Akt protects the heart against ischaemia-reperfusion injury by modulating mitochondrial morphology

Sang-Bing Ong1; Andrew R. Hall1; Rachel K. Dongworth1; Siavash Kalkhoran1; Aswin Pyakurel2; Luca Scorrano2; Derek J. Hausenloy1

1The Hatter Cardiovascular Institute, University College London Hospital, London, UK; 2Dulbecco-Telethon Institute, Venetian Institute of Molecular Medicine, Padua, Italy

Abstract

The mechanism through which the protein kinase Akt (also called PKB), protects the heart against acute ischaemia-reperfusion injury (IRI) is not clear. Here, we investigate whether Akt mediates its cardioprotective effect by modulating mitochondrial morphology. Transfection of HL-1 cardiac cells with constitutively active Akt (caAkt) changed mitochondrial morphology as evidenced by an increase in the proportion of cells displaying predominantly elongated mitochondria (73 ± 5.0 % caAkt vs 49 ± 5.8 % control; N=80 cells/group; p<0.05). This effect was associated with delayed time taken to induce mitochondrial permeability transition pore (MPTP) opening (by 2.4 ± 0.5 fold; N=80 cells/group; p<0.05); and reduced cell death following simulated IRI (32.8 ± 1.2 % caAkt vs 63.8 ± 5.6 % control; N=320 cells/group; p<0.05). Similar effects on mitochondrial morphology, MPTP opening, and cell survival post-IRI, were demonstrated with pharmacological activation of Akt using the known cardioprotective cytokine, erythropoietin (EPO). The effect of Akt on inducing mitochondrial elongation was found to be dependent on the mitochondrial fusion protein, Mitofusin-1 (Mfn1), as ablation of Mfn1 in mouse embryonic fibroblasts (MEFs) abrogated Akt-mediated mitochondrial elongation. Finally, in vivo pre-treatment with EPO reduced myocardial infarct size (as a % of the area at risk) in adult mice subjected to IRI (26.2 ± 2.6 % with EPO vs 46.1 ± 6.5 % in control; N=7/group; p<0.05), and reduced the proportion of cells displaying myofibrillar disarray and mitochondrial fragmentation observed by electron microscopy in adult murine hearts subjected to ischaemia from 5.8 ± 1.0 % to 2.2 ± 1.0 % (N=5 hearts/group; p<0.05). In conclusion, we found that either genetic or pharmacological activation of Akt protected the heart against acute ischaemia-reperfusion injury by modulating mitochondrial morphology.

Keywords

Ischaemia, reperfusion, myocardial infarction, mitochondrial morphology, Akt

Introduction

It is well recognised that the acute activation of Akt (also known as protein kinase B), whether it be by a genetic or pharmacological strategy, can protect the heart against the detrimental effects of acute ischaemia-reperfusion injury (IRI) (1–4). Furthermore, the PI3K–Akt cascade has been reported to convey the cardioprotective signal from the cell surface to the mitochondria in the setting of both ischaemic preconditioning (in which one or more cycles of brief non-lethal ischaemia and reperfusion confer protection against a lethal sustained episode of IRI) (5–8) and ischaemic post-conditioning (in which myocardial reperfusion is interrupted by short-lived episodes of ischaemia) (9, 10). However, the actual mechanism through which Akt mediates its cardioprotective effect is not fully understood, although it has been suggested that it may involve the inhibition of the mitochondrial permeability transition pore (MPTP) (11, 12), a critical mediator of cardiomyocyte death in the setting of acute IRI (13, 14).

Recently, it has been demonstrated that changes in mitochondrial morphology may influence MPTP opening susceptibility and myocardial sensitivity to acute IRI (15–17). In this regard, mitochondria have been demonstrated to undergo fission during acute IRI, thereby generating fragmented mitochondria. Interestingly, genetic or pharmacological inhibition of this process has been reported to inhibit MPTP opening and reduce myocardial infarction (MI) size (15–17), suggesting that the occurrence of mitochondrial...
fission in response to acute IRI is detrimental to mitochondrial function and cell viability. Therefore, in the current study we investigated whether Akt activation protects the myocardium against acute IRI by modulating mitochondrial morphology.

Methods

Cell transfection

HL-1 cardiac cells (derived from murine atrial cardiomyocytes) were cultured according to a previously published method (18). Wild-type and Mitofusin (Mfn) 1- or 2-deficient mouse embryonic fibroblasts (MEFs) were used as previously described (19). Using Fugene 6 (Roche Molecular Biochemicals, Basel, Switzerland), HL-1 cells or MEFs were transfected with plasmids expressing mtRFP (matrix targeted red fluorescent protein), caAkt (the constitutively active form of Akt), Akt- AA (the dominant negative mutant form of Akt), Mfn1, or Mfn2 (20, 21).

Analysis of mitochondrial morphology

Mitochondrial morphology was determined in cells co-transfected with 1) mtRFP and caAkt or Akt-AA (for HL-1 cells) or 2) mtRFP and caAkt or Mfn1 or Mfn2 (for MEFs), using a Leica TCS SP5 confocal microscope equipped with 63× oil immersion objective (Plan Apochromat, NA 1.3). Eighty randomly chosen transfected cells per treatment group (N=4 independent experiments with 20 cells per experiment) were designated as containing either predominantly (>50 %) elongated or predominantly (>50 %) fragmented mitochondria by three investigators blinded to the treatment (Figure 1 A and B for representative examples of mitochondrial elongation and fragmentation) (15, 20).

Analysis of cell death following simulated IRI in HL-1 cells

In order to determine the effect of caAkt or Akt-AA on the susceptibility to simulated IRI, HL-1 cells were subjected to 12 hours (h) simulated ischaemia in an air-tight hypoxic chamber, and 1 h of simulated reperfusion at the end of which the percentage cell death was assessed by propidium iodide staining. Twenty transfected cells were randomly selected for each treatment group, and this was repeated in at least four independent experiments giving a total of 80 cells per treatment group.

Induction and detection of MPTP opening in HL-1 cells

In order to determine the effect of caAkt and Akt-AA on the susceptibility to MPTP opening in HL-1 cells, we used a well-characterised and validated model of oxidative stress to induce and detect MPTP opening, in which confocal laser-induced activation of tetramethylrhodamine methyl ester (TMRM) generates oxidative stress within mitochondria and induces MPTP opening, which is indicated by mitochondrial membrane depolarisation (22, 23). The occurrence of MPTP opening was confirmed in this model using the known MPTP inhibitor, cyclosporin-A (0.2 μM, Sigma, St. Louis, MO, USA). Furthermore, in previous studies we have demonstrated that the collapse in mitochondrial membrane potential corresponds to the redistribution of calcein from mitochondria to cytosol, as an indicator of MPTP opening (15, 22). Twenty transfected cells were randomly selected for each treatment group, and this was repeated in at least four independent experiments giving a total of 80 cells per treatment group.

Pharmacological activation of Akt using erythropoietin

HL-1 cells were incubated with the cytokine, erythropoietin (EPO), a well-known activator of the PI3K–Akt pathway (24, 25), for a minimum of 40 minutes (min) (Roche, 10 U/ml) to investigate the effects of pharmacological Akt activation on changes in mitochondrial morphology, the susceptibility to MPTP opening, and cell death following simulated IRI, according to the methods detailed above. Treatment with Wortmannin (Sigma, 100 nM) the pharmacological inhibitor of PI3K, or transfection with Akt-AA was used to investigate whether the effects of EPO were dependent on Akt activation.

In vivo murine model of ischaemia-reperfusion injury

All animal experiments were carried out in accordance with the United Kingdom Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986.

C57BL/6 male mice were anaesthetised by intra-peritoneal injection (0.01 ml/g) of a solution containing (ketamine 10 mg/ml, xylazine 2 mg/ml and atropine 0.06 mg/ml), and were subjected to in vivo 5 min of stabilisation, 30 min of regional myocardial ischaemia followed by 120 min of myocardial reperfusion at the end of which MI size as a percentage of the area at risk was determined by dual staining with triphenyltetrazolium chloride and Evan’s Blue (26). Mice were randomly assigned to receive by intraperitoneal injection either vehicle control (0.1 ml of 0.1 % DMSO) or EPO (2,500 U/kg) (27), administered 24 h and 30 min prior to the index myocardial ischaemic event (N=7 mice per treatment group).

Electron microscopy

C57BL/6 male mice were administered (vehicle control or EPO as previous and anaesthetised as above). Hearts were subjected to 5 min stabilisation and 20 min regional ischaemia (N=4 mice) following which the hearts were excised. The excised hearts were perfused with a fixative overnight, following which a 2 mm transverse slice, from within the myocardial area-at-risk and approximately 3 mm from the apex, was obtained from each heart. Ultra-thin sections were viewed with a Jeol 1010 transition electron microscope (Jeol Ltd, Welwyn Garden City, UK). In eight randomly selected electron micrographs of longitudinally-arranged cardiomyocytes from each heart (N=5 animals/group), the number of images dis-
played predominantly myofibrillar disarray and mitochondrial fragmentation were determined by two independent observers blinded to the treatment allocation (see Figure 5 for representative examples of electron micrographs showing mitochondrial elongation and fragmentation).

Statistical analysis

All values were expressed as mean ± S.E.M. Data were analysed by an unpaired t-test where there were two groups and by one-way ANOVA followed by a Tukey’s multiple comparison post-hoc test where there were several groups. Differences were considered significant when p< 0.05.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Akt activation induced mitochondrial elongation, delayed MPTP opening, and reduced cell death following simulated IRI

Transfecting HL-1 cells with caAkt increased the proportion of cells containing predominantly elongated mitochondria (49 ± 5.8 % in the vector control vs 73 ± 5.0 % with caAkt; p< 0.05; Figure 2A); delayed the time taken to induce MPTP opening by two-fold when compared to the vector control group (p<0.05; Figure 2B); and reduced cell death following simulated IRI (63.8 ± 5.6 % in the vector control to 32.8 ± 1.2 % with caAkt; p< 0.05; Figure 2C). The percentage of dead cells under normoxic conditions was <5.0 %, and this was not significantly altered by transgene expression.

Transfection of HL-1 cells with the dominant negative mutant form of Akt (Akt-AA) had no effect on the percentage of cells con-

![Figure 1: Representative confocal images of HL-1 cardiac cells transfected with mitochondrial matrix targeted red fluorescent protein. A) Cell displaying predominantly (>50%) elongated mitochondria; B) Cell displaying predominantly (>50%) fragmented mitochondria.]
Figure 2: Effects of over-expressing constitutively active Akt (caAkt) in HL-1 cardiac cells compared to vector control and dominant negative Akt (Akt-AA). A) Increased proportion of cells displaying predominantly (>50%) elongated mitochondria; B) Increased normalised time taken to induce the opening of the MPTP opening; C) Reduced percentage cell death following simulated acute IRI. As expected the known MPTP inhibitor, Cyclosporin (CsA), also delayed the time taken to induce MPTP opening. Data represent mean±SEM of at least 4 different experiments. In each experiment, 20 cells per treatment group were scored.*P<0.05 compared to vector control. †P<0.05 compared to Akt-AA.
Akt activation using erythropoietin has similar beneficial effects

Treatment of HL-1 cells with erythropoietin (EPO) also increased the percentage of cells containing predominantly elongated mitochondria (49 ± 5.8% in the vector control vs 38 ± 16.6% with Akt-AA; p<0.05; ►Figure 2A-C). The effect of EPO on mitochondrial morphology was abrogated in cells treated with wortmannin (the PI3K inhibitor) (24 ± 10.1% with EPO+wortmannin vs 67 ± 3.4% with EPO; p<0.05; ►Figure 3A) or transfected with Akt-AA (41 ± 7.8% with EPO+Akt-AA vs 67 ± 3.4% with EPO; p<0.05; ►Figure 3A), confirming that the effect of EPO on mitochondrial morphology was mediated via the PI3K–Akt pathway. Transfection with Akt-aa or treatment with wortmannin alone had no significant effect on mitochondrial morphology (38 ± 16.6% with Akt-AA vs 43 ± 5.8% with vector control; p>0.05; ►Figure 3A). The effect of EPO on MPTP opening sensitivity (0.5 ± 0.1% with Akt-AA vs 1.8 ± 0.2% with EPO; p<0.05; ►Figure 3B) or transfected with Akt-AA (0.5 ± 0.1% with EPO+Akt-AA vs 1.8 ± 0.2% with EPO; p<0.05; ►Figure 3B) confirming that the effect of EPO on MPTP opening sensitivity is mediated via the PI3K–Akt pathway. Transfection with Akt-aa or treatment with wortmannin alone had no significant effect on MPTP opening sensitivity (0.5 ± 0.1% with Akt-AA vs 1.0 ± 0.1% with vector control; p>0.05; ►Figure 2B: 1.2 ± 0.3% with wortmannin vs 1.0 ± 0.1% with DMSO control; p>0.05; ►Figure 3B).

The effects of Akt activation on mitochondrial morphology is dependent on Mfn1

Transfection of MEFs deficient in Mfn1 with caAkt failed to induce mitochondrial elongation (32.9 ± 3.8% with caAkt vs 46.5 ± 9.6% with vector control; p>0.05; ►Figure 4A). As a positive control, Mfn1 over-expression in these Mfn1-deficient MEFs restored the proportion of cells with elongated mitochondria (72.3 ± 6.0% with Mfn1 vs 46.5 ± 9.6% with vector control; p<0.05; ►Figure 4A). However, transfection of MEFs deficient in Mfn2 with caAkt still induced mitochondrial elongation, similarly to the over-expression of Mfn2 as a positive control (71.8 ± 12.0% with caAkt and 89.3 ± 2.4% with Mfn2 vs 28.9 ± 6.6% with vector control; p<0.05; ►Figure 4B). The results obtained from the Mfn-deficient MEFs suggest that the effect of Akt on mitochondrial morphology was mediated through Mfn1 but independent of Mfn2 (►Figure 4B).

The effects of EPO on the adult heart

We found that the in vivo pre-treatment of adult murine hearts with EPO prior to myocardial ischaemia significantly reduced the proportion of randomly selected EM images displaying predominant myofibrillar disarray and mitochondrial fragmentation following 20 min myocardial ischaemia from 5.8 ± 1.0 (out of 8) in control to 2.2 ± 1.0 (out of 8) with EPO (N=8 pictures per heart with 5 hearts/group; p=0.03) (►Figure 5A).

Finally, the effect of pre-treatment with EPO was investigated in vivo in the whole murine heart subjected to MI. The area-at-risk, expressed as a percentage of the left ventricular volume, was also comparable between treatment groups: 57.8 ± 4.8% in vehicle control vs 54.5 ± 2.6% with EPO (N=7/group; p=NS). Pre-treatment with EPO prior to ischaemia significantly reduced MI size expressed as a % of the area at risk in the in vivo murine heart (26.2 ± 2.6% with EPO vs 46.1 ± 6.5% in vehicle control; N=7 /group; p<0.05) (►Figure 5B).

Discussion

The main findings from this research study are as follows: 1) We have demonstrated that genetically activating Akt or pharmacologically activating Akt using EPO in the HL-1 cardiac cell-line modulated mitochondrial morphology (as evidenced by an increase in the proportion of cells displaying elongated mitochondria), delayed the opening of the MPTP, and reduced cell death following simulated IRI; 2) The effect of Akt on inducing mitochondrial elongation appeared to be dependent on the mitochondrial fusion protein, Mitofusin 1 (Mfn1), although this was only demonstrated in MEFs; and 3) In vivo pre-treatment with EPO of the adult murine heart subjected to acute IRI reduced the extent of mitochondrial fragmentation and myofibrillar disarray, and also reduced MI size.

A number of experimental studies have demonstrated that the acute activation Akt in the heart confers protection against acute...
Figure 3: EPO treatment in HL-1 cardiac cells when compared to vehicle control. A) Increased proportion of cells displaying predominantly (>50%) elongated mitochondria; B) Increased normalised time taken to induce the opening of the MPTP; C) Reduced percentage cell death following simulated acute IRI. As expected the known MPTP inhibitor, Cyclosporin (CsA), delayed the time taken to induce MPTP opening. The PI3K inhibitor (Wortmannin) and dominant negative Akt mutant (Akt-AA) abrogated the effect of Akt on the above variables. Data represent mean ± SEM of at least four different experiments. In each experiment, 20 cells per treatment group were scored. *P<0.05 compared to vector control.
In addition, the PI3K–Akt pathway has been shown to be a major signal transduction pathway conveying the cardio-protective signal from the cell membrane to the mitochondria in the settings of both ischaemic preconditioning (6–8) and postconditioning (10, 12). The mechanism through which Akt activation mediates its cardioprotective effects is incompletely understood. A number of potential mechanisms have been proposed including: the downstream activation of a variety of anti-apoptotic pathways (including the inhibition of cytochrome c release [29] and the inhibition of BAX [30] and BAD [31]); the activation of the pro-survival kinase PKG through the activation of eNOS; and the inhibition of the MPTP.

In this research study, we provide novel evidence that Akt protects the hearts against acute IRI by modulating mitochondrial morphology. In a previous experimental study we first demonstrated that inhibiting mitochondrial fission could protect the heart against acute IRI, and that this protective effect was mediated through the inhibition of the MPTP (15). In the current, study we have found that genetic or pharmacological activation of Akt induced mitochondrial elongation possibly via the modulation of mitofusins, delayed the onset of MPTP opening, and reduced cell death post-IRI. It is important to bear in mind that in the current study we investigated the effect of acute Akt activation as a cardioprotective strategy for protecting against acute IRI, given that chronic activation of Akt has been reported to cause cardiac hypertrophy (32). Whether Akt induces mitochondrial elongation by promoting fusion or by inhibiting fission is unknown. Our data using mitofusin-deficient MEFs suggests that Mfn1 may be involved as mitochondrial elongation still occurs in the absence of Mfn2 and we can speculate that Akt promotes mitochondrial fusion by phosphorylating and increasing the activity of Mfn1, although this remains to be shown. Interestingly, a recent study has shown that insulin increases mitochondrial metabolism

**Figure 4:** Effect of over-expressing caAkt, Mfn1 or Mfn2 in MEFs deficient in either Mfn1 (A) or Mfn2 (B) on mitochondrial morphology. The results show that caAkt induced mitochondrial elongation in MEFs deficient in Mfn2 but not in those deficient in Mfn1, suggesting that the effect of Akt on mitochondrial elongation is dependent on Mfn1 but not Mfn2. Data represent mean ± SEM of at least four different experiments. In each experiment, 20 cells were scored.*P<0.05 compared to vector control.
A limitation of our study concerns the role of Mfn1 in observed Akt-mediated effects. Our data in MEFs provides preliminary data suggesting that Mfn1 may mediate the effects of Akt on mitochondrial elongation. However, to further confirm this role for Mfn1, it would be important to repeat these experiments in the HL-1 cardiac cell line, and also show that the effects of Akt on MPTP opening and cell death following simulated IRI, were also dependent on Mfn1. Similarly, it would be important to investigate whether Akt affects mRNA and protein expression of Mfn1 and Mfn2.

Of note, genetic manipulation of the Mitofusin proteins, Mfn1 and Mfn2, in the adult heart have had unexpected results with decreased susceptibility to MPTP opening and resistance to acute IRI (34, 35). This finding may possibly relate to the non-fusion pleiotropic effects of the Mitofusins such as Mfn2 acting as a tether between the sarcoplasmic reticulum and mitochondria (19). The role of other mitochondrial-shaping proteins, e.g. OPA1 and Drp1, in this process of Akt-mediated fusion was not investigated in this study and further studies should elucidate the role of the other proteins.

In summary, we have demonstrated that either genetic or pharmacological activation of Akt induces mitochondrial elongation, and this effect on mitochondrial morphology is associated with inhibition of MPTP opening and improved cell survival following simulated IRI. The effect of Akt on mitochondrial elongation appears to be dependent on Mfn1, although this data is preliminary and was performed in MEFs and should be repeated in HL-1 cardiac cells. Activation of Akt using EPO was found to modulate mitochondrial morphology and reduce MI size using an in vivo murine MI model. The discovery that one can modulate mitochondrial morphology and influence the susceptibility of the heart to acute IRI, by pharmacologically manipulating the mitochondrial fusion and fission proteins should provide novel therapeutic strategies for cardioprotection.

Figure 5: Effect of EPO pre-treatment on the adult murine heart.
A) EPO pre-treatment reduced the proportion of EM images displaying mitochondrial fragmentation and myofibrillar disarray, when compared to vehicle control. Data represent mean ± SEM of five different hearts/group, in each heart eight images were assessed. *P<0.05 compared to vector control.
B) EPO pre-treatment reduced myocardial infarct (MI) size expressed as a % of the area-at-risk (AAR), compared to vehicle control. C) Representative electron micrographs of heart slices showing longitudinally-arranged cardiomyocytes following treatment with either vehicle control or EPO at basal level and following ischaemia. Data represent mean ± SEM of at least five different hearts/group. *P<0.05 compared to vector control.
Acknowledgements
We would like to thank Mark Turmaine for the preparation of the electron micrographs.

Conflicts of interest
None declared.

References
7. Mocanu MM, Bell RM, Yellon DM. PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by ischaemic preconditioning. J Mol Cell Cardiol 2002; 34: 661–668.
32. Ong et al. Akt and mitochondrial morphology.