critical role for mitochondrial HK-II in Akt-mediated protection. This conclusion was further supported by experiments demonstrating that Akt does not show protective effects when added to liver mitochondria (Figure 6f), which lack mitochondrial-associated HK-II.[33]

To determine whether the protective effect of Akt activation is associated with increases in the amount of HK-II associated with mitochondria, neonatal rat ventricular myocytes were stimulated with LIF and fractionated. LIF treatment significantly increased mitochondrial-associated HK-II and this effect was blocked by PI3K or Akt inhibitors (Figure 7a). Previous reports have shown that the enzymatic activity of HK-II at mitochondria is increased following Akt activation.[25,27] Our studies support these findings by demonstrating increases in the amount of HK-II associated with mitochondria upon LIF treatment of cardiomyocytes. To demonstrate that

HK-II is necessary for LIF/Akt-mediated mitochondrial protection in intact cells, NRVMs were treated with a cell permeable form of the HK-II-dissociating peptide. Mitochondrial HK-II was decreased by peptide treatment, as shown in Figure 7b. Treatment of cells with the inhibitory peptide for up to 90 min did not affect the mitochondrial membrane potential (data not shown). However, in cells pretreated with the inhibitory peptide, the ability of LIF to protect against H_2O_2-induced mitochondrial depolarization was markedly attenuated (Figure 7c). These data implicate mitochondrial-associated HK-II as a mediator of the protective effects of LIF and Akt in cardiomyocytes.

Discussion

Although it is now well accepted that Akt provides a strong survival signal in many cell types, the mechanisms for Akt-mediated protection have not been fully elucidated. Recent studies have suggested the importance of mitochondrial HK-II in preservation of mitochondrial integrity.[24,27] Here we demonstrate for the first time that Akt associates with mitochondria upon its activation and that it binds to and phosphorylates HK-II, concomitantly protecting from Ca^{2+}-or H_2O_2-induced mitochondrial depolarization and cytochrome *c* release.

It is well established that H_2O_2 can induce mitochondrial Ca^{2+} overloading, which results in opening of the PT-pore, mitochondrial depolarization and cytochrome *c* release in cardiomyocytes.[28] We confirmed a role for Ca^{2+} in H_2O_2-induced cell death in our experiments since chelating Ca^{2+} with EGTA inhibited H_2O_2-induced mitochondrial depolarization (Figure 1) and DNA fragmentation (not shown). Cyclosporin-A, a PT-pore inhibitor, blocked mitochondrial depolarization induced by H_2O_2, confirming that Ca^{2+} and opening of the PT-pore are responsible for the depolarization of mitochondria induced by H_2O_2. Our finding that LIF treatment inhibits mitochondrial depolarization elicited by H_2O_2 (Figure 1) supports the protective role of agonists that activate Akt. With regard to mechanism, we postulated that the mitochondrial PT-pore was a direct target for LIF/Akt-mediated protection. This was supported by data showing that treatment with LIF inhibits mitochondrial depolarization induced by H_2O_2 (Figure 1) or induced by elevating Ca^{2+} in permeabilized cardiomyocytes (Figure 2). The observation that LIF-mediated Akt activation has a protective effect on mitochondria, which is preserved after cell permeabilization, suggests that Akt elicits stable changes in mitochondrial function, which result in decreased sensitivity of the PT-pore to Ca^{2+}. This possibility was supported by the observation that addition of recombinant active Akt to isolated mitochondria inhibited Ca^{2+}-induced cytochrome *c* release (Figure 6). Mitochondrial Ca^{2+} overloading and resultant PT-pore opening have been suggested to play a major role in ischemia/reperfusion injury in the heart. Our results imply that Akt can confer cardioprotection against ischemia/reperfusion by directly inhibiting Ca^{2+}-induced PT-pore opening.

In the unstimulated cell Akt is largely localized in the cytoplasmic compartment. In response to receptor-mediated PIP_3 formation, Akt translocates to the plasma membrane[34] where it is phosphorylated and activated. We demonstrate here that Akt activation by LIF/PI3K increases its association with the mitochondrial cell fraction. That the total mitochondrial Akt content is increased after 10 min of LIF treatment indicates that there is redistribution of Akt, although a concomitant decrease in Akt in the larger cytosolic pool cannot be easily detected (Figure 3). There is also a marked increase in the amount of phosphorylated Akt at the mitochondria, suggesting that following its activation at the plasma membrane, Akt redistributes to mitochondria. Furthermore, immunoprecipitation studies show that LIF treatment increases the amount of Akt associated with HK-II (Figure 4).

A previous study using neuroblastoma and HEK293 cells reported that there is Akt localized in mitochondria under resting condition and that mitochondrial Akt increases dramatically in response to stimulation with insulin.[30] Our fractionation studies also show a low basal level of Akt in cardiomyocyte mitochondria (although cytosolic contamination cannot be ruled out) and most remarkably that mitochondrial Akt is increased with LIF treatment. Prior evidence has suggested that the nucleus is a target for activated Akt. Thus in cardiomyocytes 17-β estradiol treatment was found to increase nuclear Akt and transgenic overexpression of nuclear-targeted Akt has been shown to protect the heart against ischemia/reperfusion injury through activation of transcriptional targets.[10] A study using a FRET-based Akt activity reporter in NIH3T3 cells clearly shows that Akt activated at the plasma membrane translocates to other compartments including the nucleus.[34] It appears, therefore, that activated Akt can translocate to multiple cellular compartments, including mitochondria and the nucleus, to afford acute and chronic protective effects on cells.

In considering the possibility that the association of Akt with mitochondrial proteins could reflect their role as Akt substrates, we searched the NCBI database for consensus phosphorylation sites. This analysis revealed that HK-II contains an Akt phosphorylation consensus sequence (RxRxxS/T) (Thr^{473}). Results presented here demonstrate that Akt activation by LIF leads to phosphorylation of HK-II in intact cardiomyocytes, as detected using the PAS antibody directed against Akt phosphorylation consensus sequences (Figure 5). Furthermore, we demonstrated that recombinant kinase active Akt (ΔPH, S473D) phosphorylates HK-II immunoprecipitated from isolated mitochondria (Figure 6a and b). This is, to our knowledge, the first experimental evidence that HK-II serves as a substrate for phosphorylation by Akt.

The ability of elevated Ca^{2+} (30 μM) to induce cytochrome *c* release from isolated mitochondria is inhibited by cyclosporine-A (Figure 6c), an established PT-pore inhibitor, as previously shown. Strikingly, pretreatment of mitochondria with recombinant kinase-active Akt not only leads to HK-II phosphorylation but also, like Cs-A, significantly inhibits Ca^{2+}-induced cytochrome *c* release (Figure 6c). The finding that recombinant Akt added to isolated mitochondria can inhibit Ca^{2+}-induced cytochrome *c* release, a sequelae of opening of the PT-pore, indicates a direct effect of Akt on PT-pore function.

Several recent seminal studies have established that Akt increases mitochondrial HK-II kinase activity and that mitochondrial HK-II protects against apoptotic Bcl-2 proteins such as Bax and Bak.[23,25,27] How Akt affects HK-II function, and

whether mitochondrial HK-II regulates the PT-pore are questions that have not been hitherto addressed. A recently published study demonstrated that Akt and HK-II were not protective against apoptosis induced by thapsigargin.[27] In contrast, ectopic HK-I has been reported to prevent Ca^{2+}-induced mitochondrial swelling and cytochrome *c* release from isolated liver mitochondria,[22] consistent with a role for HK-I in preventing PT-pore opening induced by Ca^{2+}. In this study, we show that dissociation of HK-II from mitochondria, induced by either G6P or a peptide that blocks the HK-II mitochondrial interaction, attenuates the protective effect of Akt on Ca^{2+}-induced cytochrome *c* release (Figure 6d). We also demonstrate that Akt is unable to prevent cytochrome *c* release from mitochondria purified from liver (Figure 6f), a population of mitochondria that lack HK-II.[33] Finally, we demonstrate dependence on HK-II in living cardiomyocytes in which a cell permeable HK-II-dissociating peptide markedly attenuates the ability of LIF/Akt activation to inhibit mitochondrial depolarization induced by H_2O_2. (Figure 7). Thus HK-II appears to be a critical target in Akt-mediated inhibition of mitochondrial damage.

As mentioned above, previous studies have shown that mitochondrial HK-II activity is increased in cells treated with agonists known to activate Akt.[25,27,35] Our experiments reveal that the total amount of HK-II found in the mitochondrial fraction is increased by Akt activation (Figure 7a). The current study also shows that activated Akt redistributes to mitochondria and that it associates with and phosphorylates mitochondrial HK-II. While this suggests that Akt phosphorylates HK-II in the mitochondria and decreases its dissociation, it is also possible that HK-II phosphorylation by Akt occurs in the cytoplasm and that this facilitates HK-II association with mitochondria. Indeed, as shown in Figure 5, LIF treatment increased the phosphorylation of HK-II in whole cell lysate as well as in mitochondria, suggesting that cytosolic HK-II can be phosphorylated by Akt. Interestingly ischemic preconditioning, as well as insulin and morphine (interventions known to activate Akt) were recently reported to increase mitochondrial hexokinase activity in the heart.[35] Thus it will be of interest to determine in future studies whether HK-II phosphorylation by Akt leads to changes in the avidity with which HK-II associates with cardiac mitochondria.

VDAC has been suggested to serve as the mitochondrial binding site for HK-II[16,17,36] and a recent report has shown that mitochondrial HK-II is significantly decreased in VDAC-1 knockout mice.[36] We observed that immunoprecipitated HK-II was associated with VDAC (data not shown) consistent with VDAC involvement in binding HK-II in cardiac mitochondria. VDAC was also associated with immunoprecipitated Akt; however, unlike HK-II, VDAC did not appear to serve as a substrate for phosphorylation by Akt (data not shown). We interpret these data as suggesting that Akt binds to its substrate HK-II, which is associated with VDAC at mitochondria. VDAC may be an effector of the protective function of HK-II, sensitive to its Akt-mediated phosphorylation state. However, while VDAC has been implicated by many previous studies as a component of the PT-pore,[15–17] recent genetic evidence brings its involvement into question.[18,21] Indeed, our understanding of the molecular composition of the PT-pore is rapidly evolving since the role of ANT has also been questioned.[18] Interestingly, Cyp D, which genetic evidence shows to be critical for PT-pore induction,[19,20] was recently reported to suppress apoptosis via stabilization of HK-II binding to mitochondria.[26] While our data demonstrate that mitochondrial HK-II is a key inhibitor of PT-pore (Ca^{2+}-mediated and Cs-A sensitive) induced cytochrome *c* release, and previous papers show that overexpression of HK is protective against stress.[37,38] Much additional study will be required to elucidate the mechanisms by which HK-II keeps PT pore opening in check.

The heart is enriched in mitochondria. The idea that mitochondrial death pathways are involved in cardiomyocyte loss in the heart, and that cell death plays a critical role in ischemic injury and the transition from compensatory hypertrophy to maladaptive heart failure, is well accepted. The studies presented here delineate a mechanism by which Akt can acutely protect cardiomyocytes against cell death. Our findings indicate that activated Akt translocates to mitochondria, phosphorylates HK-II and inhibits mitochondrial PT-pore opening in cardiomyocytes. We suggest that this protective pathway play a critical role in Akt-mediated protection of the heart against oxidative stress and Ca^{2+} overload.

Materials and Methods

Cell culture. Neonatal rat ventricular myocytes were isolated and cultured as described previously.[1] Briefly, hearts were obtained from 1–2-day-old Sprague–Dawley rat pups, digested with collagenase, and myocytes purified by passage through a Percoll gradient. Myocytes were maintained overnight in 4 : 1 Dulbecco's modified Eagle's medium/medium199, supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were then serum starved for 24 h prior to intervention.

Confocal imaging of mitochondrial membrane potential. Cells were loaded with 50 nM TMRE for 20 min at room temperature in the medium composed of 121 mM NaCl, 5 mM $NaHCO_3$, 10 mM Na Hepes, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 1.8 mM $CaCl_2$ and 10 mM glucose (pH = 7.4). Confocal imaging was carried out using a Bio-Rad MRC1024ES imaging system as described and validated previously.[6] TMRE was excited at 568 nm and fluorescence was recorded using a photomultiplier and emission filter of HQ598/40. Fluorescence values were background-subtracted and expressed as a relative value ($100F/F_0$, where F is the measured fluorescence and F_0 is that at the beginning of the experiment). Experiments were analyzed using Scion Image (Scion Corporation). Data were collected from 40–60 cells from at least three different experiments and averaged. Mitochondrial regions were chosen as the 'area of interest'. Changes in fluorescence in these areas were evaluated to determine mitochondrial membrane potential.

Ca^{2+} induced loss of mitochondrial membrane potential in permeabilized cells. Cells were loaded with TMRE and permeabilized with saponin (50 μg/ml, 60 s) as described previously.[6] An intracellular buffer containing magnesium methansulphonate (5.25 mM), potassium methansulphonate (40 mM), potassium chloride (75 mM), ATP (5.3 mM), PIPES (20 mM), E-64 (1 mg/ml), and EGTA (10 mM). Thapsigargin (1 μM) was also added to block sarcoplasmic reticulum function and succinate (5 mM) was added to energize mitochondria. The ionic strength was adjusted to 0.2 M, and the pH was adjusted to 7.1 at 20°C. Free Ca^{2+} concentration was changed by adding an appropriate amount of Ca^{2+}-methansulphonate.[6,39]

Western blotting and immunoprecipitation. Myocytes were washed with ice-cold PBS and lysed with lysis buffer (RIPA buffer; composed of 150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP-40, 1% of sodium deoxycholate, 0.1% of SDS, 0.2 mM EDTA, 50 mM NaF, 1 μM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM PNPP and 1 mM PMSF). Invitrogen NuPage system was used for Western blotting. Cell lysates were mixed with LDS sample buffer and reducing agent, heated at 80°C for 10 min and loaded into gels. Gels were run on ice and proteins

were transferred to PVDF membranes (Millipore). Total and phosphorylated Akt antibodies (Cell Signaling technology, rabbit, polyclonal), PAS antibody (Cell Signaling technology, rabbit, polyclonal), HK-II (Santa Cruz biotechnology, rabbit, polyclonal), VDAC (Calbiochem, rabbit, polyclonal), cytochrome *c* (BD Pharmingen, mouse, monoclonal) and rho-GDI (BD transduction laboratories, mouse, monoclonal) were all used at 1 : 1000 dilution in 5% BSA/TBS tween.

Cell lysates were precleared with Protein G-Sepharose for 30 min at 4°C and 800 μg of total protein was then incubated with 20 μl of Akt-beads (Millipore, mouse, monoclonal antibody conjugated to protein G agarose) or 4 μg HK-II antibody (Santa Cruz, goat, polyclonal) in the presence of 30 μl of 50% slurry Protein G-Sepharose, at 4°C overnight. Immunocomplexes were washed with ice-cold RIPA buffer, and beads were boiled in 2 $\times$ LDS buffer to elute captured protein.

Cytosol/mitochondria fractionation. Cytosolic and mitochondrial fractions were prepared from neonatal rat ventricular myocytes as described previously[40] (Calbiochem, Cytosol/Mitochondria Fractionation Kit). Briefly, cells were collected in ice cold PBS, spun down, resuspended in Cytosol Extraction Buffer Mix, vortexed and incubated on ice for 10 min. Samples were centrifuged at 2600 r.p.m. (700 $\times$ *g*) for 10 min and supernatant transferred and spun at 9600 r.p.m. (10 000 $\times$ *g*) for 30 min to precipitate mitochondria. Supernatant was removed as the cytosolic fraction and the pellet was resuspended in Mitochondrial Extraction Buffer Mix as the mitochondrial fraction.

Isolation of mitochondria. Hearts were removed from adult mice (Charles River, C57, male 2–4 months old) and perfused with PBS at room temperature for 5 min to remove blood. The ventricle was homogenized in isolation buffer containing 70 mM sucrose, 190 mM mannitol, 20 mM Hepes and 0.2 mM EDTA. For immunoprecipitation experiments, the isolation buffer also contained 1 μM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM PNPP and 1 mM PMSF. The homogenate was centrifuged at 600 $\times$ *g* for 10 min to remove nuclei and debris. The resulting supernatant was then centrifuged at 5000 $\times$ *g* for 15 min. The resulting mitochondrial pellet was resuspended in 500 μl isolation buffer and centrifuged three times at 5000 $\times$ *g* for 15 min, to wash the pellet. Liver was homogenized in buffer containing 250 mM sucrose, 10 mM Tris (pH 7.4) and 1 mM EGTA. The homogenate was centrifuged at 1000 $\times$ *g* for 5 min and the resultant supernatant was centrifuged at 13 000 $\times$ *g* for 10 min. The pellet was washed twice and resuspended.

Functional studies on mitochondria. To examine mitochondrial HK-II phosphorylation, the pellet was resuspended in RIPA buffer, HK-II immunoprecipitated, and the immunoprecipitate resuspended in kinase reaction buffer[34] containing 20 mM Hepes, 2 mM DTT and 5 mM $MgCl_2$ with or without 200 μM ATP and recombinant Akt (Upstate). Incubations were carried out at 30°C for 15 min, spun down, resuspended in 2 $\times$ LDS running buffer (Invitrogen) and boiled for 5 min. Western blots used PAS antibody (Cell Signaling).

For cytochrome *c* release experiments, the mitochondrial pellet was re-suspended in the same intracellular buffer used in cell permeabilization experiments (see above) (50 μg mitochondria in 50 μl buffer), incubated with or without recombinant Akt or cyclosporine-A for 10 min and centrifuged to collect mitochondria. The pellet was resuspended in the same buffer containing either 0 ($<$10 nM) or 30 μM Ca^{2+}, and incubated for 20 min at room temperature. After centrifugation, the supernatant and pellet were run on SDS gels and blotted with a cytochrome *c* antibody. To dissociate HK-II,[23,32] glucose 6-phosphate (G6P) (2 mM) or HK-II-dissociating peptide (50 μM) were added to mitochondria for 20 min at room temperature prior to Akt treatment.

Statistical analysis. Results are reported as averages $\pm$ S.E.M. Statistical significance was determined using ANOVA followed by the Tukey *post hoc* test. A *P*-value of <0.05 was considered statistically significant.

Acknowledgements. Technical assistance was provided by Jada Wang Hu and Jeffrey Smith. This work was supported by National Institutes of Health Grant HL28143 and HL085577 (to JHB) and American Heart Association Beginning Western Affiliate Grant-in-Aid 0565093Y (to SM).

1. Adams JW, Pagel AL, Means CK, Oksenberg D, Armstrong RC, Brown JH. Cardiomyocyte apoptosis induced by Gαq signaling is mediated by permeability transition pore formation and activation of the mitochondrial death pathway. *Circ Res* 2000; **87**: 1180–1187.
2. Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ Res* 2003; **93**: 292–301.
3. Matsui T, Li L, del Monte F, Fukui Y, Franke TF, Hajjar RJ *et al.* Adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes *in vitro*. *Circulation* 1999; **100**: 2373–2379.
4. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000; **101**: 660–667.
5. Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M *et al.* Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia *in vivo*. *Circulation* 2001; **104**: 330–335.
6. Miyamoto S, Howes AL, Adams JW, Dorn II GW, Brown JH. Ca^{2+} dysregulation induces mitochondrial depolarization and apoptosis: role of Na^+/Ca^{2+} exchanger and AKT. *J Biol Chem* 2005; **280**: 38505–38512.
7. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. *J Biol Chem* 1997; **272**: 5783–5791.
8. Craig R, Wagner M, McCardle T, Craig AG, Glembotski CC. The cytoprotective effects of the glycoprotein 130 receptor-coupled cytokine, cardiotrophin-1, require activation of NF-κB. *J Biol Chem* 2001; **276**: 37621–37629.
9. Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I *et al.* Sphingosine 1-phosphate $S1P_2$ and $S1P_3$ receptor-mediated Akt activation protects against *in vivo* myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2007; **292**: H2944–2951.
10. Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S *et al.* Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res* 2004; **94**: 884–891.
11. Jonassen AK, Sack MN, Mjøs OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 2001; **89**: 1191–1198.
12. del Peso L, González-García M, Page C, Herrera R, Nuñez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997; **278**: 687–689.
13. Pastorino JG, Hoek JB, Shulga N. Activation of glycogen synthase kinase 3β disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. *Cancer Res* 2005; **65**: 10545–10554.
14. Gustafsson AB, Gottlieb RA. Mechanisms of apoptosis in the heart. *J Clin Immunol* 2003; **23**: 447–459.
15. Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* 2004; **95**: 957–970.
16. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003; **4**: 552–565.
17. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; **305**: 626–629.
18. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP *et al.* The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 2004; **427**: 461–465.
19. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H *et al.* Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005; **434**: 652–658.
20. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA *et al.* Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 2005; **434**: 658–662.
21. Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 2007; **9**: 550–555.
22. Azoulay-Zohar H, Israelson A, Abu-Hamad S, Shoshan-Barmatz V. In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J* 2004; **377**: 347–355.
23. Pastorino JG, Shulga N, Hoek JB. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome *c* release and apoptosis. *J Biol Chem* 2002; **277**: 7610–7618.
24. Pastorino JG, Hoek JB. Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr Med Chem* 2003; **10**: 1535–1551.
25. Majewski N, Nogueira V, Robey RB, Hay N. Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. *Mol Cell Biol* 2004; **24**: 730–740.
26. Machida K, Ohta Y, Osada H. Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. *J Biol Chem* 2006; **281**: 14314–14320.
27. Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K *et al.* Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell* 2004; **16**: 819–830.
28. Akao M, O'Rourke B, Teshima Y, Seharaseyon J, Marbán E. Mechanistically distinct steps in the mitochondrial death pathway triggered by oxidative stress in cardiac myocytes. *Circ Res* 2003; **92**: 186–194.
29. Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI *et al.* Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res* 2004; **64**: 4394–4399.

30. Bijur GN, Jope RS. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *J Neurochem* 2003; **87**: 1427–1435.
31. Bruss MD, Arias EB, Lienhard GE, Cartee GD. Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 2005; **54**: 41–50.
32. Kabir F, Wilson JE. Mitochondrial hexokinase in brain of various species: differences in sensitivity to solubilization by glucose 6-phosphate. *Arch Biochem Biophys* 1993; **300**: 641–650.
33. Wilson JE, Felgner PL. An inverse relation between mitochondrial hexokinase content and phosphoglucomutase activity of rat tissues. *Mol Cell Biochem* 1977; **18**: 39–47.
34. Kunkel MT, Ni Q, Tsien RY, Zhang J, Newton AC. Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically-encoded fluorescent reporter. *J Biol Chem* 2004: **280**: 5581–5587.
35. Zuurbier CJ, Eerbeek O, Meijer AJ. Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution. *Am J Physiol Heart Circ Physiol* 2005; **289**: H496–499.
36. Anflous-Pharayra K, Cai ZJ, Craigen WJ. VDAC1 serves as a mitochondrial binding site for hexokinase in oxidative muscles. *Biochim Biophys Acta* 2007; **1767**: 136–142.
37. Bryson JM, Coy PE, Gottlob K, Hay N, Robey RB. Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death. *J Biol Chem* 2002; **277**: 11392–11400.
38. Ahmad A, Ahmad S, Schneider BK, Allen CB, Chang LY, White CW. Elevated expression of hexokinase II protects human lung epithelial-like A549 cells against oxidative injury. *Am J Physiol Lung Cell Mol Physiol* 2002; **283**: L573–584.
39. Miyamoto S, Izumi M, Hori M, Kobayashi M, Ozaki H, Karaki H. Xestospongin C, a selective and membrane-permeable inhibitor of IP_3 receptor, attenuates the positive inotropic effect of α-adrenergic stimulation in guinea-pig papillary muscle. *Br J Pharmacol* 2000; **130**: 650–654.
40. Del Re DP, Miyamoto S, Brown JH. RhoA/Rho kinase upregulate bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J Biol Chem* 2007; **282**: 8069–8078.