The Akt1 isoform is an
essential mediator of ischaemic preconditioning

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Abstract

Phosphatidyl-inositol-3-kinase (PI3K)-Akt pathway is essential for conferring cardioprotection in response to ischaemic preconditioning (IPC) stimulus. However, the role of the individual Akt isoforms expressed in the heart in mediating the protective response to IPC is unknown. In this study, we investigated the specific contribution of Akt1 and Akt2 in cardioprotection against ischaemia-reperfusion (I-R) injury. Mice deficient in Akt1 or Akt2 were subjected to *in vivo* regional myocardial ischaemia for 30 min. followed by reperfusion for 2 hrs with or without a prior IPC stimulus. Our results show that mice deficient in Akt1 were resistant to protection with either one or three cycles of IPC stimulus ($42.7 \pm 6.5\%$ control *versus* $38.5 \pm 1.9\%$ 1 $\times$ IPC, $N = 6$, NS; $41.4 \pm 6.3\%$ control *versus* $32.4 \pm 3.2\%$ 3 $\times$ IPC, $N = 10$, NS). Western blot analysis, performed on heart samples taken from Akt1$^{-/-}$ mice subjected to IPC, revealed an impaired phosphorylation of GSK-3β, a downstream effector of Akt, as well as Erk1/2, the parallel component of the reperfusion injury salvage kinase pathway. Akt2$^{-/-}$ mice, which exhibit a diabetic phenotype, however, were amenable to protection with three but not one cycle of IPC ($46.4 \pm 5.6\%$ control *versus* $35.9 \pm 5.0\%$ in 1 $\times$ IPC, $N = 6$, NS; $47.0 \pm 6.0\%$ control *versus* $30.8 \pm 3.3\%$ in 3 $\times$ IPC, $N = 6$; *$P = 0.039$). Akt1 but not Akt2 is essential for mediating a protective response to an IPC stimulus. Impaired activation of GSK-3β and Erk1/2 might be responsible for the lack of protective response to IPC in Akt1$^{-/-}$ mice. The rise in threshold for protection in Akt2$^{-/-}$ mice might be due to their diabetic phenotype.

Keywords: Akt isoforms • ischaemic preconditioning • *in vivo* myocardial ischaemia-reperfusion injury

Introduction

Akt/protein kinase B (PKB) is a serine–threonine protein kinase downstream to phosphatidyl-inositol-3-kinase (PI3K). It is activated in response to various physiological and stress stimuli such as growth factors [1, 2], hormones [3], hypoxia [4, 5] and oxidative stress [6]. There are three different Akt isoforms in mammals, Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, each of which is encoded by a separate gene [7, 8]. There is a high degree of sequence homology and structural similarity between the three Akt isoforms [9]. Despite these structural similarities, targeted deletion of individual Akt isoforms in mice led to different phenotypes attributing non-redundant functions to each of the isoforms [10–16]. This is in part due to their varied distribution within tissues with Akt1 being ubiquitously expressed in all tissues, Akt2 present mainly in insulin-responsive tissues and Akt3 predominantly being expressed in the brain [12, 15, 17–19]. In the heart, Akt1 and Akt2 are both present in significant amounts. Functionally, Akt1 is essential for promoting cell survival [11, 12] and cardiac growth [20] and Akt2 is implicated in maintaining glucose homeostasis in the heart [21].

Ischaemic preconditioning (IPC) is an endogenous cardioprotective phenomenon, which can protect the heart against ischaemia-reperfusion (I-R) injury through the activation of intracellular signalling pathways such as the PI3K-Akt signal transduction cascade [22, 23]. Akt in turn activates or inhibits downstream signalling molecules including metabolic enzymes, apoptotic molecules and transcription factors resulting in the inhibition of detrimental mitochondrial permeability transition (MPT) to mediate a protective response to IPC. Glycogen synthase kinase-3β (GSK-3β) and Bcl-2-associated death promoter (BAD) are two of the key

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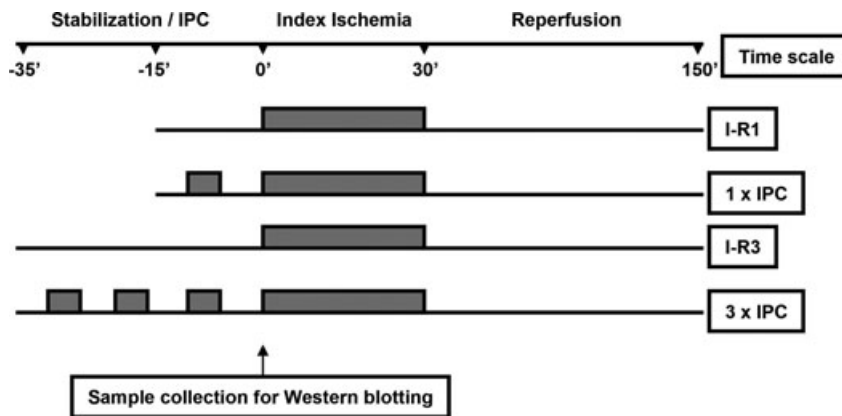Fax: +44 (0) 20 7380 9505
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Fig. 1 Protocols for experimental studies. All mice were subjected 30 min. of regional index ischaemia by occlusion of the left anterior descending coronary artery (LAD) followed by 2 hrs of reperfusion by removal of the snare. In mice subjected to the ischaemic preconditioning protocol, the index ischaemia was preceded by 5 min. of LAD occlusion followed by 5 min. of reperfusion following an initial 5-min. stabilization period (1 × IPC) while control mice received a prolonged stabilization period for 15 min. (I-R1). In some groups of mice, three cycles of IPC (3 × IPC) were administered prior to index ischaemia and the respective control mice were subjected to prolonged stabilization period of 35 min. prior to index ischaemia (I-R3). For Western blotting studies samples were collected at the end of the IPC protocol.

downstream signalling molecules of Akt involved in conferring protection against acute I-R injury. Glycogen synthase kinase-3β carries out metabolic [24] as well as apoptotic [25] functions and Akt phosphorylates and inhibits GSK-3β promoting glycogen synthesis and cell survival by increasing the threshold for MPT pore opening [26]. BAD is a pro-apoptotic molecule and acts as an inhibitor of Bcl-2/Bcl-xL, which are anti-apoptotic members of Bcl-2 family of proteins and Akt phosphorylates and inhibits BAD thereby promoting cell survival [27].

Despite the overwhelming evidence supporting the role of Akt in cardioprotection, the individual roles of the different Akt isoforms in the setting of cardiac IPC has not been investigated yet. In the current study, we examined the contribution of Akt1 and Akt2, being the two isoforms which are highly expressed in the heart, and their ability to play a role in protecting the myocardium from I-R injury following an IPC stimulus.

Materials and methods

Materials

Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail with ethylenediaminetetraacetic acid (EDTA) were purchased from Thermo Fischer Scientific, Epsom, UK. Laemmli buffer used in Western blot analysis was purchased from Sigma-Aldrich, Gillingham, UK. Primary antibodies against phospho- and total levels of Akt, Erk1/2, GSK3β and BAD were obtained from Cell-Signalling (New England Biolabs, Hitchin, UK). Primary antibody against α-tubulin was purchased from Abcam, Cambridge, UK. Secondary antibodies were purchased from Li-Cor Biosciences, Cambridge, UK. All PCR reagents were purchased from Qiagen, Crawley, UK.

Generation of transgenic animals

Generation of transgenic animals was carried out in Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. Akt1 and Akt2 single isoform gene-knockout mice were generated as previously described [12] and [18], respectively. Akt1 mutant mice (129/Ola strain) and Akt2 mutant mice (129SvJae strain) were back-crossed with C57Bl/6 mice until F4 and F2 generations, respectively. Heterozygous male and female mice expressing Akt1 and Akt2 were bred to obtain respective littermate and knockout mice of Akt1 and Akt2, respectively. Genotyping was carried out to check the genetic identity of the pups using multiplex PCR. Additional information on genotyping including the primers used in PCR is provided in the supporting information.

In vivo murine model for myocardial ischaemia-reperfusion injury

All procedures on animals were carried out in accordance with the Guidance on Operation of the Animals (Scientific Procedures) act 1986. Male experimental mice (8–12 weeks) were anaesthetized by intraperitoneal injection using a mixture of ketamine, xylazine and atropine with final concentrations of 10 mg/ml, 2 mg/ml and 0.06 mg/ml, respectively, administered in a final volume of 0.01 ml/g. The left anterior descending (LAD) coronary artery was occluded for 30 min. followed by 120 min. of reperfusion at the end of which myocardial infarct size was measured [28]. Ischaemic preconditioning comprised either one or three cycles of 5 min. of LAD occlusion followed by 5 min. of reperfusion immediately prior to the sustained episode of I-R (Fig. 1). Detailed methods on surgical procedures are provided in the supporting information.

Infarct size measurement

At the end of the reperfusion period, the hearts were differentially stained with triphenyl-tetrazolium chloride (TTC) and Evans blue to demarcate the infarct area (IA) and the area at risk (AAR), respectively [29]. Planimetry was then performed to calculate the infarct area as a percentage of area at risk using Image J software from NIH Image. Infarct area was expressed as a percentage of the AAR (%IA/AAR).

Tissue fractionation and Western blot analysis

For Western blot analysis, the mice were subjected to one or three cycles of IPC followed by immediate excision of heart tissue from the AAR for

Fig. 2 Physiological characteristics of Akt1 and Akt2 transgenic mice. (**A**) Total bodyweight of Akt1 mice. (**B**) Total bodyweight of Akt2 mice. (**C**) Glycated haemoglobin levels measured as a percentage of total haemoglobin in Akt2 mice aged (i) 8–12 weeks and (ii) 52 weeks. All values are expressed as means ± S.E.M.; N = 6–12 per group, *$P \leq 0.05$ versus +/+, †$P \leq 0.05$ versus +/−.
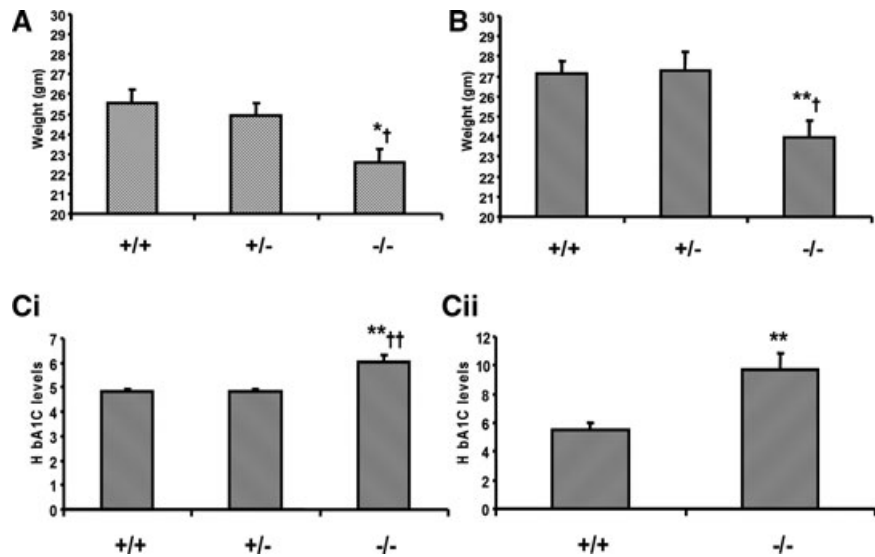
subsequent analysis. The tissue was immediately snap-frozen in liquid nitrogen and stored at −80°C until further processing. Cytosolic tissue fractions were prepared by differential centrifugation after homogenizing the whole-heart tissue in lysis buffer (250 mM Sucrose, 10 mM Tris–HCl, pH 7.4) containing protease, phosphate cocktails and EDTA (pH 8.0). Either whole-heart homogenates or cytosolic fractions were used for detection of proteins. Total protein was electrophoresed on SDS-PAGE and transferred on to nitrocellulose membrane and the membrane was probed for either total or phosphorylated levels of Akt, GSK3β, BAD and Erk1/2. Phosphorylated levels were normalized to loading control, α-tubulin. Secondary antibodies with infrared fluorescent reporter tags were used for antibody detection using the Odyssey imaging system from Li-Cor Biosciences. Detailed methods on western blotting are provided in the supporting information.

Statistical analysis

All values are expressed as means ± SEM. Data were analysed either using unpaired Student's *t*-tests or one-way ANOVA followed by *post hoc* analysis using Turkey multiple-comparisons. Differences were considered to be significant if $P \leq 0.05$.

Results

Physiological characteristics of Akt1 and Akt2 transgenic mice

Growth retardation in Akt1 and Akt2 knockout mice

Akt1 and Akt2 transgenic mice have been extensively characterized in previous studies [10–14]. Akt1 has previously been shown to be expressed in most tissues within the body [12], and has

been shown to contribute to both physiological as well as pathological growth [20]. Hence, it is expected that lack of Akt1 would result in whole body growth retardation. In our study, Akt1$^{-/-}$ mice exhibited significant growth retardation with an average bodyweight of 22.7 ± 0.8 g compared to 25.5 ± 0.7 g in wild-type littermate Akt1$^{+/+}$ mice (n = 8–12, P = 0.013, Fig. 2A). As such our results appear to be in line with previous studies, which have also shown that Akt1 knockout mice exhibit significant growth retardation [10, 12]. However, there is contradictory evidence as to the role of Akt2 in whole body growth [13, 14]. Our data show that Akt2$^{-/-}$ mice in common with Akt1$^{-/-}$ mice also exhibited significant growth retardation weighing 24.0 ± 0.8 g compared to 27.1 ± 0.6 g in Akt2$^{+/+}$ mice (n = 12, P = 0.006, Fig. 2B).

Akt1 knockout mice exhibit poor survival

Additionally, Akt1 plays an important role in survival [10] and any lack of Akt1 may result in the increased mortality [12, 17]. In the current study, mating between Akt1 heterozygous male and female mice resulted in much fewer Akt1 homozygous knockout mice than would be expected under Mendelian ratio. On examination, of 104 pups aged between 8 and 12 weeks and spanning over 15 litters, 37.5% were Akt1$^{+/+}$ mice, 54.8% were Akt1$^{+/-}$, however, only 7.7% of the surviving pups were Akt1$^{-/-}$ mice. On the contrary, in relation to Akt2 transgenic mice, out of 98 pups examined over 7 litters, 27.6% of them were Akt2$^{+/+}$, 39.8% were Akt2$^{+/-}$ and 31.6% were Akt2$^{-/-}$ exhibiting a near Mendelian ratio.

Akt2 knockout mice exhibit progressive hyperglycaemia with age

It has previously been shown that Akt2$^{-/-}$ mice exhibit a diabetic phenotype [13, 14]. In the current study to confirm this observation we measured HbA1C levels in Akt2 transgenic mice, and

noticed that Akt2$^{-/-}$ mice show significantly higher glycated haemoglobin of 6.0 ± 0.3% compared to 4.8 ± 0.1% in Akt2$^{+/+}$ ($n = 6$, $P = 0.006$, Fig. 2Ci) and 4.9 ± 0.1% in Akt2$^{+/-}$ mice ($n = 6$, $P = 0.06$, Fig. 2Ci), which increases to 9.7 ± 1.1% in the Akt2$^{-/-}$ compared to 5.5 ± 0.5% in Akt2$^{+/+}$ examined at 1 year of age ($n = 6$, $P = 0.006$, Fig. 2Cii).

In situ murine myocardial infarction studies

Akt1 knockout mice are not amenable to ischaemic preconditioning

In order to study the role of Akt1 in cardioprotection, we subjected Akt1$^{+/-}$ and Akt1$^{-/-}$ mice as well as wild-type littermate control Akt1$^{+/+}$ mice to a sustained episode of I-R injury. There were no differences between areas at risk across the groups studied (Fig. S4). Mean arterial blood pressure measurements taken at specific time points did not show any significant differences between the experimental groups studied (Table 1A). There were no significant differences in the heart rate across groups at baseline (Table 1A). However, heart rate was significantly lower following the ischaemic insult in the IPC group of Akt1$^{-/-}$ mice compared to I-R group (Table 1A).

Ischaemia-reperfusion injury did not result in larger myocardial infarct areas in mice lacking either one or both alleles for the Akt1 gene compared to their littermate control mice (Fig. 3A). We then proceeded to examine whether Akt1 plays a role in mediating the ischaemic preconditioning stimulus. Ischaemic preconditioning resulted in significant protection in Akt1$^{+/+}$ mice against I-R injury (28.9 ± 1.4% with IPC *versus* 45.5 ± 2.6% in control, $n = 6$, $P = 0.0001$, Fig. 3A, first panel). However, neither Akt1$^{+/-}$ nor Akt1$^{-/-}$ mice were amenable to protection induced by IPC (40.5 ± 7.8% with IPC *versus* 45.3 ± 5.1% in control, 38.5 ± 1.9 with IPC *versus* 42.7 ± 6.5% in control, respectively, $n = 6$, Fig. 3A, middle panels). In addition, increasing the number of IPC cycles from one to three did not result in significant protection in Akt1$^{+/-}$ mice (32.4 ± 3.2% with IPC *versus* 41.4 ± 6.3% in control, $n = 10$, NS, Fig. 3A, last panel).

Akt2 knockout mice require a stronger ischaemic preconditioning stimulus

To investigate if Akt2 also contributes to the IPC-mediated cardioprotective response, we subjected Akt2 transgenic mice to I-R injury with or without an IPC stimulus. There were no differences between areas at risk across the groups studied (supplementary Fig. S4). There were no significant differences in the haemodynamic parameters studied across different experimental groups amongst Akt2 mice (Table 1B). Similar to Akt1, there were no differences in the %IA/AAR across the three genotypes of Akt2 mice subjected to I-R injury (Fig. 3B). On administration of one cycle of IPC Akt2$^{+/-}$ mice were amenable to cardioprotection along with Akt2$^{+/+}$ mice (20.7 ± 2% with IPC *versus* 46.2 ± 4.7% in control, $n = 6$, $P = 0.0005$, 30.2 ± 2.3% with IPC *versus*

41.8 ± 4.0% in control, $n = 6$, $P = 0.03$, respectively, Fig. 3B, first two panels). However, the Akt2$^{-/-}$ mice were resistant to cardioprotection elicited by one cycle of IPC (35.9 ± 5.0% *versus* 46.4 ± 5.6%, $n = 6$, $P = $ NS; Fig. 3B, last panel) and required three cycles of IPC to elicit cardioprotection (30.8 ± 3.3% *versus* 47.0 ± 6.0% I-R, $n = 6$, $P = 0.03$; Fig. 3B, last panel).

Downstream signalling to Akt is attenuated in Akt1 knockout mice

In order to explore which downstream effectors of Akt1 might be involved in mediating the IPC stimulus we carried out immunoblotting studies. Previous study from our laboratory examining the different time points of Akt activation has clearly demonstrated biphasic response of Akt activation in hearts subjected to acute I-R injury with IPC stimulus. The first peak was observed immediately following IPC stimulus and the second at the onset of reperfusion. There was a slight reduction in the level of phosphorylation at the onset of reperfusion compared to that immediately following IPC. However, this difference was not significant [23]. It has also been shown that blocking the Akt activation immediately following IPC using pharmacological agents eliminates activation of Akt at reperfusion and abrogates protection against I-R injury [30–32]. Hence, we proceeded to examine the activation of the reperfusion injury salvage kinase (RISK) pathway following IPC stimulus.

Using antibodies non-selective for a particular Akt isoform, we initially studied the phosphorylation of Akt at serine-threonine residues as well as its total protein levels. Our results show that there was a significant decrease in Akt phosphorylation in Akt1$^{-/-}$ compared to wild-type Akt1$^{+/+}$ mice at Ser473 (0.026 ± 0.002 *versus* 0.054 ± 0.005, $n = 4$, $P = 0.00256$) but not at Thr308 residue (0.0005 ± 0.00 *versus* 0.0005 ± 0.00, $n = 4$) in response to IPC (Fig. 4A and B, respectively). As expected, IPC induced phosphorylation of Akt at both Thr308 (0.0005 ± 0.00 *versus* 0.00006 ± 0.00, $n = 4$, $P = 0.0002$) as well as Ser473 (0.054 ± 0.005 *versus* 0.017±0.008, $n = 4$, $P = 0.008$) in Akt1$^{+/+}$ mice. Total levels of Akt were significantly lower in Akt1$^{-/-}$ hearts compared to Akt1$^{+/+}$ (0.09 ± 0.005 *versus* 0.23 ± 0.02, $n = 8$, $P = 0.000$, Fig. 4C). The marginal increase in Akt phosphorylation at Ser473 in response to IPC in Akt1$^{-/-}$ mice might suggest that IPC-induced activation of Akt is not isoform-specific.

Downstream of Akt we studied the phosphorylation of GSK-3β as well as the pro-apoptotic protein, BAD, in response to IPC. We found that GSK-3β phosphorylation was significantly reduced in response to IPC in the Akt1$^{-/-}$ compared to Akt1$^{+/+}$ mice (0.017 ± 0.0009 *versus* 0.034 ± 0.002, $n = 4$, $P = 0.0002$, Fig. 5A) whereas the total levels of GSK-3β remain the same (Fig. 5B). Preconditioning did not result in increased phosphorylation of BAD in the control Akt1$^{+/+}$ mice (0.023 ± 0.007 with IPC *versus* 0.025 ± 0.004 in control, $n = 4$, Fig. 5C). However, there was a significant decrease in basal-level phosphorylation of BAD in the

Table 1 Haemodynamic measurements in (A) Akt1 and (B) Akt2 mice at one or two time points during stabilization, 35 min. (−35-S) or 15 min. (−15-S) prior to onset of index ischaemia; at the onset of index ischaemia (0-I), 15 min. (15-I) or 30 min. (30-I) into index ischaemia; at 60 (60R) or 120 min. (120R) into reperfusion, in all experimental groups.

(A)

Time point	I-R$^{+/+}$	IPC$^{+/+}$	I-R$^{+/-}$	IPC$^{+/-}$	I-R$^{-/-}$	IPC$^{-/-}$	I-R3$^{+/-}$	IPC3$^{+/-}$
Akt1 mean arterial blood pressure (mmHg)								
−35-S	–	–	–	–	–	–	154 ± 7	145 ± 6
−15-S	150 ± 9	118 ± 13	135 ± 16	124 ± 10	129 ± 7	136 ± 9	153 ± 5	131 ± 4
0-I	135 ± 6	105 ± 13	129 ± 12	124 ± 5	129 ± 10	126 ± 8	102 ± 8	103 ± 4
15-I	105 ± 5	83 ± 10	100 ± 7	95 ± 4	98 ± 8	104 ± 6	98 ± 4	90 ± 3
30-I	94 ± 4	74 ± 9	90 ± 6	86 ± 4	86 ± 9	97 ± 4	88 ± 4	83 ± 3
60R	83 ± 7	67 ± 6	75 ± 5	81 ± 4	72 ± 4	75 ± 7	80 ± 3	71 ± 4
150R	67 ± 8	49 ± 5	52 ± 6	49 ± 5	48 ± 7	49 ± 12	56 ± 6	52 ± 4
Akt1 heart rate (beats/min.)								
−35-S	–	–	–	–	–	–	406 ± 19	454 ± 23
−15-S	391 ± 20	396 ± 38	403 ± 24	396 ± 9	364 ± 19	359 ± 17	324 ± 14	381 ± 18*
0-I	332 ± 11	331 ± 11	342 ± 14	394 ± 10	336 ± 15	335 ± 19	324 ± 14	385 ± 19*
15-I	331 ± 7	331 ± 16	336 ± 5	360 ± 11	382 ± 24	321 ± 9*	342 ± 11	373 ± 14
30-I	334 ± 6	342 ± 9	335 ± 3	357 ± 4	372 ± 14	325 ± 6*	335 ± 12	374 ± 11
60R	339 ± 10	351 ± 18	358 ± 19	380 ± 17	374 ± 18	325 ± 16*	356 ± 10	379 ± 8
150R	363 ± 21	345 ± 19	383 ± 25	343 ± 8	414 ± 48	321 ± 9*	361 ± 15	395 ± 15

(B)

Time point	I-R$^{+/+}$	IPC$^{+/+}$	I-R$^{+/-}$	IPC$^{+/-}$	I-R$^{-/-}$	IPC$^{-/-}$	I-R3$^{-/-}$	IPC3$^{-/-}$
Akt2 mean arterial blood pressure (mmHg)								
−35-S	–	–	–	–	–	–	154 ± 7	157 ± 16
−15-S	148 ± 11	148 ± 10	148 ± 9	127 ± 4	147 ± 7	148 ± 11	146 ± 7	129 ± 11
0-I	139 ± 7	134 ± 10	145 ± 8	120 ± 2	138 ± 5	137 ± 6	112 ± 4	109 ± 10
15-I	111 ± 5	106 ± 11	107 ± 7	88 ± 5	120 ± 6	116 ± 7	105 ± 2	94 ± 10
30-I	95 ± 4	94 ± 11	95 ± 4	76 ± 3	105 ± 7	100 ± 7	92 ± 4	81 ± 7
60R	78 ± 3	68 ± 4	72 ± 3	69 ± 4	81 ± 3	75 ± 1	71 ± 4	60 ± 6
150R	55 ± 4	45 ± 4	42 ± 4	47 ± 8	63 ± 3	48 ± 6	45 ± 7	46 ± 6
Akt2 heart rate (beats/min.)								
−35-S	–	–	–	–	–	–	478 ± 16	534 ± 19
−15-S	474 ± 18	447 ± 10	490 ± 11	458 ± 13	478 ± 16	494 ± 12	414 ± 7	459 ± 24
0-I	417 ± 8	429 ± 5.4	439 ± 9	427 ± 13	419 ± 10	458 ± 11	404 ± 8	449 ± 19
15-I	410 ± 11	394 ± 15	411 ± 7	412 ± 12	414 ± 3	417 ± 12	407 ± 12	431 ± 23
30-I	390 ± 13	400 ± 16	410 ± 8	405 ± 9	416 ± 6	430 ± 11	400 ± 6	430 ± 17
60R	402 ± 17	389 ± 21	383 ± 16	405 ± 14	391 ± 12	406 ± 11	435 ± 28	410 ± 11
150R	414 ± 15	438 ± 32	378 ± 18	433 ± 34	407 ± 31	428 ± 5	473 ± 26	496 ± 30

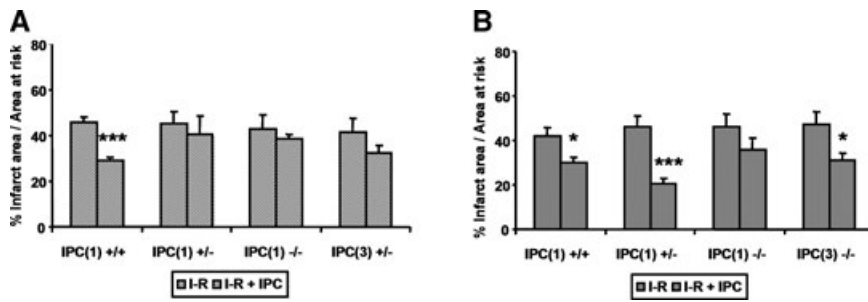*$P \leq 0.05$ *versus* respective I-R group.

Fig. 3 Quantitative analysis of myocardial infarct areas expressed as a percentage of areas at risk in (**A**) Akt1 and (**B**) Akt2 transgenic mice. Backward slashes denote values from mice subjected to ischaemia-reperfusion (I-R) injury without an ischaemic preconditioning (IPC) stimulus and forward slashes denote those subjected to either 1 cycle of IPC (IPC1) or three cycles of IPC (IPC3). Values are expressed as means ± S.E.M.; $N = 6$–10; $*P \leq 0.05$ compared to I-R of the same genotype.
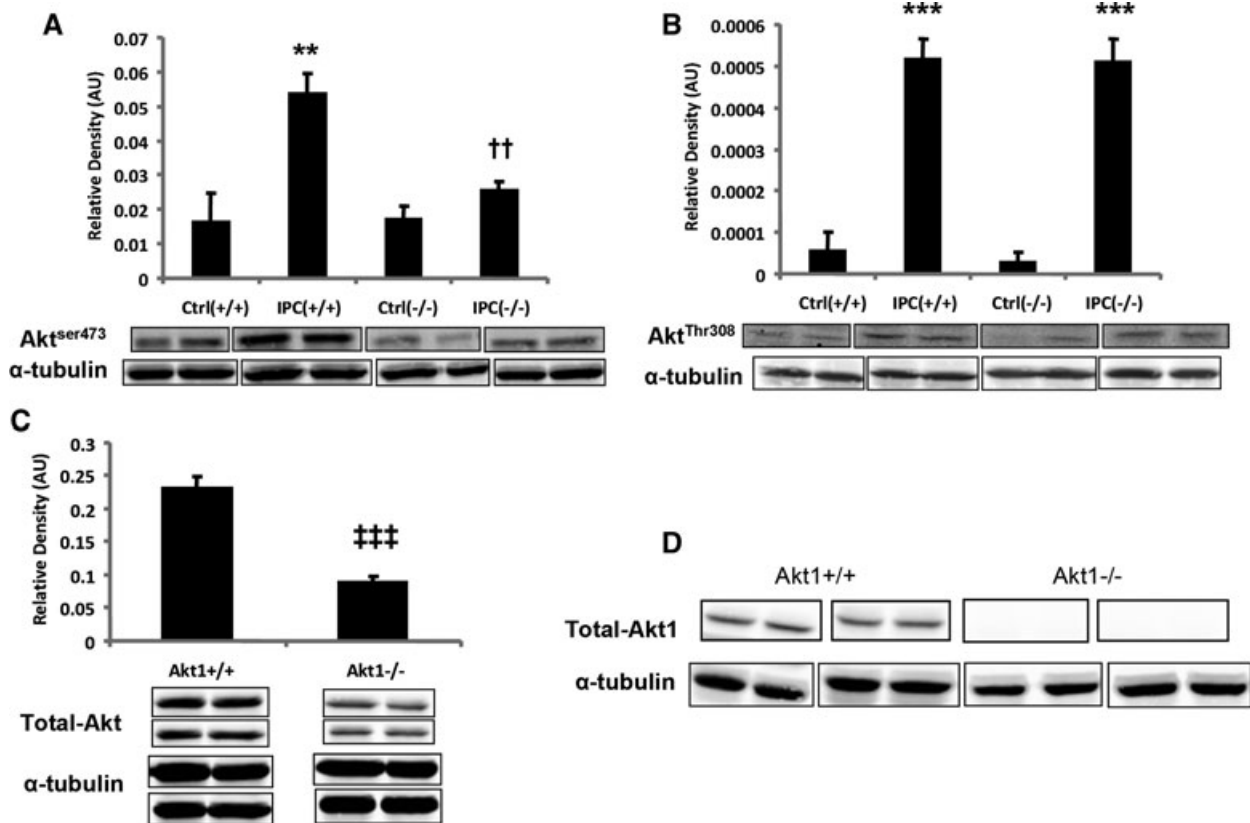

Fig. 4 Immunoblot analysis of heart samples from Akt1$^{+/+}$ and Akt1$^{-/-}$ mice subjected to either 15 min. of stabilization (Control, Ctrl) or one cycle of IPC stimulus. Representative Western blot images show levels of (**A**) phospho-Akt^{ser473}, (**B**) phospho-Akt^{Thr308} and (**C**) total Akt. (**D**) Representative blots showing presence of Akt1 in Akt1$^{+/+}$ and absence of any trace of Akt1 in Akt1$^{-/-}$ hearts. Representative bands were cut from a single blot for display purposes. Bottom panel of the bands represent the levels of α-tubulin used as loading control. Average densitometric values are shown as histograms. All values are represented as means ± SEM, $n = 4$–8. $*P \leq 0.05$ *versus* Ctrl; $^{†}P \leq 0.05$ *versus* IPC (+/+); $^{‡}P \leq 0.05$ *versus* Akt1$^{+/+}$.

Akt1$^{-/-}$ compared to Akt1$^{+/+}$ hearts (0.009 ± 0.003 *versus* 0.025 ± 0.004, $n = 4$, $P = 0.016$, Fig. 5C). Interestingly, the total level of BAD protein was significantly lower in the Akt1$^{-/-}$ hearts compared to Akt1$^{+/+}$ hearts (0.015 ± 0.002 *versus* 0.033 ± 0.004, $n = 8$, $P = 0.002$, Fig. 5D).

In addition to the PI3K-Akt pathway, Erk1/2, the other component of the reperfusion injury salvage kinase pathway, has also been shown to mediate protective effects in response to IPC and a few studies have suggested crosstalk between the two pathways [23, 33]. In the current study, to investigate the relationship
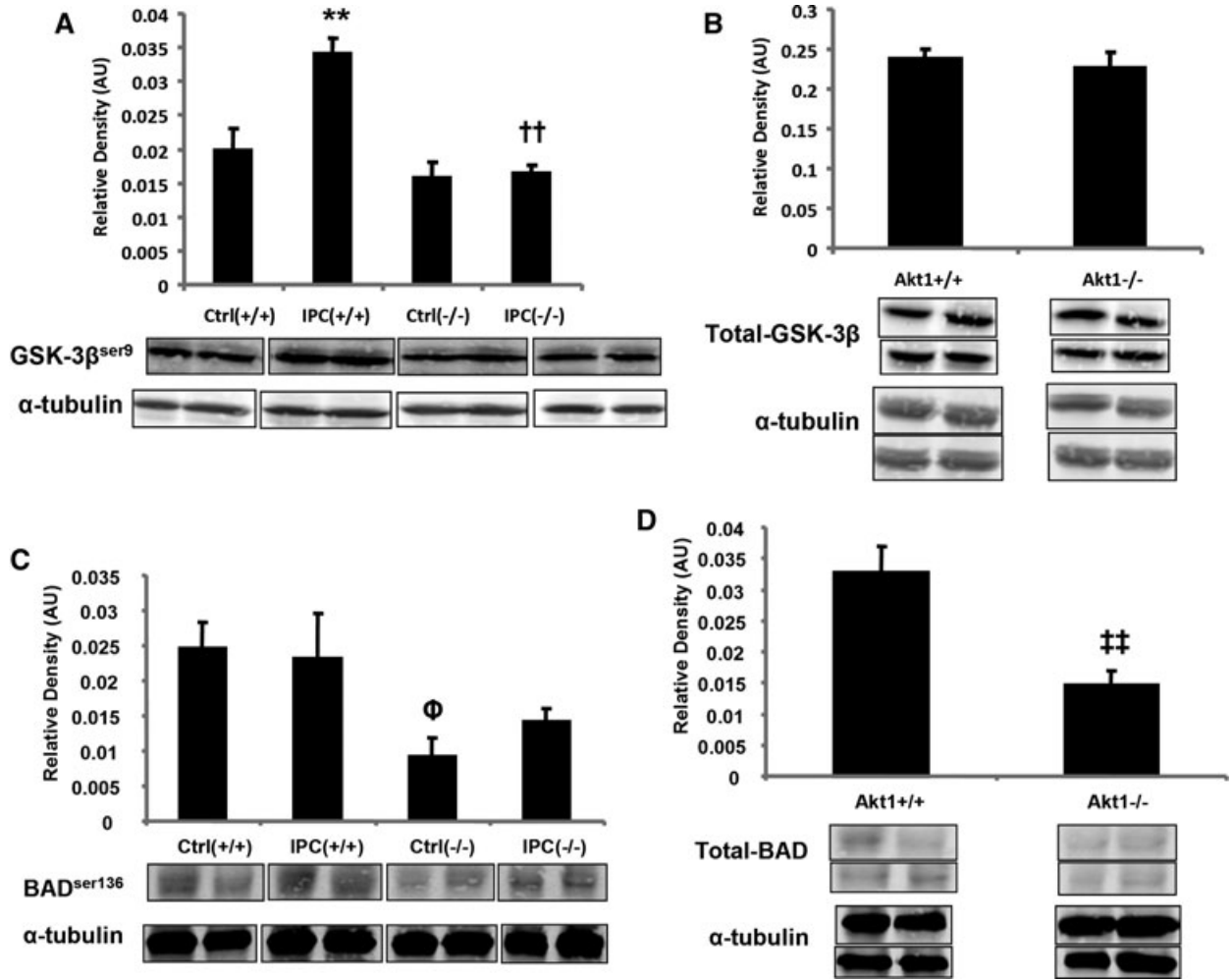
Fig. 5 Immunoblot analysis of heart samples from Akt1^{+/+} and Akt^{−/−} mice subjected to either 15 min. of stabilization (Control, Ctrl) or one cycle of IPC stimulus. Representative Western blot images show levels of (**A**) phospho-GSK-3β[ser9], (**B**) total GSK-3β, (**C**) phospho-BAD[Ser136] and (**D**) total BAD. Representative bands were cut from a single blot for display purposes. Bottom panel of the bands represent the levels of α-tubulin used as loading control. Average densitometric values are shown as histograms. All values are represented as means ± S.E.M., $n = 4$–8. *$P \leq 0.05$ *versus* Ctrl; [†]$P \leq 0.05$ *versus* IPC $(+/+)$; [Φ]$P \leq 0.05$ *versus* Ctrl $(+/+)$; [‡]$P \leq 0.05$ *versus* Akt1^{+/+}.

between Erk and Akt in the setting of IPC we looked at the phosphorylation status of Erk1/2 in response to IPC in Akt1^{−/−} mice. Our results have shown that Erk1/2 phosphorylation is significantly lower in Akt1^{−/−} mice in response to IPC compared to Akt1^{+/+} mice (Erk1: 0.0038 ± 0.0005 with IPC *versus* 0.012 ± 0.001 in control, $n = 4$, $P = 0.0017$, Fig. 6A; Erk2: 0.0047 ± 0.0009 *versus* 0.020 ± 0.002, $n = 4$, $P = 0.0002$, Fig. 6B). However, there was significant phosphorylation of Erk1/2 in response to IPC in Akt^{+/+} mice (Erk1: 0.012 ± 0.001 with IPC *versus* 0.003 ± 0.0006 in control, $n = 4$, $P = 0.001$, Erk2: 0.020 ± 0.002 IPC *versus* 0.005 ± 0.0007 control, $n = 4$, $P = 0.0001$, Fig. 6A and B). There were no significant differences in total levels of Erk1/2 between Akt1^{−/−} and wild-type mice (Fig. 6C and D).

Discussion

Our study shows for the first time that mice deficient in Akt1 were resistant to the myocardial infarct limitation induced by ischaemic preconditioning despite increasing the IPC stimulus, suggesting that Akt1 is an obligatory mediator of IPC. In contrast, although mice deficient in Akt2 were also resistant to the protection induced by ischaemic preconditioning, this could be overcome by increasing the IPC stimulus, suggesting that Akt2 is not an obligatory mediator of IPC.

Akt1 plays a key role in the regulation of physiological cardiac growth, maladaptive pathological hypertrophy [20] and apoptosis, with mice lacking Akt1 exhibiting increased spontaneous apoptosis
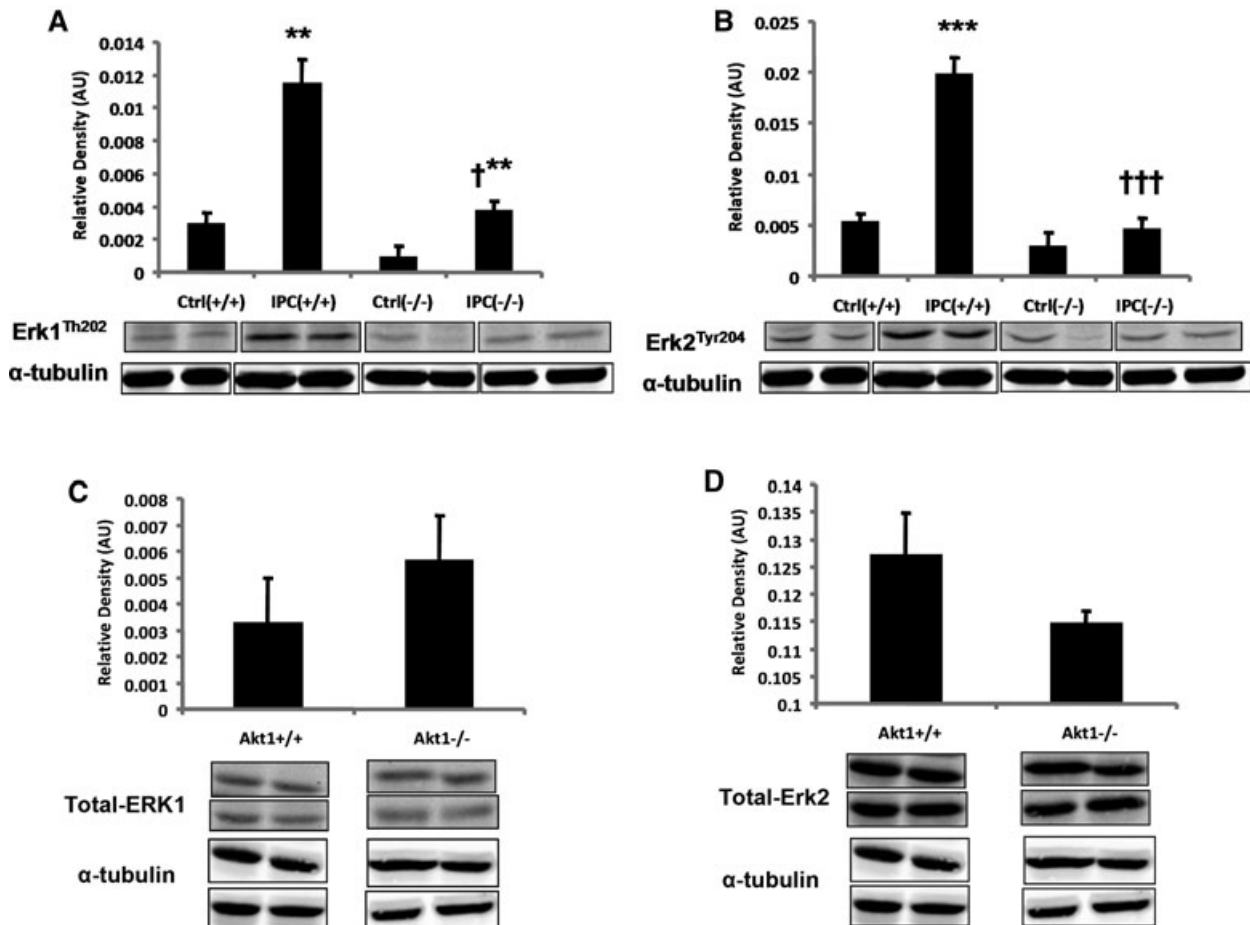
Fig. 6 Immunoblot analysis of heart samples from Akt1^{+/+} and Akt1^{−/−} mice subjected to either 15 min. of stabilization (Control, Ctrl) or one cycle of IPC stimulus. Representative Western blot images show levels of (**A**) phospho-Erk1[Thr202], (**B**) phospho-Erk2[Tyr204], (**C**) total Erk1 and (**D**) total Erk2. Representative bands were cut from a single blot for display purposes. Bottom panel of the bands represent the levels of α-tubulin used as loading control. Average densitometric values are shown as histograms. All values are represented as means ± S.E.M., $n = 4$–8. *$P \leq 0.05$ *versus* Ctrl; †$P \leq 0.05$ *versus* IPC (+/+).

in their tissues [10]. It has been shown that acute activation of Akt1 in cardiomyocytes *in vivo* and *in vitro* protects against apoptosis subsequent to injurious ischaemia [34]. However, paradoxically the chronic activation of Akt1 leads to detrimental effects resulting in larger infarct areas and poor functional recovery in mice when subject to I-R injury thereby leading to the conclusion that the level of activation of Akt should be optimal for conferring cardioprotection [35]. One study exploring the sex differences in the mechanism of Met[5]-enkephalin (ME)-induced cardioprotection observed that the three Akt isoforms play different roles in response to ME. The results from this study showed that neonatal cardiomyocytes from male mice primarily utilized Akt1/2 whereas those from female mice used Akt3 for mediating ME-induced protection [36]. It has been previously shown that during the developmental stages of mice all the three Akt isoforms are present to significant levels in the heart [17]. However, the levels of Akt3 in

the adult heart are significantly low compared with Akt1/2 [12, 16, 19]. In addition, in retinal ischaemic preconditioning the protection observed is blocked when Akt2 and Akt3 were knocked down using small interfering RNA (siRNA) implicating their role in retinal neuroprotection [37]. The data resulting from our study suggest that Akt1, which is highly expressed in the heart, might be selectively involved in triggering a protective signalling cascade in response to IPC.

There are many downstream effectors of Akt that could potentially mediate the cardioprotective effect elicited by IPC. Two of the most significant targets in the repertoire of Akt substrates involved in survival signalling are GSK3β and the pro-apoptotic protein BAD, which are phosphorylated and inhibited by Akt at Serine9 and Serine136 positions, respectively, to promote cell survival [25] and [27], respectively. Several studies have proposed GSK3β as a point of convergence, a common target of multiple signal

pathways and a regulator of the mitochondrial permeability transition pore [26, 38, 39]. By combining the additive effects of ischaemic preconditioning and pharmacological treatment it was suggested that the level of GSK3β phosphorylation determines the myocardial infarct size in hearts following myocardial injury [40]. In our study GSK3β phosphorylation was significantly lower in Akt1$^{-/-}$ mice in response to IPC than was seen in Akt1$^{+/+}$ mice, whereas total levels of GSK3β remain unchanged. Hence, it is probable that the lack of protection seen in Akt1$^{-/-}$ mice is due to insufficient phosphorylation of GSK3β suggesting that Akt1, in response to IPC, might selectively phosphorylate and inhibit GSK3β to confer cardioprotection in mice.

Interestingly, investigating the effect of IPC on both total BAD as well as its phosphorylated levels in Akt1$^{-/-}$ mice revealed that there was a significantly lower amount of total BAD protein resulting in lower amounts of phosphorylated BAD protein in response to IPC. The significance of this observation is unclear. Certainly as a key component of the apoptotic mitochondrial death pathway, a reduction in total levels of BAD should release anti-apoptotic Bcl-2 and Bcl-xL from the inhibitory complex with BAD leading to a reduction in overall apoptotic cell death. However, the actual cause for the reduction in the levels of BAD in Akt1$^{-/-}$ mice is unclear. It is possible that Akt1, owing to one of the key cellular functions of Akt in protein translation, might play a role in expression of BAD protein. Additionally, there was no significant increase in the phospho-BAD^{Ser136} in response to IPC in Akt1$^{+/+}$ mice. Although the phosphorylation of BAD is seen as an essential signalling effect in response to Akt activation in the setting of IPC [41–43], analysis of experimental data from other studies indicating such a change reveals that the timing of tissue extraction during the procedure may play a key role in detecting the phosphorylation status of BAD. Studies showing a positive increase in BAD phosphorylation, subsequent to IPC, have detected a significant change either following injurious ischaemia [43, 44] or at the onset of reperfusion subsequent to injurious ischaemia [44, 45]. Therefore, in relation to our study, it is possible that detectable differences in the phosphorylation of BAD between control and IPC-treated hearts might take place only after being subjected to injurious ischaemia or at the onset of reperfusion and not immediately after the induction of IPC.

Previous studies from our laboratory have suggested crosstalk between the PI3K-Akt and the MEK-Erk pathways in the setting of IPC using pharmacological inhibitors [33, 46]. In the current study the use of the Akt transgenic mice has enabled us to confirm that there appears to be a crosstalk between the two components of the RISK pathway in response to IPC. We found that there was significant reduction in the Erk1/2 phosphorylation in response to IPC in Akt1$^{-/-}$ mice when compared to its wild-type control mice. There are a number of studies showing abrogation of protection seen with IPC on inhibition of MEK/Erk1/2 pathway and resulting in a reduction in Erk1/2 phosphorylation [47–49]. It is therefore possible that Erk1/2 is activated either in parallel or downstream of Akt1 in response to IPC and lack of activation of both pathways together results in lack of protection in Akt1$^{-/-}$ mice.

The Akt2 isoform has been shown to be pivotal for maintaining glucose homeostasis and as such plays a significant role in regulating cardiac metabolism as well as cardiomyocyte survival [13, 14, 21]. Lack of Akt2 in mice results in significantly larger infarct areas 7 days following surgery when subjected to pressure overload [21]. In our study acute I-R injury did not result in larger infarct areas in Akt2$^{-/-}$ mice compared with Akt2$^{+/+}$. However, a more potent IPC stimulus was required to confer protection against I-R injury in these mice. In addition, it is shown that Akt2$^{-/-}$ mice exhibit a diabetic phenotype with progressive hyperglycaemia and insulin resistance [13, 14, 50]. It was shown for the first time by Tosaki *et al.*, in isolated working heart preparation that diabetic hearts exhibit resistance to protection elicited by preconditioning [51]. This study also highlighted that in the early stages of diabetes, the hearts exhibit a protected phenotype. Previously published studies from our laboratory in both animal and human muscle have indicated that in the diabetic myocardium the threshold for protection is raised, therefore a stronger IPC stimulus to protect against severe I-R injury is required [32] and [52], respectively. It is possible that the higher threshold for cardioprotection exhibited by Akt2$^{-/-}$ mice in the current study could be due to their diabetic phenotype. However, we were unable to test this hypothesis in the present study by administering an anti-diabetic agent to the mice and investigate if the threshold for protection is lowered as all of the anti-diabetic drugs available can also be cardioprotective [53–57]. Hence, the limitation of the current study is that a direct comparison of the merits of the two isoforms in conferring cardioprotection has not been possible due to the diabetic phenotype exhibited by the Akt2$^{-/-}$ mice.

In conclusion, our study has showed for the first time that Akt1 but not Akt2 is an essential mediator of myocardial IPC. It appears that although both Akt1 and Akt2 are activated in response to IPC, Akt1 plays a key role in transmitting the cardioprotective signal resulting in amelioration of myocardial injury as a result of severe ischaemia-reperfusion injury. Although a definite candidate amongst the downstream effector(s) of Akt has not as yet been identified, which would be involved in transmitting the protective signal; it is likely that a complex interaction between more than one substrate might likely lead to the protective response to IPC *via* Akt1.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Representative agarose gel electrophoresis showing genomic DNA bands. Genomic DNA isolated from ear snips of Akt1 and Akt2 transgenic mice was used for PCR analysis. Akt1 mice are expected to show a PCR product at 285 base pairs (bp) for wild-type, 204 bp for knockout and both for heterozygote mice. Akt2 mice are expected to show a PCR product at 360 bp for wild-type, 600 bp for knockout and both for heterozygote mice.

Fig. S2 (**A**) Representative trace of rapid fall in blood pressure immediately following left anterior descending coronary artery (LAD) occlusion. (**B**) Electrocardiogram recording before and after LAD occlusion with the latter representing a clear ST-segment elevation.

Fig. S3 Typical representative heart sections from Akt mice subjected to I-R injury (top row) and with IPC (bottom row) in (**A**) Akt1$^{+/+}$, (**B**) Akt1$^{+/-}$, (**C**) Akt1$^{-/-}$, (**D**) Akt1$^{+/-}$ (three cycles of IPC), (**E**) Akt2$^{+/+}$, (**F**) Akt2$^{+/-}$, (**G**) Akt2$^{-/-}$ and (**H**) Akt2$^{-/-}$ (three cycles of IPC). Blue area represents non-area-at-risk, red area represents viable area within area-at-risk and white area is infarcted tissue.

Fig. S4 Areas at risk represented as a percentage of total area of left ventricle in (**A**) Akt1, (**B**) Akt2 mice subjected to ischaemia-reperfusion (I-R) injury without ischaemic preconditioning (IPC) stimulus (black bars) or with either one cycle of IPC (IPC1) or three cycles of IPC (IPC3) (grey bars). Values are expressed as means $\pm$ SEM; $N = 6$–10.

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