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Isoflurane Postconditioning Prevents Opening of the Mitochondrial Permeability Transition Pore through Inhibition of Glycogen Synthase Kinase 3β

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Background: Postischemic administration of volatile anesthetics activates reperfusion injury salvage kinases and decreases myocardial damage. However, the mechanisms underlying anesthetic postconditioning are unclear.

Methods: Isolated perfused rat hearts were exposed to 40 min of ischemia followed by 1 h of reperfusion. Anesthetic postconditioning was induced by 15 min of 2.1 vol% isoflurane (1.5 minimum alveolar concentration) administered at the onset of reperfusion. In some experiments, atractyloside (10 μM), a mitochondrial permeability transition pore (mPTP) opener, and LY294002 (15 μM), a phosphatidylinositol 3-kinase inhibitor, were coadministered with isoflurane. Western blot analysis was used to determine phosphorylation of protein kinase B/Akt and its downstream target glycogen synthase kinase 3β after 15 min of reperfusion. Myocardial tissue content of nicotinamide adenine dinucleotide served as a marker for mPTP opening. Accumulation of MitoTracker Red 580 (Molecular Probes, Invitrogen, Basel, Switzerland) was used to visualize mitochondrial function.

Results: Anesthetic postconditioning significantly improved functional recovery and decreased infarct size (36 ± 1% in unprotected hearts *vs.* 3 ± 2% in anesthetic postconditioning; *P* < 0.05). Isoflurane-mediated protection was abolished by atractyloside and LY294002. LY294002 inhibited isoflurane-induced phosphorylation of protein kinase B/Akt and glycogen synthase kinase 3β and opened mPTP as determined by nicotinamide adenine dinucleotide measurements. Atractyloside, a direct opener of the mPTP, did not inhibit phosphorylation of protein kinase B/Akt and glycogen synthase kinase 3β by isoflurane but reversed isoflurane-mediated cytoprotection. Microscopy showed accumulation of the mitochondrial tracker in isoflurane-protected functional mitochondria but no staining in mitochondria of unprotected hearts.

Conclusions: Anesthetic postconditioning by isoflurane effectively protects against reperfusion damage by preventing opening of the mPTP through inhibition of glycogen synthase kinase 3β.

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POSTCONDITIONING is an effective therapeutic strategy of attaining myocardial protection against ischemia–reperfusion damage. Ischemic postconditioning is elicited by brief episodes of ischemia right at the onset of reperfusion and modifies the conditions of reperfusion,[1] whereas pharmacologic postconditioning by volatile anesthetics ("anesthetic postconditioning") modifies postischemic cellular signaling.[2,3] From a clinical point of view, postconditioning is particularly promising because no previous knowledge of the onset of the ischemic event is required to provide effective protection.

An increasing body of evidence supports the concept that preconditioning and postconditioning enhance activation of multiple prosurvival reperfusion injury salvage kinases during early recovery from ischemia.[3–6] Chiari *et al.*[3] reported in an *in vivo* rabbit model that isoflurane administered during early reperfusion enhances activation of protein kinase B (PKB)/Akt. Similarly, in anesthetic preconditioning, da Silva *et al.*[4] observed increased activity of extracellular signal–regulated kinase 1/2 after ischemia–reperfusion. Of note, enhanced activation of reperfusion injury salvage kinases was causally related and tightly linked to cytoprotection in these studies.

Mitochondrial permeability transition pore (mPTP) is a critical determinant of cell death in ischemia–reperfusion injury.[7,8] Opening of mPTP induces apoptosis and necrosis. Recently, glycogen synthase kinase 3β (GSK3β), a direct PKB/Akt downstream target, has been demonstrated to mediate convergence of myocyte protection signaling through inhibition of mPTP opening.[9] Although prevention of mPTP opening has been reported in anesthetic preconditioning[10] and ischemic postconditioning,[11] the role of mPTP in anesthetic postconditioning–induced cytoprotection has not been evaluated so far. Therefore, this strongly prompted us to investigate whether inhibition of the mPTP is involved in anesthetic postconditioning by isoflurane. Specifically, we hypothesized that isoflurane administration during early reperfusion would prevent mPTP opening. In addition, we investigated whether the activity of the known mPTP modulator and PKB/Akt downstream target GSK3β are inhibited by isoflurane postconditioning (fig. 1).

The data presented in this study provide strong evidence that PKB/Akt-dependent phosphorylation and inhibition of GSK3β prevents mPTP opening during early reperfusion and thereby mediates cytoprotection in anesthetic postconditioning.
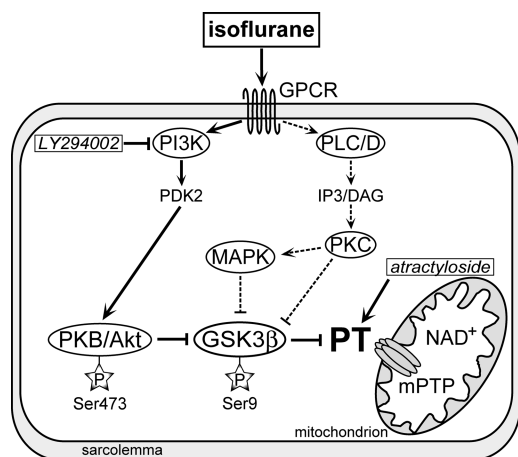
Fig. 1. Investigated signaling pathways (*full lines*). During early reperfusion, multiple signaling cascades inhibit the master switch kinase glycogen synthase kinase 3β (GSK3β), which converges the prosurvival pathways and prevents permeability transition (PT) in mitochondria. Beside other kinases, protein kinase B (PKB)/Akt represents a key enzyme in the reperfusion injury salvage kinase cascade requiring phosphorylation at Ser473 for full activation. Phosphorylated PKB/Akt subsequently inactivates its downstream target GSK3β by phosphorylation at Ser9. LY294002 specifically inhibits phosphatidylinositol 3-kinase (PI3K). Atractyloside induces opening of the mitochondrial permeability transition pore (mPTP). *Arrows* indicate positive activity, and *lines with blunted ends* indicate inhibition. DAG = diacylglycerol; GPCR = G protein–coupled receptor; IP3 = inositol triphosphate; MAPK = mitogen-activated protein kinases; NAD$^+$ = nicotinamide adenine dinucleotide; PDK2 = phosphatidylinositol-dependent kinase 2, also called Ser473 kinase; PKC = protein kinase C; PLC/D = phospholipase C/D.

Materials and Methods

This study was conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Zurich, Zurich, Switzerland.

Isolated Perfused Rat Heart Preparation

Male Wistar rats (250 g) were heparinized (500 U intraperitoneal) and 15 min later decapitated without previous anesthesia. The hearts were removed and perfused in a noncirculating Langendorff apparatus with Krebs-Henseleit buffer (155 mM Na$^+$, 5.6 mM K$^+$, 138 mM Cl$^-$, 2.1 mM Ca^{2+}, 1.2 mM PO$_4$ $^{3-}$, 25 mM HCO$_{3-}$, 0.56 mM Mg^{2+}, 11 mM glucose) gassed with 95% O$_2$–5% CO$_2$ (pH 7.4, temperature 37°C). Perfusion pressure was set to 80 mmHg. Left ventricular developed pressure and derivatives, end-diastolic pressure, epicardial electrocardiogram, coronary flow, and perfusion pressure were recorded on a personal computer, as previously described.[12]

Available at: http://rsb.info.nih.gov/ij/. Accessed December 1, 2004.

Experimental Protocols, Analysis of Functional Parameters, and Determination of Infarct Size

Spontaneously beating hearts were equilibrated for 10 min. After 40 min of test ischemia, anesthetic postconditioning was induced by isoflurane administered for 15 min at 1.5 minimum alveolar concentration (MAC; 2.1 vol%) immediately at the onset of reperfusion. The buffer solution was equilibrated with isoflurane using an Isotec 3 vaporizer (Datex-Ohmeda, Tewksbury, MA) with an air bubbler. Isoflurane concentrations were also measured in the buffer solution right before entering the aorta using a gas chromatograph (Perkin-Elmer, Norwalk, CT): 2.1% isoflurane (vol/vol) (1.5 MAC in rats at 37°C), 0.51 ± 0.05 mM. Care was taken that all reservoirs were filled with buffer saturated with 1.5 MAC isoflurane by adding isoflurane to the perfusate 10 min before opening the stopcock for reperfusion. In some experiments, 15 μM LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride; Alexis, Lausen, Switzerland), a specific inhibitor of phosphatidylinositol 3-kinase, was coadministered with isoflurane.[13] Similarly, 10 μM of the specific mPTP opener atractyloside (Sigma, St. Louis, MO), a kaurene-type diterpene glycoside originally isolated from the Mediterranean thistle *Atractylis gummifera*, was also coadministered with isoflurane to induce mPTP opening. Previous studies used 20 μM atractyloside to inhibit ischemic postconditioning.[14] However, preliminary experiments showed that 10 μM atractyloside was sufficient to block isoflurane postconditioning. Both LY294002 and atractyloside were dissolved in dimethyl sulfoxide at a final concentration of less than 0.1%. For each experimental group, five hearts (n = 5) were prepared, and functional parameters were recorded (fig. 2) and used to determine infarct size by 2,3,5-triphenyltetrazolium chloride staining, as previously described.[15] Briefly, hearts were frozen at −20°C for 2 h at the end of the experiment and subsequently sliced into five 2-mm cross-sections. The sections were incubated at 37°C for 30 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer with pH adjusted to 7.4. Slices were fixed overnight in 10% formaldehyde and digitally photographed. Planimetric analysis was performed using ImageJ 1.33.# Because the entire left ventricle was at risk (global ischemia), infarct size was determined by dividing the total necrotic area of the left ventricle by the total left ventricular slice area (percent necrotic area). Hearts subjected to ischemia and reperfusion alone served as ischemic control. Separate experiments (n = 5 for each experimental group) served to determine PKB/Akt and GSK3β activity and nicotinamide adenine dinucleotide (NAD$^+$) tissue content after 15 min of reperfusion. Preliminary experiments in our model showed that PKB/Akt and GSK3β phosphorylation was consistently increased after 15 min of reperfusion and that differences between experimental groups were most pronounced at this time point.
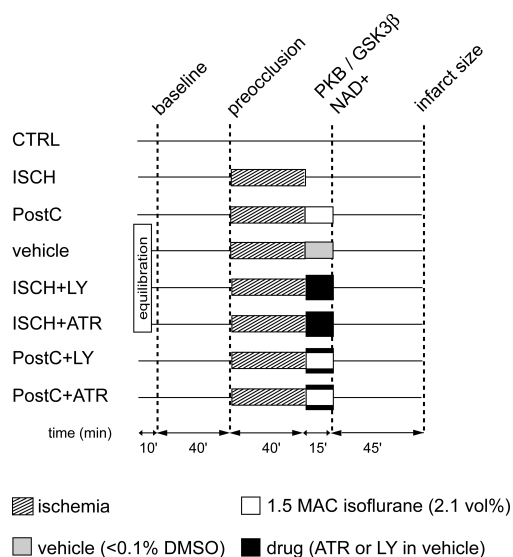
Fig. 2. Scheme of treatment protocols. Anesthetic postconditioning (PostC): after 40 min of test ischemia, isoflurane was administered for 15 min (1.5 minimum alveolar concentration [MAC]) immediately at the onset of reperfusion. Atractyloside and LY294002 were coadministered with isoflurane during early reperfusion. In each group, five hearts were used. ATR = atractyloside (10 μM in < 0.1% DMSO); CTRL = time-matched perfusion; DMSO = dimethyl sulfoxide; GSK3β = glycogen synthase kinase 3β; ISCH = unprotected hearts exposed to 40 min of ischemia only; LY = LY294002 (15 μM in < 0.1% DMSO [vehicle]); NAD$^+$ = nicotinamide adenine dinucleotide; PKB = protein kinase B.

Similarly, tissue NAD$^+$ content was shown to be markedly reduced after 20 min of reperfusion.[16]

Western Blot Analysis

Polyclonal antibody specific for phospho-PKB/Akt (Ser473), phospho-GSK3β (Ser9), and polyclonal GSK3β were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal anti-PKB/Akt antibody (Ab10) was a gift from Dr. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland).[17] Monoclonal antiactin was purchased from Chemicon (Temecula, CA). Left ventricular tissue was powdered and homogenized in lysis buffer containing 50 mM Tris-hydrochloride at pH 7.5, 1% Triton-100, 120 mM NaCl, 10 mM sodium fluoride, 40 mM β-glycerol phosphate, 0.1 mM sodium orthopervanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM microcystin-LR (Alexis). Extracts were centrifuged for 30 min at 12,000g, and protein concentrations in the supernatants were determined by the Bradford method. Subsequently, extracts were resolved by 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were blocked with phosphate-buffered saline containing 5% nonfat dry milk and 0.05% Tween-20 for 1 h and then incubated with the primary antibody. The membranes were washed and incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL). After extensively washing, the blots were exposed to films (Fuji

Photo Film Co., Kanakawa, Japan) for various times to obtain a linear response by the enhanced chemiluminescence method (Pierce). The quantity of the immunoreactive bands was determined by densitometry (MCID; Imaging Inc., Fonthill, Ontario, Canada).

Determination of NAD$^+$, a Marker of mPTP Opening

In separate experiments, NAD$^+$ was extracted from left ventricular tissue after 15 min of reperfusion using perchloric acid, as previously described.[16,18] NAD$^+$ is released from inactive and dysfunctional mitochondria upon opening of the mPTP pore and is washed out during reperfusion. Therefore, low concentrations of NAD$^+$ in postischemic cardiac tissue indicate mPTP opening. For these determinations, 30 mg freeze-clamped tissue was powdered in a mortar and thoroughly mixed with 150 μl perchloric acid, 0.6 M. The mixture was then homogenized and centrifuged after neutralization with 3 M potassium hydroxide. NAD$^+$ concentrations were determined fluorometrically using alcohol dehydrogenase (Roche, Rotkreuz, Switzerland) at a wavelength of 340 nm (Biomate 3; Fisher Scientific, Schwerte, Germany).

Visualization of Postischemic Mitochondrial Dysfunction

In additional experiments, postischemic hearts were reperfused for 15 min and subsequently exposed to 200 nM of the red-fluorescent MitoTracker Red 580, a mitochondrion-selective fluorescent probe (Molecular Probes, Invitrogen, Basel, Switzerland) for 5 min and washed out for 10 min. MitoTracker Red passively diffuses cell membranes and highly accumulates in active viable mitochondria, whereas dysfunctional mitochondria do not accumulate the staining. Hearts were embedded in optimal cutting temperature medium (Tissue-Tek; Sakura, Finetek Inc., Torrance, CA) and immediately frozen in liquid nitrogen. Cryosections of 20 μm thickness were prepared with a cryostat (Cryo-star HM 560M, Microtom, Kalamazoo, MI) and examined for mitochondrial staining using an epifluorescence microscope (Axiovert M200; Zeiss, Jena, Germany).

Statistics

Data are presented as mean ± SD. For hemodynamic data, repeated-measures analysis of variance was used to evaluate differences over time between groups. An unpaired t test was used to compare groups at identical time points, and a paired t test was used to compare within groups over time. P values were multiplied by the number of comparisons that were made (Bonferroni correction), and corrected $P < 0.05$ was considered statistically significant. For all other data, one-way anal-

ysis of variance with *post hoc* Tukey test for multiple comparisons was used, and $P < 0.05$ was considered statistically significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL) was used for statistical analysis.

Results

Cardioprotection by Anesthetic Postconditioning Depends on Phosphatidylinositol 3-kinase Signaling and Is Mediated by Preventing Opening of Mitochondrial Permeability Transition Pore

After 40 min of test ischemia, anesthetic postconditioning with isoflurane (1.5 MAC) for 15 min immediately administered at the onset of reperfusion significantly improved functional recovery and decreased infarct size when compared with unprotected hearts (table 1 and fig. 3). Cardioprotection by isoflurane postconditioning was completely abolished by coadministration of the phosphatidylinositol 3-kinase inhibitor LY294002 and the mPTP opener atractyloside during early reperfusion. LY294002 and atractyloside administered alone during reperfusion did not further deteriorate postischemic recovery or increase infarct size (table 1 and fig. 3). These results suggest that activation of the phosphatidylinositol 3-kinase signaling pathway and inhibition of the mPTP are directly involved in the cytoprotection observed after isoflurane postconditioning.

Isoflurane Postconditioning Mediates Phosphorylation and Inhibition of GSK3β, a Direct Regulator of the Mitochondrial Permeability Transition Pore

It has been well demonstrated that GSK3β is a major downstream target of PKB/Akt, and phosphorylation of an N-terminal serine residue (Ser9) leads to inhibition of GSK3β.[9] Here we show that 40 min of test ischemia alone increased phosphorylation of PKB/Akt and GSK3β to a certain extent when compared with time-matched perfusion (figs. 4A and B). However, when isoflurane was administered during the early reperfusion phase, a significant increase in phosphorylation of PKB/Akt and GSK3β was observed (figs. 4A and B). Both ischemia- and isoflurane-induced phosphorylation of PKB/Akt and GSK3β were strongly suppressed by administration of LY294002. These results indicate that isoflurane-induced cardiac protection is indeed mediated by activation of PKB/Akt signaling and subsequent inhibition of GSK3β. In contrast, atractyloside, the direct mPTP opener, did not suppress phosphorylation of PKB/Akt and GSK3β sufficiently, but completely abrogated isoflurane-induced protection, indicating that isoflurane-induced cardiac protection is completely mediated through inhibition of mPTP opening.

Activation of PKB/Akt and Inhibition of GSK3β by Isoflurane Postconditioning Prevents Postischemic NAD+ Loss in the Myocardium

To determine mPTP opening, NAD+, which is released from damaged mitochondria upon opening of mPTP and subsequently washed out from cardiac tissue, was measured from whole tissue extracts in the different treatment protocols.[16] Isoflurane clearly prevented the release of NAD+ from myocardial tissue indicating inhibition of the mPTP (fig. 5). In contrast, in the presence of LY294002 postischemic NAD+ release and washout were not inhibited by isoflurane indicating that mPTP opening is mediated by PKB/Akt signaling pathway. Similar to LY294002, atractyloside also completely reversed the protective effects of isoflurane on mPTP. These data suggest that isoflurane postconditioning prevents mPTP opening *via* PKB/Akt-GSK3β signaling.

Mitochondria from Postconditioned Hearts Accumulate MitoTracker Red 580 and Remain Functional

Separate experiments served to visualize activity of mitochondria after anesthetic postconditioning, as compared with unprotected hearts. For this purpose, the cell membrane permeable red-fluorescent MitoTracker Red 580 was added to postconditioned or unprotected hearts after 15 min of reperfusion. In unprotected hearts, MitoTracker Red was exclusively found in the epicardial outer layers of the hearts, whereas deeper layers of the myocardium exhibited virtually no mitochondrial staining. In contrast, postconditioned mitochondria showed clear accumulation of the staining (figs. 6A–F).

Discussion

The current study shows several salient findings. First, isoflurane administered during early reperfusion induces phosphorylation and inhibition of the downstream master switch kinase GSK3β *via* PKB/Akt signaling in isolated rat hearts. These results confirm and extend recently reported observations demonstrating enhanced postischemic PKB/Akt activation in anesthetic postconditioning.[3] Second, administration of the mPTP opener atractyloside completely reversed functional and structural protection by isoflurane postconditioning, implying that isoflurane exerts its cellular protection by preventing opening of mPTP. The inhibitory effects of isoflurane on mPTP opening could be corroborated by measuring NAD+ tissue content, a surrogate marker of mPTP opening.[16] Finally, coadministration of LY294002 during isoflurane postconditioning annihilated phosphorylation and inhibition of GSK3β and completely abolished cardioprotection. Collectively, the data presented provide evidence that anesthetic postconditioning prevents mPTP opening *via* the PKB/Akt-dependent master

Table 1. Hemodynamics

| | Baseline | Preocclusion Value | Reperfusion | | |
			15 min	30 min	60 min
LVDP, mmHg					
CTRL	101 ± 13	100 ± 10	93 ± 6	93 ± 6	91 ± 4
ISCH	90 ± 3	95 ± 5	12 ± 6*†	20 ± 7*†	22 ± 4*†
ISCH + LY	89 ± 1	96 ± 4	10 ± 5*†	16 ± 5*†	25 ± 10*†
ISCH + ATR	89 ± 2	95 ± 4	11 ± 7*†	18 ± 5*†	27 ± 9*†
PostC	94 ± 5	94 ± 10	38 ± 5*†	64 ± 9*†	67 ± 11*†
PostC + LY	93 ± 9	95 ± 5	13 ± 4*†	25 ± 8*†	19 ± 5*†
PostC + ATR	96 ± 7	92 ± 8	15 ± 6*†	25 ± 8*†	24 ± 10*†
ISCH + DMSO	92 ± 5	96 ± 4	15 ± 3*†	21 ± 6*†	22 ± 7*†
LVEDP, mmHg					
CTRL	2 ± 1	2 ± 1	3 ± 2	3 ± 2	2 ± 1
ISCH	3 ± 1	3 ± 1	45 ± 3*†	35 ± 6*†	38 ± 7*†
ISCH + LY	3 ± 1	3 ± 1	50 ± 5*†	35 ± 6*†	38 ± 8*†
ISCH + ATR	2 ± 1	2 ± 2	48 ± 4*†	37 ± 3*†	34 ± 6*†
PostC	2 ± 1	3 ± 1	20 ± 4*†	9 ± 4*†	11 ± 2*†
PostC + LY	2 ± 1	2 ± 1	42 ± 13*†	37 ± 10*†	34 ± 6*†
PostC + ATR	3 ± 1	3 ± 1	46 ± 4*†	36 ± 2*†	39 ± 11*†
ISCH + DMSO	3 ± 1	3 ± 2	45 ± 5*†	36 ± 3*†	32 ± 8*†
+dp/dt, mmHg/s					
CTRL	3,280 ± 258	3,200 ± 310	3,470 ± 480	3,470 ± 480	3,340 ± 280
ISCH	3,240 ± 340	3,340 ± 400	300 ± 170*†	560 ± 270*†	600 ± 254*†
ISCH + LY	3,200 ± 220	3,400 ± 400	420 ± 100*†	500 ± 287*†	540 ± 240*†
ISCH + ATR	3,320 ± 300	3,200 ± 210	390 ± 250*†	470 ± 178*†	680 ± 240*†
PostC	3,480 ± 340	3,160 ± 200	2,100 ± 320*†	2,740 ± 207*†	2900 ± 651*†
PostC + LY	3,000 ± 300	3,200 ± 170	250 ± 150*†	500 ± 339*†	600 ± 200*†
PostC + ATR	3,200 ± 400	3,100 ± 200	300 ± 100*†	680 ± 192*†	680 ± 190*†
ISCH + DMSO	3,260 ± 180	3,280 ± 160	290 ± 170*†	580 ± 238*†	680 ± 192*†
−dp/dt, mmHg/s					
CTRL	2,540 ± 100	2,580 ± 300	2,454 ± 150	2,454 ± 150	2,580 ± 140
ISCH	2,580 ± 300	2,560 ± 421	120 ± 100*†	320 ± 194*†	380 ± 200*†
ISCH + LY	2,620 ± 304	2,400 ± 200	180 ± 150*†	280 ± 192*†	340 ± 150*†
ISCH + ATR	2,520 ± 414	2,750 ± 500	200 ± 105*†	270 ± 205*†	380 ± 170*†
PostC	2,640 ± 340	2,480 ± 200	1,170 ± 400*†	1,460 ± 200*†	1400 ± 160*†
PostC + LY	2,660 ± 300	2,680 ± 500	160 ± 110*†	360 ± 200*†	340 ± 140*†
PostC + ATR	2,440 ± 200	2,430 ± 200	120 ± 120*†	320 ± 220*†	300 ± 152*†
ISCH + DMSO	2,400 ± 200	2,500 ± 400	140 ± 133*†	340 ± 222*†	170 ± 150*†
CF, ml/min					
CTRL	14.0 ± 1.8	13.8 ± 1.1	12.2 ± 1.6	12.2 ± 1.6	12.2 ± 1.0
ISCH	12.4 ± 2.0	13.4 ± 1.1	2.3 ± 1.3*†	3.4 ± 1.1*†	3.8 ± 0.8*†
ISCH + LY	12.0 ± 2.0	12.8 ± 0.8	2.2 ± 1.4*†	2.8 ± 1.4*†	3.2 ± 1.4*†
ISCH + ATR	13.2 ± 1.4	12.4 ± 2.0	2.1 ± 1.1*†	3.0 ± 1.2*†	3.2 ± 1.4*†
PostC	13.0 ± 1.3	13.2 ± 1.3	9.5 ± 0.9*†	10.8 ± 0.8*†	11.6 ± 1.1*†
PostC + LY	13.4 ± 2.0	12.4 ± 2.5	1.9 ± 1.6*†	3.4 ± 1.1*†	2.6 ± 0.9*†
PostC + ATR	12.0 ± 3.0	12.0 ± 3.0	2.2 ± 1.3*†	3.2 ± 1.0*†	3.8 ± 0.5*†
ISCH + DMSO	13.4 ± 1.1	11.8 ± 1.5	2.3 ± 1.3*†	3.0 ± 1.0*†	3.8 ± 0.8*†
HR, beats/min					
CTRL	287 ± 9	310 ± 20	288 ± 13	288 ± 13	299 ± 16
ISCH	298 ± 17	294 ± 11	170 ± 50*†	200 ± 30*†	210 ± 20*†
ISCH + LY	292 ± 13	294 ± 15	166 ± 40*†	198 ± 40*†	202 ± 43*†
ISCH + ATR	290 ± 13	300 ± 15	182 ± 44*†	212 ± 14*†	206 ± 35*†
PostC	295 ± 26	287 ± 29	222 ± 20*†	259 ± 10*†	256 ± 17*†
PostC + LY	302 ± 24	294 ± 11	140 ± 29*†	190 ± 27*†	204 ± 21*†
PostC + ATR	292 ± 24	300 ± 15	170 ± 27*†	200 ± 23*†	218 ± 16*†
ISCH + DMSO	302 ± 125	298 ± 16	142 ± 51*†	202 ± 31*†	217 ± 10*†

Data are presented as mean ± SD (n = 5/group).

* Significantly ($P < 0.05$) different from baseline (intragroup comparison).   † Significantly ($P < 0.05$) different from respective value in CTRL and ISCH (intergroup comparison).

ATR = atractyloside; CF = coronary flow; CTRL = control (time-matched perfusion); DMSO = dimethyl sulfoxide (vehicle; < 0.1%), used to dissolve LY294002 and atractyloside; +dp/dt = inotropy; −dp/dt = lusitropy; HR = heart rate; ISCH = test ischemia without postconditioning; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; LY = LY294002; PostC = isoflurane postconditioning; Preocclusion values = before test ischemia.
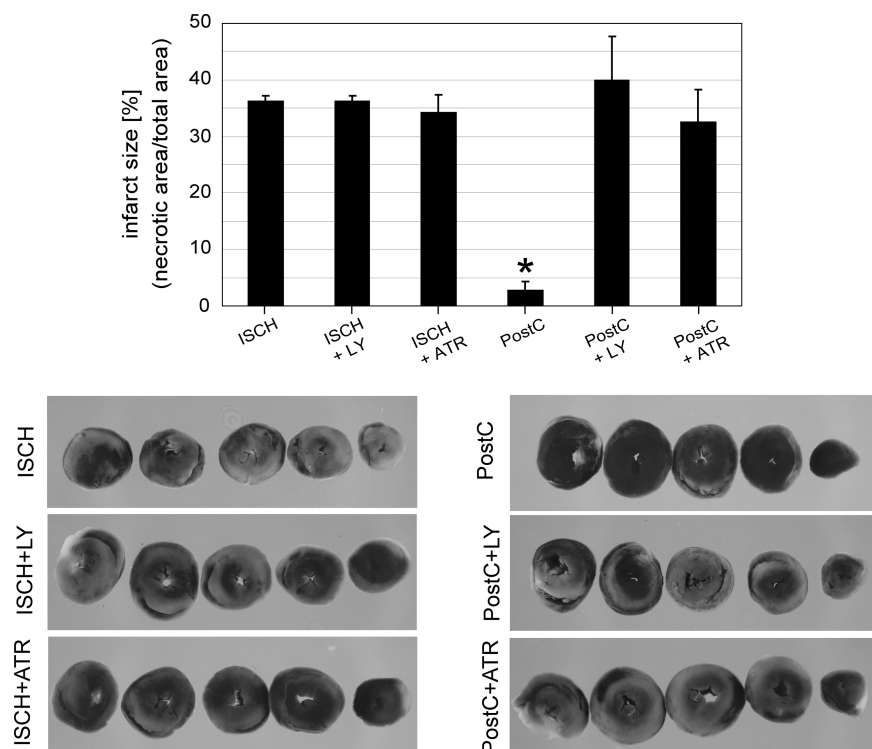
Fig. 3. Infarct size was determined using 1% triphenyltetrazolium chloride staining, as described in the Materials and Methods section. In each group, five hearts were used. Data are given as mean ± SD. ATR = atractyloside (10 μM in < 0.1% DMSO); CTRL = time-matched perfusion; ISCH = unprotected hearts exposed to ischemia–reperfusion; LY = LY294002 (15 μM in < 0.1% DMSO); PostC = anesthetic postconditioning. * P < 0.05 *versus* ISCH.

switch kinase GSK3β, thereby exerting mitochondrial protection against reperfusion injury.

Inhibition of the mPTP is a general mechanism of cardiomyocyte protection against ischemia–reperfusion.[19] mPTP is a large protein complex, which spans from the inner to the outer mitochondrial membrane consisting of the key compounds adenine nucleotide transporter (ANT), the voltage-dependent anion channel, cyclophilin-D, and the mitochondrial creatine kinase. Interestingly, the ANT located in the inner mitochondrial membrane has been recognized as one of the main components forming the mitochondrial adenosine triphosphate–sensitive potassium channel,[20] which is known to be activated by volatile anesthetics in the context of pharmacologic preconditioning.[21] Recently, isoflurane postconditioning but not preconditioning was shown to inhibit ANT transcription.[22] Whether the pore itself is formed by the ANT alone or in conjunction with the voltage-dependent anion channel is not clear at present. mPTP remains closed under ischemic "de-energized" conditions but opens at the onset of reperfusion triggered by the Ca^{2+} overload and the excessive formation of reactive oxygen species.[23,24] Pore opening results in the collapse of the mitochondrial membrane potential $\Delta\Psi$m, uncoupling of the respiratory chain, and release of death inducing factors into the cytosol such as cytochrome *c*, apoptosis-inducing factor AIF, Smac/DIABLO, and endonuclease G.[25] Recently, Piriou *et al.*[10] demonstrated that anesthetic preconditioning by isoflurane delays Ca^{2+}-induced mPTP opening. The findings of the
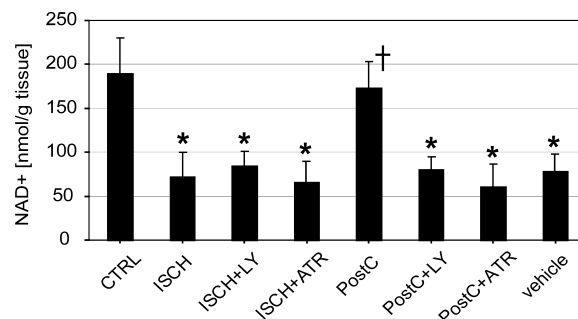
current study now provide for the first time evidence that similar to anesthetic preconditioning, cytoprotection by anesthetic postconditioning is achieved by inhibition of the mPTP. Like Bax, Ca^{2+}, and thiol oxidants, atractyloside, the mPTP opener used in this study, mediates pore opening *via* binding to the intermembrane face of the ANT,[26] thereby opposing the inhibitory effects of isoflurane. According to the model described by Crompton *et al.*,[27] the ANT changes into the pore-forming c-conformation at the onset of reperfusion to which atractyloside binds and thereby promotes opening of the mPTP. Nonetheless, infarct size was not further increased in hearts treated with atractyloside alone in our experiments, as previously reported.[14] One might speculate that atractyloside exclusively opens pores that have been potentially closed *via* the activation of PKB/Akt-GSK3β signaling. This is further supported by the observation that atractyloside when given alone did not further enhance the release of NAD$^+$ from mitochondria at reperfusion.

Di Lisa *et al.*[28] and Halestrap *et al.*[19] have recently reviewed the advantages and limitations of various methods to determine mPTP opening. mPTP opening could be assessed by the radioactively labeled 2-deoxyglucose in the isolated heart model. This compound is trapped within the cytosol and unable to cross intracellular membranes, so that mitochondrial accumulation is regarded as an indicator for mPTP opening. However, 2-deoxyglucose is an inhibitor of glycolysis and may interfere with bioenergetics. Therefore, to quantify mPTP opening in

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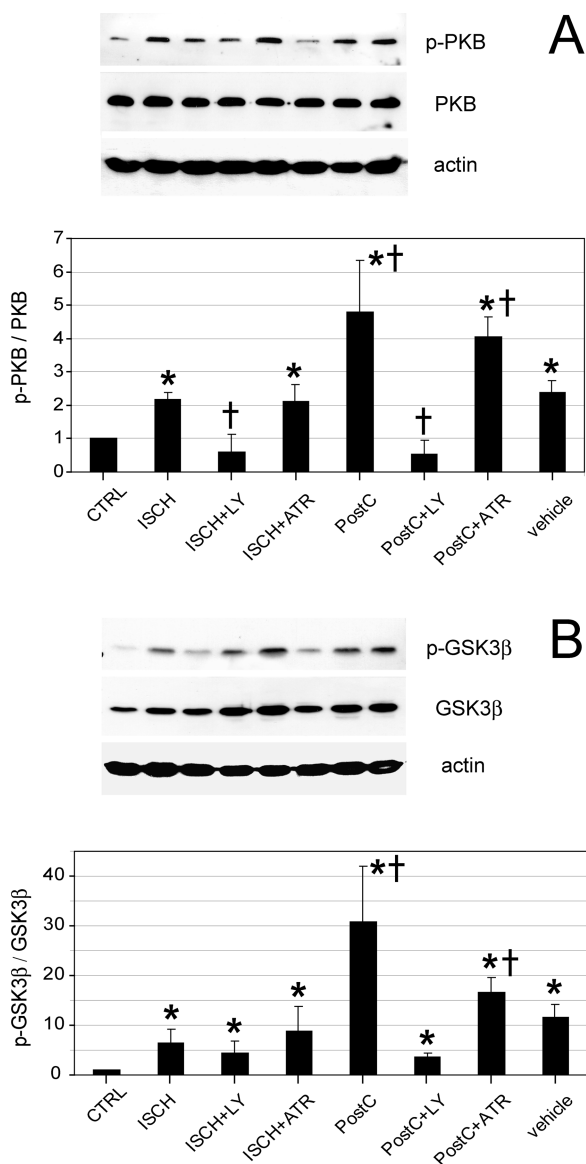
Fig. 4. Western blot analysis. Phosphorylation status of protein kinase B (PKB)/Akt (60 kd) (*A*) and glycogen synthase kinase 3β (GSK3β) (47 kd) (*B*) were analyzed by Western blot with specific phospho-PKB (Ser473) and phospho-GSK3β (Ser9) antibodies in the various treatment groups. Equal loading was controlled by Western blot with antiactin antibody. Data are given as mean ± SD (n = 5/group). ATR = atractyloside (10 μM in < 0.1% DMSO); CTRL = time-matched perfusion; ISCH = unprotected hearts exposed to ischemia–reperfusion; LY = LY294002 (15 μM in < 0.1% DMSO [vehicle]); PostC = anesthetic postconditioning. * *P* < 0.05 *versus* CTRL. † *P* < 0.05 *versus* ISCH.

Fig. 5. Nicotinamide adenine dinucleotide (NAD$^+$) measurements in the various treatment groups. NAD$^+$ content was determined after 15 min of reperfusion (n = 5/group). Data are given as mean ± SD. ATR = atractyloside (10 μM in < 0.1% DMSO); CTRL = time-matched perfusion; CTRL = time-matched perfusion; LY = LY294002 (15 μM in < 0.1% DMSO [vehicle]); PostC = anesthetic postconditioning. * *P* < 0.05 *versus* CTRL. † *P* < 0.05 *versus* ISCH.

chondrial release of NAD$^+$ *per se* may aggravate reperfusion damage because NAD$^+$ becomes substrate of the cytosolic glycohydrolase forming cyclic adenosine diphosphate-ribose and nicotinic acid adenine dinucle-
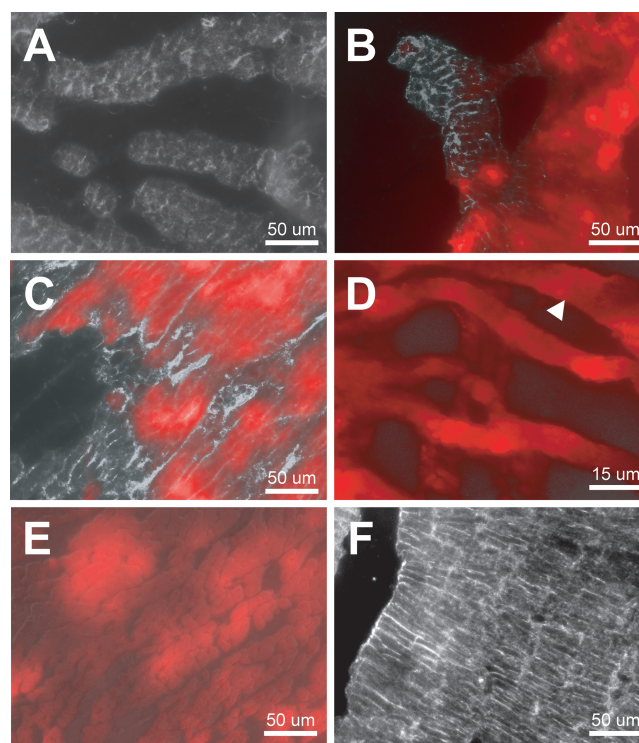
Fig. 6. Postischemic uptake of MitoTracker Red 580 in mitochondria of isolated perfused rat hearts. Unprotected hearts show no staining (*A*) except for the superficial epicardial layers of the myocardium (*B*). In contrast, postconditioned protected hearts exhibit marked accumulation of MitoTracker Red 580 in the tissue (*C* and *D*) similar to control hearts not subjected to ischemia–reperfusion (time-matched perfusion) (*E*). Atractyloside, a direct opener of the mitochondrial permeability transition pore, completely abolished the isoflurane effects (*F*). *A*, *B*, *C*, *E*, and *F* are epifluorescence micrographs (*red channel*) merged with phase contrast images. *D* shows at higher magnification the syncytial net of postconditioned myocytes containing brightly staining mitochondria (*red channel*). *Arrow* indicates intercalated disks.

our study, NAD$^+$ tissue concentrations were determined during reperfusion. Cellular loss of NAD$^+$ during reperfusion results from mPTP opening, because it can be selectively blocked by cyclosporin A and analogs, which bind to cyclophilin D and inhibit pore formation.[16] Mitochondria represent the major stores of NAD$^+$, possessing more than 90% of the total cellular content. Therefore, NAD$^+$ tissue content can be used as surrogate indicator of mPTP pore opening.[29] Interestingly, mito-

otide phosphate, which in turn promote Ca^{2+} release from the sarcoplasmic reticulum.[30]

The reperfusion injury salvage kinases afford marked protection against ischemia and are known to phosphorylate $GSK3\beta$ during reperfusion.[31,32] $GSK3\beta$ was originally identified as an enzyme that regulates glycogen synthesis in response to insulin but, in contrast to many other protein kinases, is active in resting cells in its dephosphorylated state on regulatory Ser9.[33] Conversely, phosphorylation on Ser9 by PKB/Akt leads to inactivation of $GSK3\beta$. Pharmacologic inhibition of $GSK3\beta$ reduces infarct size and improves postischemic function.[31] Using a transgenic mouse model with cardiac-specific expression of a constitutively active signal-resistant form of $GSK3\beta$ (Ser9 to Ala9 mutation), a recent comprehensive study[9] demonstrated that the protection signaling in cardiomyocytes integrates through $GSK3\beta$, which in turn inhibits the mPTP. Consistent with this concept of a master switch kinase is the notion that hypoxic preconditioning and a wide variety of protective agents including diazoxide, insulin, and Li^+ require a functionally inhibitable $GSK3\beta$. Although not yet completely elucidated, possible mPTP-modulating targets of $GSK3\beta$ could be Bcl-2, Bis, and serine/threonine protein phosphatase 2A. The current study now demonstrates phosphorylation and inhibition of $GSK3\beta$ in response to isoflurane and suggests that protection by isoflurane postconditioning could be mediated, at least in part, through the master switch kinase $GSK3\beta$ by inhibiting mPTP. Similar to previous reports,[3] in our study, phosphorylation of PKB/Akt and $GSK3\beta$ was increased to a certain extent in even unprotected hearts when compared with time-matched perfused hearts, indicating that a partial or delayed phosphorylation is insufficient to prevent myocardial damage.

Study Limitations

The following specific comments should be added. (1) The results of our study are largely dependent on the specificity of the pharmacologically active agents LY294002 and atractyloside. However, the observed specificity and potential toxicity of these drugs are difficult to appreciate. (2) We cannot completely rule out that additional PKB/Akt targets besides $GSK3\beta$ may contribute to the observed mPTP inhibition by isoflurane. In fact, Tsang *et al.*[13] showed that pharmacologic inhibition of endothelial nitric oxide synthase, another PKB signaling downstream target, prevented infarct size limitation in ischemic postconditioning, and isoflurane is known to modulate endothelial nitric oxide synthase activity.[34] Nonetheless, $GSK3\beta$ is a known major regulator of mPTP activity. (3) Our postconditioning regimen dramatically reduced infarct size. This may be due to the relatively high dose of isoflurane administered for a rather extended period (15 min) to elicit anesthetic postconditioning. Another explanation for this finding might be

that infarct size was determined relatively early after reperfusion (1 h), resulting in a less pronounced demarcation of the necrotic from the surviving tissue by triphenyltetrazolium chloride staining. However, a dramatic reduction in infarct size by anesthetic postconditioning was previously reported,[35] and reperfusion for 1 h or more is regarded as optimal for acute assessment of infarct size.[36] (4) In this study, NAD^+ was not directly measured in mitochondria. However, using the same experimental model, Di Lisa *et al.*[16] clearly demonstrated that loss of mitochondrial NAD^+ closely parallels loss of NAD^+ in whole tissue extracts. (5) Although genomics has demonstrated that there is more than 85% similarity in coding regions of the rat genome compared with the human genome, data from rodent studies must be always interpreted with caution, particularly with respect to cellular signaling. In addition, in contrast to an *in vivo* model, buffer-perfused hearts have a limited long-term biologic stability and may undergo short confounding ischemic periods during the isolation procedure, which could potentially activate PKB/Akt. (6) Opening of mPTP does not always inevitably lead to cell death. In fact, transient opening of mPTP during the triggering phase of preconditioning may even elicit cytoprotection.[37]

In summary, many signaling elements previously documented to be involved in anesthetic preconditioning have been now shown also to be important in anesthetic postconditioning. The current study provides evidence that isoflurane postconditioning inhibits opening of the mPTP *via* PKB/Akt-$GSK3\beta$ signaling, thereby providing a powerful anti-ischemic protection.

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