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Isoflurane-induced myocardial preconditioning is dependent on phosphatidylinositol-3-kinase/Akt signalling

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Background. Isoflurane and other volatile anaesthetics have a cardioprotective effect and limit myocardial infarct size to the same extent as ischaemic preconditioning. Phosphatidylinositol-3-kinase (PI3K) was found to play a key role in myocardial protection by ischaemic preconditioning. The aim of the present investigation was to evaluate whether isoflurane-induced myocardial preconditioning is dependent on PI3K signalling.

Methods. Using a model of regional myocardial ischaemia and reperfusion, New Zealand White rabbits were subjected to 40 min of regional myocardial ischaemia followed by 120 min of reperfusion. The rabbits were randomly assigned to one of the following six experimental groups: sham-operated controls (n=5); ischaemia and reperfusion controls (n=8); isoflurane preconditioning (n=8); a PI3K inhibitor, wortmannin (0.6 mg kg^{-1} i.v.) + isoflurane (n=8); and wortmannin+ischaemia and reperfusion (n=8). An additional control group of sham operation+ wortmannin (n=5) was also included. Myocardial injury was assessed by measuring the serum concentration of the MB fraction of creatine kinase (CK-MB) and infarct size was assessed by 2,3,5-triphenyl tetrazolium chloride staining. Phosphorylation of Akt, a downstream target of PI3K, was assessed by western blotting.

Results. Isoflurane preconditioning was seen as reduced infarct size compared with control animals: 24 (4) and 41 (5)% respectively (P<0.05). Wortmannin inhibited this cardioprotective effect with myocardial infarct size at 44 (3)% (not significant). Akt phosphorylation was increased after isoflurane preconditioning, but administration of wortmannin blocked this effect.

Conclusions. Our data demonstrate that isoflurane protects the heart against ischaemia and decreases myocardial infarction by activation of PI3K.

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Repeated brief episodes of myocardial ischaemia protect the myocardium against subsequent prolonged ischaemic insults. This phenomenon, which has been termed 'ischaemic preconditioning', has been described in various animal models, as well as in humans.[1–3] In addition to ischaemia, myocardial preconditioning can be achieved by several pharmacological agents, including volatile anaesthetics.[4–6] The extent of protection produced by volatile anaesthetics is similar to that observed during classic ischaemic preconditioning.[7 8]

The mechanisms by which volatile anaesthetics protect the heart have been investigated extensively and are believed to involve activation of adenosine receptors[9 10] and protein kinase C,[11 12] release of reactive oxygen species[13–15] and opening of ATP-regulated potassium channels.[6 16 17] However, despite extensive research the precise mechanisms responsible for volatile anaesthetic-induced protection against ischaemic injury are still incompletely understood. Identifying the mechanisms by which isoflurane and other volatile anaesthetic agents mediate their anti-ischaemic actions may be of special clinical significance in protection against the ischaemic events that frequently occur in patients with coronary artery disease in the perioperative period.

Activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway has been shown to be essential for the anti-apoptotic effects of hypoxic preconditioning in cardiomyocytes,[18–20] as well as for infarct size reduction.[21 22]

However, whether PI3K/Akt signalling is essential in anaesthetic-induced myocardial preconditioning is unknown.

Since the signal transduction pathways that are involved in anaesthetic-induced preconditioning share many common steps with the pathways that are activated by ischaemic preconditioning, the purpose of the present study was to evaluate whether isoflurane-induced myocardial preconditioning is mediated via activation of the PI3K/Akt pathway.

Methods

All experiments were conducted with the approval of the institutional Committee for Animal Care and Laboratory Use, and in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

General preparation

New Zealand White male rabbits, weighing 3 (0.5) kg, were anaesthetized with i.v. pentobarbital 30 mg kg^{-1} via a 20-G catheter in a marginal ear vein, followed by a 5 mg kg^{-1} h^{-1} infusion. Isoflurane was used according to the study protocol. Neuromuscular blocking agents were not administered in order to assess anaesthetic depth. A tracheostomy was performed using a ventral midline incision and the rabbits' lungs were ventilated mechanically with positive pressure ventilation using 30–40% air/oxygen mixture to maintain an arterial oxygen partial pressure of 100–150 mm Hg. Ventilation rate was 30–35 bpm and tidal volume was approximately 15 ml kg^{-1}. The respiratory rate was adjusted to maintain blood pH in the range of 7.35–7.45. End-expiratory carbon dioxide tension was monitored continuously. Catheters filled with heparinized saline 10 U ml^{-1} were inserted in a carotid artery for arterial pressure monitoring and blood sampling, and in an internal jugular vein for i.v. drug administration. Maintenance fluids (NaCl 0.9%) were administered at 15 ml kg^{-1} h^{-1} during the experiment. Core body temperature was measured with a rectal temperature probe and maintained at 38.5 (0.2)°C (normothermia for rabbits) with a radiant heat and a warming blanket. A three-lead electrocardiogram was recorded continuously. A left thoracotomy was performed in the fourth intercostal space, the pericardium was opened and the heart was suspended in a pericardial cradle. A 4–0 silk suture was passed around the left anterior descending (LAD) coronary artery just distal to the first diagonal branch with a tapered needle and the ends of the suture were threaded through a small vinyl tube to form a snare. Coronary artery occlusion was performed by tightening the snare around the coronary artery. Myocardial ischaemia was confirmed by regional epicardial cyanosis and ST-segment elevation in the electrocardiogram. Reperfusion was achieved by releasing the snare and confirmed by visual observation of reactive hyperaemia.

Ventricular fibrillation, if it occurred, was reversed using direct mechanical stimulation: an index finger was flicked directly against the right ventricle side of the fibrillating heart one to three times to achieve defibrillation. Failure to convert to an organized rhythm after three attempts was defined as refractory ventricular fibrillation.

Experimental protocol

The experimental design is illustrated in Figure 1. After a 30-min stabilization period, heparin 500 U was administered i.v. to all rabbits. All animals (except for the rabbits in the sham groups) were subjected to 40 min of regional myocardial ischaemia followed by 120 min of reperfusion. Rabbits were randomly assigned to one of the following groups according to a computer-generated number schedule: Group 1, a non-ischaemic control group of sham-operated rabbits (Sham, n=5); Group 2, an ischaemia–reperfusion control group (40 min of myocardial ischaemia and 120 min of reperfusion) (I/R, n=8); Group 3, isoflurane group (Iso, n=8). A minimal alveolar concentration of isoflurane of 1.0 (2.1%)[23] was started at the end of the stabilization period and administered for 30 min, followed by 30 min of washout before coronary occlusion. End-tidal concentrations of isoflurane were measured at the tip of the tracheostomy tube using an infrared anaesthetic analyser (Dräger Medical, Lübeck, Germany) that was calibrated with known standards.

To evaluate the role of the PI3K/Akt pathway in anaesthetic preconditioning, the PI3K inhibitor wortmannin (Sigma, St Louis, MO, USA) was administered i.v. to the next three experimental groups. To rule out any direct effects of wortmannin on the heart during baseline conditions or during ischaemia and reperfusion, two control groups were evaluated. In Group 4 (Sham+Wort, n=5), rabbits were treated with wortmannin (0.6 mg kg^{-1})[24] but did not undergo any coronary intervention. In Group 5, wortmannin (0.6 mg kg^{-1}) was administered 70 min before coronary ischaemia and reperfusion (Wort+I/R, n=8). In Group 6, wortmannin was administered i.v. (0.6 mg kg^{-1}) 10 min before isoflurane preconditioning (Wort+Iso, n=8) to test the hypothesis that isoflurane-induced cardioprotection is mediated by the PI3K/Akt pathway.

Blood pressure, heart rate and temperature were recorded continuously. Blood samples were obtained and analysed for serum concentrations of the MB fraction of creatine kinase (CK-MB) by an enzymatic assay method using a CK assay kit (Sigma Diagnostics, St Louis, MO, USA). Samples were collected at the following time points: immediately after anaesthetizing the animals (baseline sample), after 20 min and 40 min during coronary occlusion, and after 30, 60 and 120 min of reperfusion (Fig. 2).

Determination of infarct size and area at risk

Determination of infarct size was performed as described previously,[25] with slight modifications. Briefly, at the end of

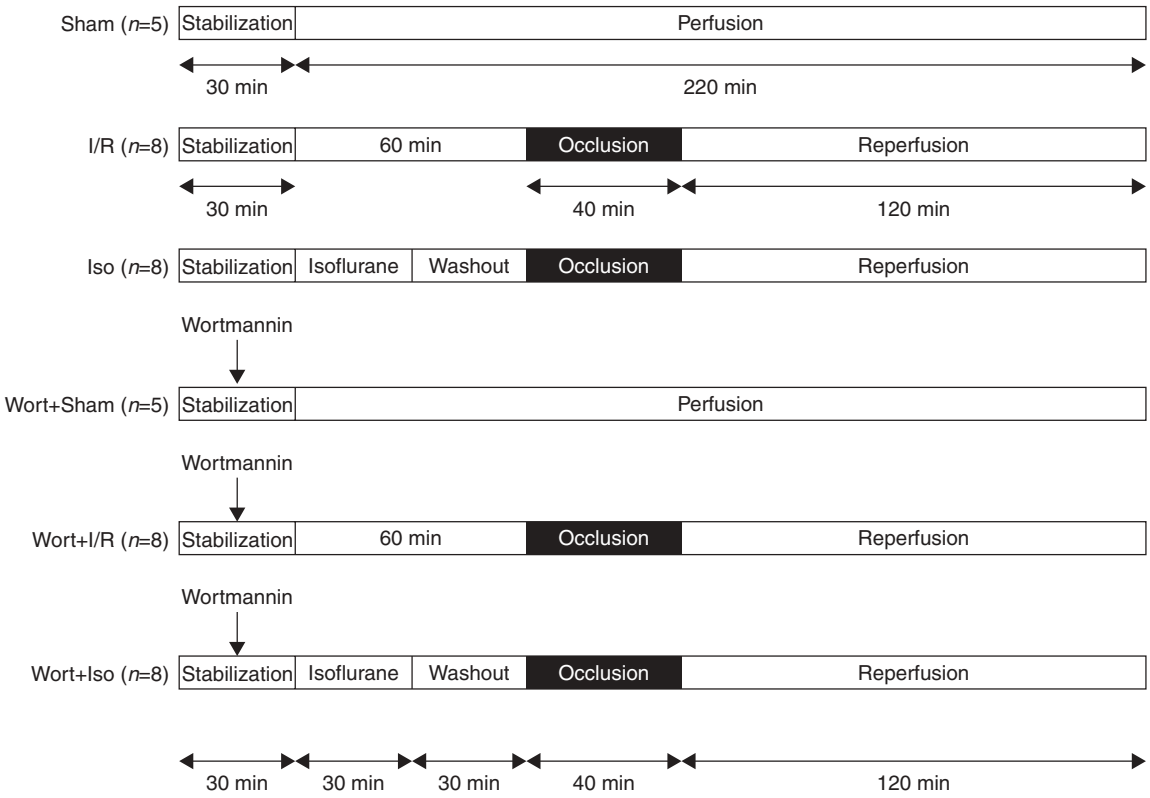

Fig 1 Diagram of the experimental protocol. Animals were subjected to 40 min of regional myocardial ischaemia and 120 min of reperfusion. One minimal alveolar concentration of isoflurane was administered for 30 min followed by 30 min of washout before coronary occlusion. Wortmannin (0.6 mg kg^{-1}, i.v.) was administered 10 min before isoflurane preconditioning. I/R, ischaemia–reperfusion; Iso, isoflurane; Wort+I/R, wortmannin+ischaemia–reperfusion; Wort+Iso, Wortmannin+isoflurane.

the experimental protocol, hearts were excised, mounted on a Langendorff apparatus, and perfused with phosphate-buffered saline at 100 cm H_2O for 1 min in order to wash out intravascular blood. The coronary artery was re-occluded and methylene blue 0.1%, 10 ml was infused into the aortic root to label the normally perfused zone with a deep blue colour, thereby delineating the risk zone as a non-stained area. The hearts were then removed from the Langendorff apparatus, trimmed of atria and great vessels, weighed and frozen (in a cold chamber at −18°C). Hearts were then cut into 2 mm transverse slices. The slices were incubated in 2,3,5-triphenyl tetrazolium chloride (TTC) 1% in pH 7.4 buffer for 20 min at 37°C. The slices were then placed in 10% neutral buffered formalin for 10 min to increase the contrast between stained and non-stained tissue. Since TTC stains viable tissue a deep red colour, non-stained tissue was presumed to be infarcted. Slices were then photographed, and the risk and infarct areas in each slice were measured by computed planimetry. The mass-weighted average of the ratio of infarct area to the area at risk of the ventricle from each slice was determined (percent infarction).

Western immunoblotting

In a second set of experiments, six similar experimental groups of rabbits (n=5 in each group) were subjected to the same experimental procedures. At the end of reperfusion the animals were killed by an i.v. injection of 10% KCl solution and myocardial samples were collected from ischaemic left ventricle regions for evaluation of tissue abundance of total Akt and phospho-Akt. Samples were frozen in liquid nitrogen and kept at −80°C until further processed. Tissue was homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.4), NaCl 150 mM, Na_2EDTA 1 mM, EGTA 1 mM, Nonidet P40 1%, sodium pyrophosphate 2.5 mM, Na_3VO_4 1 mM, and complete proteinase inhibitor cocktail (one tablet per 10 ml; Roche Diagnostics, Indianapolis, IN, USA). The homogenate was centrifuged at 10 000 *g* for 15 min at 4°C to remove cellular debris and isolate total protein. Protein concentrations were determined using a modified Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. Equivalent amounts (50 μg) of protein samples were loaded and separated on a 6% SDS-PAGE gel and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween-20, nitrocellulose membranes were incubated overnight at 4°C in 0.1% Tween-20 containing 5% milk and a 1:1000 dilution of monoclonal antibody against Ser^{473} of phospho-Akt (Cell Signaling Technology, Beverly, MA, USA). Membranes were then washed three times with 0.1% Tween-20 for 5 min and subsequently incubated

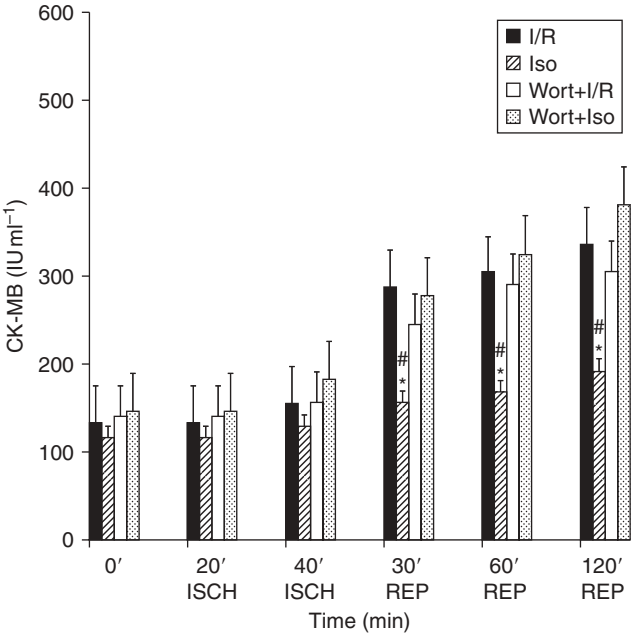

Fig 2 CK-MB concentrations. Serum CK-MB concentrations were analysed by an enzymatic assay using a CK assay kit. The increase in CK-MB concentrations was lower in rabbits pretreated with isoflurane than in controls (I/R group). Wortmannin abolished this protective effect and the increase in CK-MB was similar to that seen in the control group. Data are mean and SEM. ISCH, ischaemia; REP, reperfusion; I/R, ischaemia–reperfusion; Iso, isoflurane; Wort+I/R, wortmannin+ischaemia–reperfusion; Wort+Iso, wortmannin+isoflurane. *P<0.05 *vs* I/R; #P<0.05 *vs* Wort+Iso.

with a 1:3000 dilution of horseradish peroxidase-labelled anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1% Tween-20 containing 5% milk. Bound antibody signals were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA), followed by exposure to hyperfilm (Amersham Pharmacia Biotech). β-Actin (Santa Cruz Biotechnology; 1:2000) was detected on immunoblots as a loading control for protein quantitation. For determination of total Akt abundance, the membrane was stripped with stripping buffer (Pierce, Rockford, IL, USA) and reprobed with polyclonal goat Akt antibody (Santa Cruz Biotechnology). Then the membrane was subjected to interventions identical to those described above. Optical density for each western blot band was quantified with ImageQuant 5.0 Windows NT software (Molecular Dynamics, Sunnyvale, CA, USA).

Each western blot assay was repeated three times.

Data analysis and statistics

Changes in haemodynamics were analysed using two-way analysis of variance (ANOVA) to determine the main effect of time, group, and time × group interaction, followed by the Student–Newman–Keuls *post hoc* test. Intergroup comparisons for infarct sizes were made with one-way ANOVA and group differences were detected with the Student–Newman–Keuls *post hoc* test. Difference between groups in CK-MB concentrations were assessed by ANOVA with the Student–Newman–Keuls *post hoc* test. Differences between groups in phospho-Akt tissue abundance were calculated by ANOVA with the Student–Newman–Keuls *post hoc* test. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). Data are presented as mean (SEM). Significance level was set at P<0.05.

Results

Seventy-two rabbits were used to successfully complete 67 experiments. Five animals were excluded because of refractory ventricular fibrillation. The incidence of refractory ventricular fibrillation was not significantly different among the groups (I/R, 2/8; Iso, 1/8; Wort+Iso, 1/8; Wort+I/R, 1/8). There was no mortality in the sham-operated animals.

Systemic haemodynamics

Haemodynamic data are presented in Table 1. There were no differences in baseline haemodynamic parameters between groups, nor there were any differences recorded during the time course of the experiment in the two sham-operated animal groups. Mean arterial pressure decreased in the isoflurane group during administration of the volatile anaesthetic. After the washout period the pressure returned to baseline values. A transient increase in heart rate (not significant) was also noted during isoflurane administration. LAD occlusion significantly decreased (P<0.05) mean arterial pressure and the rate–pressure product in the I/R, Wort+Iso and Wort+I/R groups. Heart rate, mean arterial pressure and rate–pressure product decreased over time during reperfusion in all experimental groups. This reduction, which can be explained at least partially by a decrease in the surgical stimuli, was significant compared with baseline values (P<0.05). However, there were no significant differences in the haemodynamic parameters between groups during reperfusion.

CK-MB concentrations and myocardial infarct size

In the I/R group the CK-MB concentration increased by 237 (12)% after 120 min of reperfusion, whereas isoflurane attenuated this increase to only 107 (6)% at the same time point (Fig. 2; P<0.05). Administration of the PI3K inhibitor wortmannin inhibited the cardioprotective effect of isoflurane, and CK-MB concentrations increased maximally by 267 (9)% (Fig. 2; P<0.05 compared with the Iso group). In the Wort+I/R group CK-MB increased by 217 (11)% above baseline concentrations after 120 min of reperfusion.

The ratio of area at risk to left ventricular mass did not differ significantly among the groups: 51 (2)% in the I/R group, 54 (3)% in the Iso group, 47 (4)% in the Wort+I/R group and 48 (3)% in the Wort+Iso group. These data

Table 1 Systemic haemodynamics. Five animals were excluded from the study because of refractory ventricular fibrillation (I/R group, 2/8; Iso group, 1/8; Wort+I/R group, 1/8; Wort+Iso group, 1/8). The sizes of the groups in the table are after exclusion of the five rabbits that did not complete the experiments. Data are mean (SEM). I/R, ischaemia–reperfusion; Iso, isoflurane; Wort+I/R, wortmannin+ischaemia–reperfusion; Wort+Iso, wortmannin+isoflurane; MAP, mean arterial pressure; RPP, rate–pressure product. *$P<0.05$ *vs* baseline values

	No.	Baseline	Coronary occlusion (min)		Reperfusion (min)			
			20	40	5	30	60	120
Heart rate (beats min^{-1})								
I/R	6	253 (6)	257 (4)	260 (8)	233 (12)*	228 (5)*	227 (6)*	220 (9)*
Iso	7	247 (4)	261 (11)	266 (7)	241 (8)*	236 (3)*	233 (7)*	215 (6)*
Wort+I/R	7	259 (7)	254 (8)	250 (3)	244 (12)*	229 (8)*	226 (4)*	221 (9)*
Wort+Iso	7	255 (8)	263 (13)	269 (10)	239 (10)*	231 (7)*	230 (5)*	219 (4)*
MAP (mm Hg)								
I/R	6	84 (3)	66 (5)*	62 (9)*	64 (7)*	58 (4)*	55 (2)*	54 (4)*
Iso	7	87 (6)	82 (7)	79 (5)	61 (3)*	60 (6)*	58 (7)*	55 (6)*
Wort+I/R	7	83 (5)	75 (2)*	77 (11)	70 (2)*	61 (8)*	60 (5)*	60 (9)*
Wort+Iso	7	86 (8)	71 (8)*	74 (10)	66 (6)*	59 (3)*	52 (4)*	53 (6)*
RPP (min^{-1} mm Hg 10^3)								
I/R	6	21.6 (0.8)	17 (1)*	16.1 (1.3)*	14.9 (0.7)*	13.2 (0.6)*	12.5 (0.5)*	11.9 (0.7)*
Iso	7	22 (1.1)	20.6 (0.7)*	19.9 (0.4)*	14.7 (1.4)*	14.2 (0.4)*	13.5 (1.1)*	11.8 (0.9)*
Wort+I/R	7	21.5 (1.1)	19.1 (1.2)*	19.2 (0.9)	17.1 (1.1)*	14 (1.2)*	13.5 (0.4)*	13.3 (1.1)*
Wort+Iso	7	21.9 (0.6)	18.7 (0.6)*	19.9 (1.3)	15.8 (0.9)*	13.6 (1.5)*	12 (1.3)*	11.6 (1.4)*

suggest that changes in the infarct sizes observed in the various experimental groups cannot be related to the percentage of the left ventricular myocardium that was occluded.

In the I/R group the infarct size was 41 (5)% of the area at risk. Pretreatment with isoflurane had a cardioprotective effect and the infarct size was reduced to 24 (4)% ($P<0.05$ compared with the I/R group). Administration of wortmannin before myocardial ischaemia and reperfusion did not affect infarct size [38 (3)%; not significant compared with the I/R group]. Wortmannin eliminated the cardioprotection produced by 1 MAC of isoflurane [44 (3)%; $P<0.05$ compared with the Iso group] (Fig. 3).

In the various groups of sham-operated animals there was no increase in CK-MB concentration above baseline, nor were any myocardial infarctions observed (data not shown).

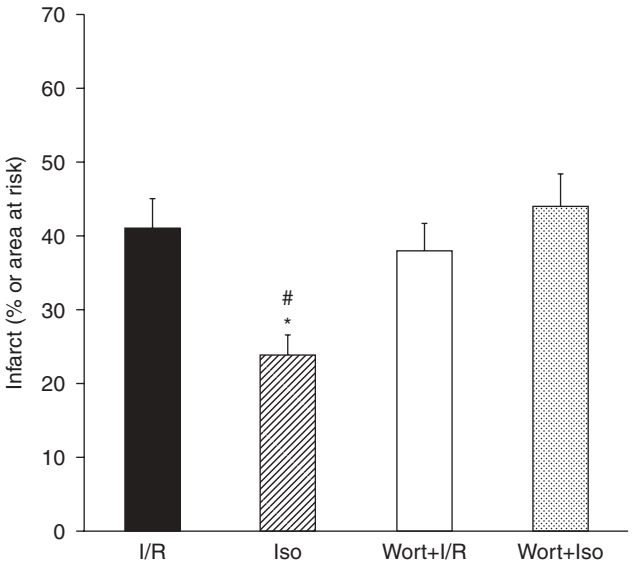

Fig 3 Effects of isoflurane preconditioning with and without wortmannin administration on infarct size. Bar graph shows infarct size as percentage of the area at risk. Isoflurane significantly decreased infarct size compared with control (I/R) animals, whereas wortmannin abolished this protective effect and infarct size was similar to that of the controls. Data are mean and SEM. I/R, ischaemia–reperfusion; Iso, isoflurane; Wort+I/R, wortmannin+ischaemia–reperfusion; Wort+Iso, wortmannin+isoflurane. *$P<0.05$ *vs* I/R group; #$P<0.05$ *vs* Wort+Iso.

Isoflurane preconditioning increases the expression of phosphorylated Akt

To evaluate the expression of total Akt and its activated, phosphorylated form (phospho-Akt) during myocardial ischaemia and reperfusion and during anaesthetic-induced preconditioning, western blot analysis of total Akt and of phospho-Akt at Ser^{473} (Fig. 4) was used. Total Akt expression was comparable in all experimental groups. Phospho-Akt expression was significantly increased after isoflurane preconditioning. Pretreatment with wortmannin inhibited the phosphorylation of Akt in isoflurane-treated and non-treated rabbits to a degree comparable to that in sham-operated animals.

Discussion

The results of the present investigation confirm our hypothesis that activation of the PI3K/Akt signalling pathway is involved in anaesthetic-induced preconditioning and that inhibition of the PI3K cascade by a selective inhibitor, wortmannin, caused loss of isoflurane-mediated cardioprotection. Our findings are important, therefore, because it has been known for several years that anaesthetic preconditioning confers protection against ischaemia and reperfusion in various organs, including the heart[4,6–8] and the brain.[26 27] However, the mechanisms involved in this phenomenon are not completely understood despite extensive research.

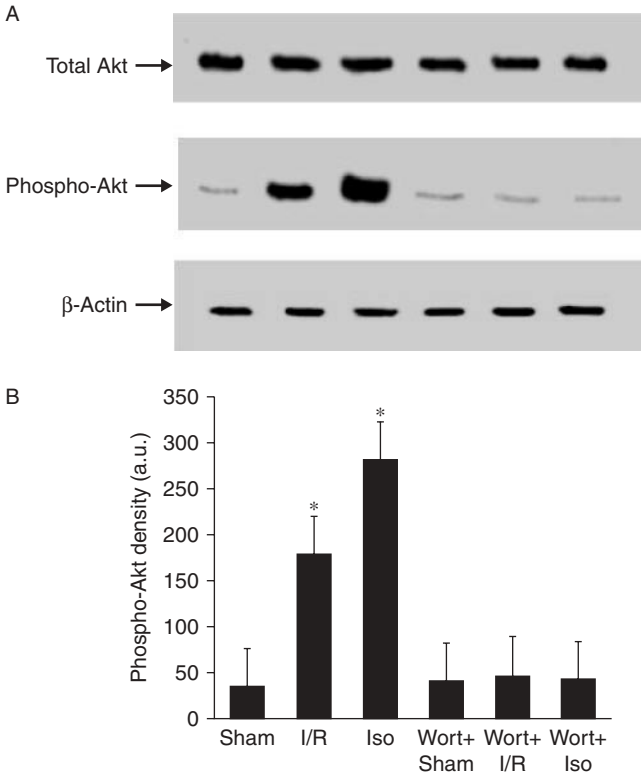

Fig 4 Effects of isoflurane with and without administration of wortmannin on Akt phosphorylation. (A) Representative western blot analysis of total Akt (top lanes) and phosphorylation of Akt at Ser^{473} (middle lanes). In each lane the protein concentration was 50 μg. β-Actin (lower lanes) was used to demonstrate equal protein loading. (B) Graphic presentation of the phospho-Akt quantified by integrating the volume of autoradiograms from three separate experiments. Values in the graph are expressed as fold changes over the control and are presented as mean and SEM. I/R, ischaemia–reperfusion; Iso, isoflurane; Wort+I/R, wortmannin+ischaemia–reperfusion; Wort+Iso, wortmannin+isoflurane. n=5 in each group. *P<0.05 *vs* sham-operated group.

Activation of the PI3K/Akt pathway has been demonstrated to play a key role in both early and delayed myocardial preconditioning.[19–22 28] PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate.[28] Phosphatidylinositol-3,4,5-trisphosphate-stimulated phosphorylation of the serine-threonine kinase Akt by phosphoinositide-dependent kinase 1 subsequently inhibits formation of the proapoptotic proteins Bad, Bax and caspase 9.[29] Moreover, Akt has been shown to increase the formation of nitric oxide.[30] The protective actions of nitric oxide during myocardial preconditioning have already been demonstrated.[31–34] Furthermore, a recent study by Chiari and colleagues has also shown that endothelial nitric oxide synthase has an important role in isoflurane-induced delayed preconditioning in rabbits.[35] In addition, phosphoinositide-dependent kinase 1 is a potent activator of other protein kinases, including protein kinase C, that have been implicated in the protection of myocardium against ischaemia and reperfusion injury produced by ischaemic[36] and anaesthetic[37 38] preconditioning. Volatile anaesthetic-induced preconditioning involves activation of A_1 adenosine receptors and G_i proteins,[9 10] protein kinase C,[11 12] release of reactive oxygen species[13–15] and opening of ATP-regulated potassium channels.[6 16 17] Furthermore, as already mentioned, nitric oxide is believed to play a major role in anaesthetic-induced cardioprotection, both as a mediator and as an effector of isoflurane-induced preconditioning.[20 35 39] The regulatory role of the PI3K/Akt survival pathway in nitric oxide synthesis is well established.[36] Therefore, it seems that the PI3K signalling cascade may contribute to the recruitment of multiple endogenous cardioprotective pathways to reduce myocardial damage after ischaemia and reperfusion.

We used a well established model of regional myocardial ischaemia and reperfusion in rabbits. This model has also been used by others with similar favourable results when infarct size is the end-point.[4 5] The question of whether the timing of isoflurane administration could have influenced the results of the present investigation may be raised. In a study that was performed by Preckel,[40] isoflurane did not affect infarct size in an *in vivo* rabbit model when administered a few minutes before reperfusion and continued for 15 min during the reperfusion period. However, the study of Chiari and colleagues[41] demonstrated a favourable effect of isoflurane when administered during reperfusion—an effect called 'anaesthetic postconditioning'. This effect was mediated by activation of the PI3K/Akt pathway. Therefore, in our opinion, changing the timing of isoflurane administration will probably not alter the isoflurane-induced myocardial protection. Although a beneficial effect of isoflurane has already been demonstrated in postconditioning, the present study demonstrates for the first time that anaesthetic preconditioning by isoflurane is mediated by activation of the PI3K/Akt signalling pathway and that isoflurane causes an increase in the expression of phospho-Akt.

The present results must be interpreted with caution. The PI3K/Akt signalling pathway has been clearly implicated as protective in apoptosis that is triggered by reperfusion.[28 42] Our results suggest that activation of PI3K mediates salvage of myocardium against infarction during preconditioning by the volatile anaesthetic agent isoflurane, but further investigations are needed to ascertain whether these beneficial actions also involve a decrease in apoptosis. Wortmannin has been shown to be a selective PI3K inhibitor at the dose used in the present investigation.[24 43] Nevertheless, the possibility that wortmannin may have inhibited other protein kinases involved in myocardial protection cannot be completely excluded.

Myocardial infarct size is determined primarily by the size of the area at risk and the extent of coronary collateral perfusion. The area at risk, expressed as a percentage of total left ventricle mass, was similar between groups in the present investigation. Coronary collateral blood flow was not specifically quantified in the present investigation. However, rabbits have been shown to possess little if any coronary collateral blood flow.[44] Thus, it appears unlikely that

differences in collateral perfusion between groups account for the observed results.

In the present investigation we used CK-MB to evaluate the extent of myocardial injury. Although troponins are more specific, CK-MB is still in wide use for evaluation of myocardial injury.[45 46] One may comment that a part of our measured CK-MB concentrations may originate from muscle injury during the surgical procedure. Indeed, striated muscle damage can result in elevated CK-MB concentrations. However, considering the fact that all animals went through the same surgical procedure, we assume that the extent of muscle injury was similar in all the experimental groups. Therefore, the differences in CK-MB concentrations probably correlate with differences in the extent of myocardial injury. Furthermore, the increase in CK-MB concentrations occurred mainly during the reperfusion phase, which is more compatible with myocardial injury than with muscle damage.

Phosphorylation of Akt by isoflurane and its inhibition by the PI3K antagonist wortmannin provides strong supportive evidence for the involvement of PI3K in isoflurane-induced preconditioning. Nevertheless, the possibility that another unrelated protein kinase was responsible for phosphorylation of Akt cannot be entirely excluded.

In summary, the present investigation indicates that preconditioning by administration of 1.0 MAC isoflurane before myocardial ischaemia and reperfusion salvages myocardium from infarction. These beneficial effects of anaesthetic-induced preconditioning are mediated by activation of the PI3K signalling pathway. Additional research will be required to identify other signalling elements involved in preconditioning by anaesthetics and clarify the mechanisms responsible for this phenomenon.

References

1 Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74**: 1124–36

2 Li YW, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* 1992; **123**: 346–53

3 Alkhulaifi AM. Preconditioning the human heart. *Ann R Coll Surg Engl* 1997; **79**: 49–54

4 Cason BA, Gamperl AK, Slocum RE, Hickey RF. Anesthetic-induced preconditioning: previous administration of isoflurane decreases myocardial infarct size in rabbits. *Anesthesiology* 1997; **87**: 1182–90

5 Ismaeil MS, Tkachenko I, Gamperl AK, Hickey RF, Cason BA. Mechanisms of isoflurane-induced myocardial preconditioning in rabbits. *Anesthesiology* 1999; **90**: 812–21

6 Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC. Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels: reduction of myocardial infarct size with an acute memory phase. *Anesthesiology* 1997; **87**: 361–70

7 Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC. Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth* 2003; **91**: 551–65

8 Chiari PC, Pagel PS, Tanaka K, *et al*. Intravenous emulsified halogenated anesthetics produce acute and delayed preconditioning against myocardial infarction in rabbits. *Anesthesiology* 2004; **101**: 1160–6

9 Kersten JR, Orth KG, Pagel PS, Mei DA, Gross GJ, Warltier DC. Role of adenosine in isoflurane-induced cardioprotection. *Anesthesiology* 1997; **86**: 1128–39

10 Roscoe AK, Christensen JD, Lynch C 3rd. Isoflurane, but not halothane, induces protection of human myocardium via adenosine A1 receptors and adenosine triphosphate-sensitive potassium channels. *Anesthesiology* 2000; **92**: 1692–701

11 Uecker M, Da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M. Translocation of protein kinase C isoforms to subcellular targets in ischemic and anesthetic preconditioning. *Anesthesiology* 2003; **99**: 138–47

12 Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF. Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. *Anesthesiology* 2003; **99**: 421–8

13 Tanaka K, Weihrauch D, Kehl F, *et al*. Mechanism of preconditioning by isoflurane in rabbits: a direct role for reactive oxygen species. *Anesthesiology* 2002; **97**: 1485–90

14 Mullenheim J, Ebel D, Frassdorf J, Preckel B, Thamer V, Schlack W. Isoflurane preconditions myocardium against infarction via release of free radicals. *Anesthesiology* 2002; **96**: 934–40

15 Novalija E, Varadarajan SG, Camara AK, *et al*. Anesthetic preconditioning: triggering role of reactive oxygen and nitrogen species in isolated hearts. *Am J Physiol Heart Circ Physiol* 2002; **283**: H44–52

16 Pain T, Yang XM, Critz SD, *et al*. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000; **87**: 460–6

17 Kersten JR, Schmeling TJ, Hettrick DA, Pagel PS, Gross GJ, Warltier DC. Mechanism of myocardial protection by isoflurane. Role of adenosine triphosphate-regulated potassium (KATP) channels. *Anesthesiology* 1996; **85**: 794–807; discussion 27A

18 Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation* 2004; **109**: 3042–9

19 Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. *Am J Physiol Heart Circ Physiol* 2005; **288**: H971–6

20 Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol* 2004; **37**: 449–71

21 Kis A, Yellon DM, Baxter GF. Second window of protection following myocardial preconditioning: an essential role for PI3 kinase and p70S6 kinase. *J Mol Cell Cardiol* 2003; **35**: 1063–71

22 Murphy E. Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. *Circ Res* 2004; **94**: 7–16

23 Drummond JC. MAC for halothane, enflurane, and isoflurane in the New Zealand white rabbit: and a test for the validity of MAC determinations. *Anesthesiology* 1985; **62**: 336–8

24 Kim CH, Cho YS, Chun YS, Park JW, Kim MS. Early expression of myocardial HIF-1alpha in response to mechanical stresses: regulation by stretch-activated channels and the phosphatidylinositol 3-kinase signaling pathway. *Circ Res* 2002; **90**: E25–33

25 Gozal Y, Wolff RA, Van Winkle DM. Manipulations in glycogen metabolism and the failure to influence infarct size in the ischaemic rabbit heart. *Eur J Anaesthesiol* 2002; **19**: 495–503

26 Zheng S, Zuo Z. Isoflurane preconditioning reduces purkinje cell death in an *in vitro* model of rat cerebellar ischemia. *Neuroscience* 2003; **118**: 99–106

27 Zhao P, Zuo Z. Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase-dependent in neonatal rats. *Anesthesiology* 2004; **101**: 695–703

28 Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. *Cardiovasc Res* 2004; **61**: 448–60

29 Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002; **296**: 1655–7

30 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; **399**: 601–5

31 Berges A, Van Nassauw L, Bosmans J, Timmermans JP, Vrints C. Role of nitric oxide and oxidative stress in ischaemic myocardial injury and preconditioning. *Acta Cardiol* 2003; **58**: 119–32

32 Cho S, Park EM, Zhou P, Frys K, Ross ME, Iadecola C. Obligatory role of inducible nitric oxide synthase in ischemic preconditioning. *J Cereb Blood Flow Metab* 2005; **25**: 493–501.

33 Dawn B, Bolli R. Role of nitric oxide in myocardial preconditioning. *Ann N Y Acad Sci* 2002; **962**: 18–41

34 Novalija E, Hogg N, Kevin LG, Camara AK, Stowe DF. Ischemic preconditioning: triggering role of nitric oxide-derived oxidants in isolated hearts. *J Cardiovasc Pharmacol* 2003; **42**: 593–600

35 Chiari PC, Bienengraeber MW, Weihrauch D, *et al*. Role of endothelial nitric oxide synthase as a trigger and mediator of isoflurane-induced delayed preconditioning in rabbit myocardium. *Anesthesiology* 2005; **103**: 74–83

36 Tong H, Chen W, Steenbergen C, Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ Res* 2000; **87**: 309–15

37 Obal D, Weber NC, Zacharowski K, *et al*. Role of protein kinase C-epsilon (PKCepsilon) in isoflurane-induced cardioprotection. *Br J Anaesth* 2005; **94**: 166–73

38 Toma O, Weber NC, Wolter JI, Obal D, Preckel B, Schlack W. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart *in vivo*. *Anesthesiology* 2004; **101**: 1372–80

39 Uruno A, Sugawara A, Kanatsuka H, *et al*. Upregulation of nitric oxide production in vascular endothelial cells by all-*trans* retinoic acid through the phosphoinositide 3-kinase/akt pathway. *Circulation* 2005; **112**: 727–36

40 Preckel B, Schlack W, Thamer V. Enflurane and isoflurane, but not halothane, protect against myocardial reperfusion injury after cardioplegic arrest with HTK solution in the isolated rat heart. *Anesth Analg* 1998; **87**: 1221–7

41 Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR, Warltier DC. Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: evidence for anesthetic-induced postconditioning in rabbits. *Anesthesiology* 2005; **102**: 102–9

42 Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 1994; **94**: 1621–8

43 Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000; **351**: 95–105

44 Maxwell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc Res* 1987; **21**: 737–46

45 Korbmacher B, Klein KK, Sunderdiek U, Gams E, Schipke JD. Does adenosine pharmacologically precondition human myocardium during coronary bypass surgery? *J Cardiovasc Surg (Torino)* 2005; **46**: 285–90

46 Nayeem MA, Matherne GP, Mustafa SJ. Ischemic and pharmacological preconditioning induces further delayed protection in transgenic mouse cardiac myocytes over-expressing adenosine A1 receptors (A1AR): role of A1AR, iNOS and K(ATP) channels. *Naunyn Schmiedebergs Arch Pharmacol* 2003; **367**: 219–26