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Infarct-remodeled Myocardium Is Receptive to Protection by Isoflurane Postconditioning

Role of Protein Kinase B/Akt Signaling

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Background: **Postinfarct remodeled myocardium exhibits numerous structural and biochemical alterations. So far, it is unknown whether postconditioning elicited by volatile anesthetics can also provide protection in the remodeled myocardium.**

Methods: **Myocardial infarct was induced in male Wistar rats by ligation of the left anterior descending coronary artery. Six weeks later, hearts were buffer-perfused and exposed to 40 min of ischemia followed by 90 min of reperfusion. Anesthetic postconditioning was induced by 15 min of 2.1 vol% isoflurane. In some experiments, LY294002 (15 μM), a phosphatidylinositol 3-kinase inhibitor, was coadministered with isoflurane. Masson's trichrome staining, immunohistochemistry, Western blot analysis, and reverse-transcription polymerase chain reaction served to confirm remodeling. In buffer-perfused hearts, functional recovery was recorded, and acute infarct size was measured using 1% triphenyltetrazolium chloride staining and lactate dehydrogenase release during reperfusion. Western blot analysis was used to determine phosphorylation of reperfusion injury salvage kinases including protein kinase B/Akt and its downstream targets after 15 min of reperfusion.**

Results: **Infarct hearts exhibited typical macroscopic and molecular changes of remodeling. Isoflurane postconditioning improved functional recovery and decreased acute infarct size, as determined by triphenyltetrazolium (35 ± 5% in unprotected hearts *vs.* 8 ± 3% in anesthetic postconditioning; $P < 0.05$) and lactate dehydrogenase release. This protection was abolished by LY294002, which inhibited phosphorylation of protein kinase B/Akt and its downstream targets glycogen synthase kinase 3β, endothelial nitric oxide synthase, and p70S6 kinase.**

Conclusions: **Infarct-remodeled myocardium is receptive to protection by isoflurane postconditioning *via* protein kinase B/Akt signaling. This is the first time to demonstrate that anesthetic postconditioning retains its marked protection in diseased myocardium.**

REMODELING is a maladaptive response to chronic hemodynamic sequelae occurring after large myocardial infarcts and leading to left ventricular dilatation and compensatory hypertrophy of the residual intact cardiac tissue. Apoptosis and fibrosis participate in this chronic process, which has a high propensity for arrhythmogenesis, including sudden cardiac death, and ultimately leads to congestive heart failure.[1,2] In remodeled hearts, left ventricular dysfunction does not only occur in infarcted cicatrized areas, but also occurs in residual intact myocardium. Beside many dramatic macroscopic and microscopic structural changes associated with remodeling, significant alterations in metabolism were previously reported, including a shift toward anaerobic metabolism and a depletion in high-energy phosphates putting the diseased myocardium at particular risk for further ischemic injury.[3]

Postconditioning by volatile anesthetics ("anesthetic postconditioning") is a most effective therapeutic strategy of reducing infarct size after prolonged ischemia. Similar to ischemic postconditioning,[4,5] anesthetic postconditioning enhances the activity of the reperfusion injury salvage kinase (RISK) protein kinase B (PKB)/Akt at the time of reperfusion,[6–8] thereby preventing mitochondrial permeability transition through inhibition of glycogen synthase kinase 3β (GSK3β) and reducing infarct size.[7] Because the onset of reperfusion is predictable and under the control of the operator, this novel therapeutic strategy is particularly promising for the clinical setting.

To date, all experimental studies have evaluated the phenomenon of cardiac postconditioning in healthy juvenile hearts. However, this is far from clinical reality, because diseased myocardium would benefit most from protection by postconditioning. On the other side, some clinical[9,10] and experimental[11] studies provide evidence that diseased myocardium may be less amenable to protection by preconditioning, the most powerful endogenous protective mechanism, which is at the opposite site of ischemia but shares many signaling steps with postconditioning.[12] Therefore, we tested the hypothesis whether protection by

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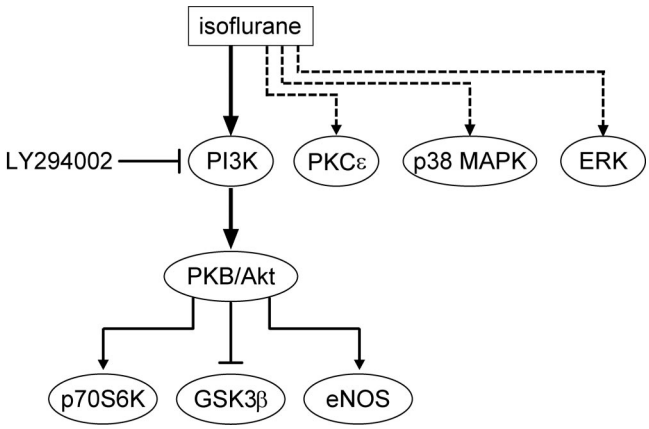

Fig. 1. Investigated signaling components in isoflurane postconditioning in the infarct-remodeled rat heart. Protein kinase B (PKB)/Akt is downstream of phosphatidylinositol 3-kinase (PI3K), a key enzyme in the reperfusion injury salvage kinase cascade (*full arrows*). Phosphorylated PKB/Akt activates 70-kd ribosomal protein S6 kinase (p70S6K) and endothelial nitric oxide synthase (eNOS) and inactivates glycogen synthase kinase 3β (GSK3β) by phosphorylation. Activation of protein kinase C ε (PKC-ε), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular signal–regulated protein kinases (ERKs), potentially involved in cardioprotection from reperfusion injury (*dashed arrows*), were also assessed. LY294002: specific inhibitor of PI3K.

pharmacologic postconditioning elicited by isoflurane would be diminished or lost in markedly remodeled postinfarct myocardium. Additional experiments served to delineate the specific role of PKB/Akt among other RISK components in the protection by isoflurane postconditioning (fig. 1).

The data provided in this study show for the first time that protection by anesthetic postconditioning is fully preserved in postinfarct remodeled myocardium. They further underscore the unique role of PKB/Akt in the cardioprotection by isoflurane postconditioning in the severely diseased heart.

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.

Induction of Myocardial Infarct In Vivo *to Promote Cardiac Remodeling*

Infarct or sham operations were performed in 200-g male Wistar rats (8–9 weeks old) kept in a 12-h light–dark cycle on commercial rat chow and water *ad libitum*. Ligation of the left anterior descending coronary artery was performed in the intubated rats during isoflurane anesthesia, as previously described.[13] Briefly, the fourth intercostal space was opened, the heart was exteriorized, and the pericardium was cut. The left anterior descending coronary artery was ligated between the left atrium and the pulmonary outflow tract using a 6.0 silk suture. Successful ligation was verified by regional cyanosis of the myocardial surface and ischemic ST-segment changes in the electrocardiogram. The heart was replaced into the thoracic cavity, which was drained from remaining air, and the chest was immediately closed. At the end of operation, all animals received 0.02 mg/kg subcutaneous buprenorphine for postoperative analgesia (with subsequent doses every 12 h for the first 3 postoperative days), and 10 mg/kg subcutaneous enrofloxacin as an antibiotic prophylaxis. A microchip was implanted subcutaneously for identification. Sham-operated animals underwent the same procedure except that the suture was passed under the coronary artery without ligation. A series of separate experiments served to verify the occurrence of an effective remodeling process 6 weeks after ligation. Because successful remodeling and impairment of left ventricular function can be expected only if infarct size reaches approximately 30% of the left ventricular mass,[13,14] acute infarct size was determined 12 h after coronary artery ligation using 1% 2,3,5-triphenyltetrazolium chloride (n = 5). In additional experiments, sham-operated hearts and infarcted hearts were evaluated for their function on the Langendorff apparatus, as well as for morphologic changes and biochemical markers of remodeling on the messenger RNA (mRNA) and protein level (n = 5).

Langendorff Preparation of Rat Hearts and Experimental Protocols

Six weeks after ligation of the left anterior descending coronary artery, rats were heparinized (500 U intraperitoneal) and 15 min later decapitated without previous anesthesia. 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, and left ventricular end-diastolic pressure was set at 10 mmHg after equilibration. Left ventricular developed pressure and derivatives ($\pm$dp/dt), left ventricular end-diastolic pressure, epicardial electrocardiogram, coronary flow, and perfusion pressure were recorded, as previously described.[15] After equilibration, spontaneously beating hearts were exposed to 40 min of global test ischemia (fig. 2). Anesthetic postconditioning was induced by isoflurane administered for 15 min at 1.5 minimum alveolar concentration (MAC; 2.1 vol%) right at the onset of reperfusion. The buffer 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 before entering the aortic cannula using a gas chromatograph (Perkin-Elmer, Norwalk, CT): 0.50 $\pm$ 0.04 mM. In the blocker experiments, 15 μM of the phosphatidylinositol 3-kinase (PI3K) LY294002 (Alexis, Lausen, Switzerland),

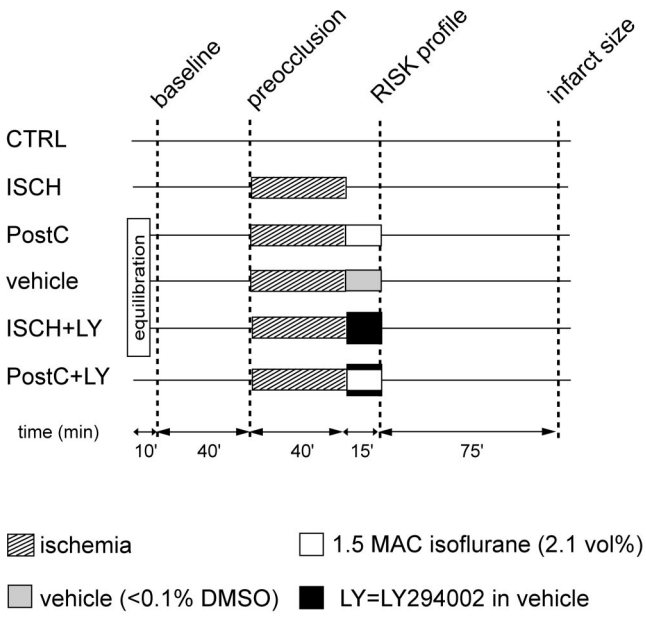

Fig. 2. Treatment protocols. Anesthetic postconditioning (PostC) = isoflurane administered for 15 min (1.5 minimum alveolar concentration [MAC]) right at the onset of reperfusion after 40 min of test ischemia; CTRL = time-matched perfusion of remodeled hearts; ISCH = unprotected remodeled hearts exposed to 40 min of ischemia alone; LY = LY294002. LY294002 dissolved in dimethyl sulfoxide (DMSO; < 0.1%) was coadministered with isoflurane during early reperfusion. Reperfusion injury salvage kinases (RISKs) were assayed after 15 min of reperfusion, and infarct size was determined after 90 min of reperfusion. Five hearts were used in each group.

dissolved in dimethyl sulfoxide at a final concentration of less than 0.1%, was coadministered with isoflurane or given alone during the first 15 min of reperfusion.[5,7] Dimethyl sulfoxide alone did not affect functional recovery, infarct size, or the phosphorylation status of any of the measured kinases. Hearts subjected to ischemia and reperfusion alone served as ischemic control. For each experimental group, five hearts (n = 5) were prepared, and functional parameters were recorded (fig. 2). To characterize myocardial function 6 weeks after induction of infarct, additional Langendorff perfusion studies were performed as described to asses cardiac function *ex vivo* in infarct-remodeled and sham-operated hearts (n = 5 in each group).

Determination of Infarct Size and Myocardial Damage

Infarct size was determined by 2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion. Briefly, hearts were frozen at −20°C for 2 h at the end of the experiment and 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 (pH = 7.4), fixed in 10% formaldehyde, and digitally photographed. Planimetric analysis was performed using ImageJ 1.33.§§ Briefly, the chronic infarct (bright white) was subtracted from the total left ventricular slice area to obtain the area at risk. Infarct size was determined by dividing the freshly necrotic area (salmon pink) of the left ventricle by the area at risk. Therefore, areas infarcted *in vivo* by coronary ligation (chronic infarct) were excluded from subsequent infarct size analysis. In addition, myocardial damage was estimated by measuring the release of lactate dehydrogenase (LDH) from necrotic tissue. For this purpose, the perfusate was collected, and LDH was determined by the Roche/Hitachi 917 (sensitivity 6 U/l, intraassay and interassay coefficients of variance < 1%; Hitachi Corp., Tokyo, Japan).

Western Blot Analysis

Separate experiments served to determine the activities of kinases after 15 min of reperfusion (n = 5 in each group; fig. 1). The following antibodies were used for Western blot analysis: polyclonal antibody specific for atrial natriuretic peptide and protein kinase C ε (PKC-ε) (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA); polyclonal antibodies specific for phospho-PKB/Akt (Ser-473), GSK3β, phospho-GSK3β (Ser-9), p70S6 kinase (p70S6K), phospho-p70S6K (Thr-389), extracellular signal–regulated kinase1/2 (ERK1/2), phospho-ERK1/2 (Thr-202/Tyr-204), p38 mitogen-activated protein kinase, phospho-p38 mitogen-activated protein kinase (Thr-180/Tyr-182), endothelial nitric oxide synthase (eNOS), and phospho-eNOS (Ser-1177) (Cell Signaling Technology, Beverly, CA); polyclonal phospho-PKC-ε (Ser-729) antibody (Upstate, Milton Keynes, United Kingdom); monoclonal anti–pan-actin (Chemicon, Temecula, CA); monoclonal anti–α-tubulin (Sigma, St. Louis, MO). Monoclonal antibodies against myosin heavy chain α and β (α- and β-MHC) were a gift from Simon M. Hughes, Ph.D. (Division of Cell and Molecular Biology, The Randall Institute, King's College London, London, United Kingdom). Polyclonal anti-PKB/Akt antibody was a gift from Brian A. Hemmings, Ph.D. (Friedrich Miescher Institute, Basel, Switzerland). Polyclonal antibody against α-skeletal actin was a gift from Christine Chapponier, Ph.D. (Department of Pathology, University of Geneva, Geneva, Switzerland). After 15 min of reperfusion, left ventricular tissue at risk was taken and frozen in liquid nitrogen. Subsequently, it was powdered and homogenized in lysis buffer containing 62.5 mM Tris-HCl at pH 6.8, 2% sodium dodecyl sulfate, 4 mM EDTA, and 7% sucrose. Extracts were boiled at 95°C for 5 min followed by 30 min centrifugation at 12,000*g*. Protein concentrations in the supernatants were determined by the Bradford method. Extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene diflouride membranes (Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.05%

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

Tween-20 for 1 h and then incubated with the primary antibody. The membranes were washed and incubated with horseradish peroxidase–conjugated goat antirabbit immunoglobulin G (Pierce, Rockford, IL). After extensive washing, the blots were exposed to films (Fuji Photo Film Co., Kanakawa, Japan) for 10 s up to 1 min to obtain a linear response by the enhanced chemiluminescence method (Pierce, Rockford, IL). The quantity of the immunoreactive bands was determined by densitometry using ImageJ 1.33.§§

mRNA Extraction and Real-time Quantitative Polymerase Chain Reaction

Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). First strand complementary DNA (cDNA) was synthesized from 1.0 μg total RNA using SuperScript II reverse transcriptase (Invitrogen, Basel, Switzerland) and oligo-dT as a primer. Real-time polymerase chain reaction was accomplished with the specific primers at an annealing temperature of 55°C on a Stratagene MX3000 real-time sequence detection instrument using the Brilliant SYBR Green QPCR kit (Stratagene Europe, Amsterdam, The Netherlands). The following primers were used: atrial natriuretic peptide: 5′-ATCACCAAGGGCTTCTTCCT-3′ (sense) and 5′-TGTTGGACACCGCACTGTAT-3′ (antisense); brain natriuretic peptide: 5′-GGAAATGGCTCAGAGACAGC-3′ (sense) and 5′-CGGTCTATCTTCTGCCCAAA-3′ (antisense); β-MHC: 5′-TCTTGTGCTACCCAACCCTAA-3′ (sense) and 5′-TTGGCTTGAAGGAAAATTGC-3′; α-MHC: 5′-GCAGAAAATGCACGATG-3′ (sense) and 5′-TACAGGCAAAGTCAAGC-3′ (antisense); α-skeletal-actin: 5′-CACGGCATTATCACCAACTG-3′ (sense) and 5′-CCGGAGGCATAGAGAGACAG-3′ (antisense). The expression levels were measured in triplicates of the first strand cDNA, and α-tubulin was used for normalization of the data. The sense and antisense primers for α-tubulin were 5′-CCATGCGTGAGTGTATCTCC-3′ and 5′-GTGCCAGTGCGAACTTCATC-3′, respectively.

Histologic Assessment

Left ventricular tissue samples were placed in optimal cutting temperature medium (Tissue-Tek; Sakura Finetek Inc., Torrance, CA), frozen in liquid nitrogen, and stored at −70°C. Cryosections (5 μm) were collected on gelatin-precoated slides and air dried. The sections were fixed in precooled methanol at −20°C for 10 min. After washing with PBS, the sections were blocked with 5% normal goat serum at room temperature for 1 h. The sections were incubated at 4°C overnight with the following primary antibodies in 1% normal goat serum and PBS: mouse monoclonal anti–N-cadherin (Zymed, Basel, Switzerland), mouse monoclonal antidesmin (DakoCytomation AG, Baar, Switzerland), mouse monoclonal anti–α-MHC and anti–β-MHC (a gift from Simon M. Hughes, Ph.D., The Randall Institute, King's College London, United Kingdom). Subsequently, the sections were washed in PBS and incubated with Alexa Fluor-488–labeled anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) and 4′-6-diamidino-2-phenylindole (DAPI, 10 ng/ml; Sigma-Aldrich, Buchs, Switzerland) in 1% normal goat serum with PBS at room temperature in the dark for 1 h. After several steps of washing, the sections were mounted in Lisbeth's mounting medium (33 mM Tris, pH 9.5, 70% glycerol, 50 mg/ml n-propyl gallate) and examined using an epifluorescence microscope Axiovert M200 (Zeiss, Jena, Germany). Additional conventional histologic staining including hematoxylin–eosin and Masson's trichrome was performed. Hearts were perfused with 4% paraformaldehyde and embedded in paraffin. Sections of 3 μm were cut, mounted, and stained.

Statistics

All data are presented as mean ± SD. For hemodynamic data and LDH release, 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). For all other data, one-way analysis of variance with *post hoc* Tukey test was used for multiple comparisons. $P < 0.05$ was considered significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL) was used for analysis.

Results

Postinfarct Remodeled Hearts Exhibit Characteristic Macroscopic and Ultrastructural Changes

Ligation of the left anterior descending coronary artery induced extended infarcts (35 ± 5%), which were sufficient to initiate and promote myocardial remodeling in

Table 1. Characteristics of Sham-operated and Infarcted Hearts

	Sham Rats	Infarct Rats
Body weight, g*	296 ± 17	288 ± 20
Heart weight (wet), g*	1.21 ± 0.07	1.66 ± 0.11‡
Heart weight/body weight, g/kg	4.07 ± 0.37	5.70 ± 0.56‡
Infarct size, % of left ventricular wall	—	35 ± 5†
Heart rate, beats/min	302 ± 13	294 ± 10
LVDP, mmHg	100 ± 6	73 ± 8‡
Coronary flow, ml/min	14.5 ± 1.0	15.0 ± 0.8

Baseline hemodynamics were determined on the Langendorff apparatus 20 min after initiating buffer perfusion. Cardiac function was measured under similar conditions of 10 mmHg left ventricular end-diastolic pressure.

* Body and heart weights as determined 6 weeks after ligation of the left anterior descending coronary artery (n = 5). † Individual infarct sizes were as follows: heart 1 = 41%; heart 2 = 32%; heart 3 = 37%; heart 4 = 28%; heart 5 = 36%. ‡ $P < 0.05$.

LVDP = left ventricular developed pressure.

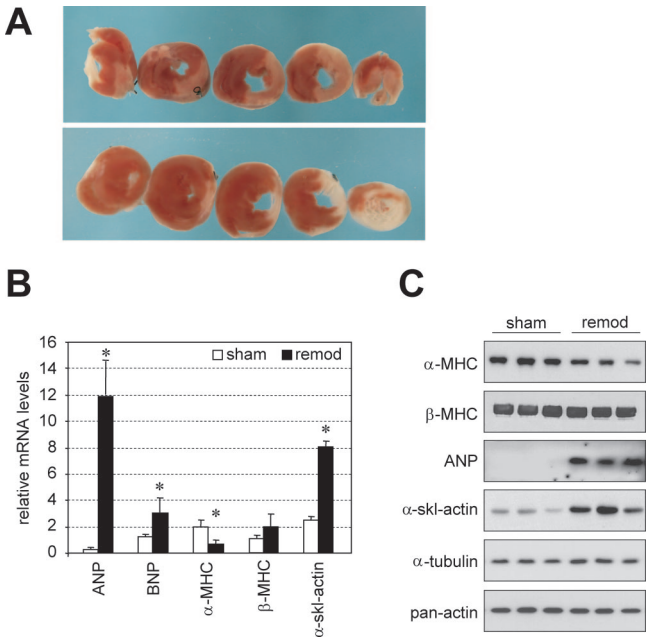

Fig. 3. Cardiac remodeling. Left anterior descending coronary artery was ligated, and infarct size was determined after 12 h using 1% triphenyltetrazolium chloride staining (*A*, *upper panel*). Viable myocardium is stained *brick-stone red*, whereas freshly infarcted myocardium is stained *salmon pink*. Note the *black ties* on the two basal transverse heart sections. A mean infarct size of 35 ± 5% was obtained (see also table 1). Six weeks after ligation, necrosis was replaced by scar tissue, and compensatory hypertrophy developed (*A*, *lower panel*). Chronic infarct is stained *bright white*. Transcript levels of remodeling markers (*B*): atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α- and β-myosin heavy chains (α- and β-MHC), α-skeletal-actin (α-skl-actin). Western blot analysis of remodeling markers (*C*): ANP, BNP, α- and β-MHC, α-skl-actin. Alpha-tubulin and total actin (pan-actin) were used as loading controls. Remod = postinfarct remodeled hearts; sham = sham-operated hearts. Data are given as mean ± SD (n = 5 in each group). * *P* < 0.05 *versus* sham.

the postinfarct healing period (table 1). The ratio of heart weight over body weight was significantly increased 6 weeks after ligation in the infarcted hearts (table 1) compared with sham-operated hearts, indicating compensatory hypertrophy, predominantly in the septal area of the heart (fig. 3A). Of note, heart weight (1.63 ± 0.13 g) and heart weight/body weight ratio (5.80 ± 0.33 g/kg) were also markedly increased in all animals used for experimentation. Consistent with loss of viable myocardium and hypertrophy was the observed reduced contractile function of the remodeled hearts as determined *ex vivo* on the Langendorff apparatus under control conditions (left ventricular diastolic pressure set at 10 mmHg) (table 1). At the mRNA level, transcript levels for atrial natriuretic peptide, brain natriuretic peptide, and α-skeletal-actin were increased, whereas the transcript for α-MHC was nearly absent (fig. 3B). Similarly, at the protein level, atrial natriuretic peptide and α-skeletal-actin were overexpressed, and α-MHC was markedly reduced (fig. 3C). Masson's trichrome staining of whole heart longitudinal and transverse sections exhibited increased amounts of collagen in the chronic infarct scar and compensatory hypertrophy with dilated spherical left ventricular cavities (fig. 4). Detailed immunohistochemical analysis including markers of cell–cell interaction (N-cadherin), contractile filaments α- and β-MHC, and extracellular matrix (desmin) revealed differences between sham-operated and infarcted hearts (fig. 4). Comparable to previous studies with similar models,[16–18] there was no difference in the phosphorylation status of PKB/Akt and ERK1/2 between sham-operated and infarcted hearts 6 weeks after operation (data not shown). Together, the results of these experiments demonstrate significant morphologic changes at the macroscopic and microscopic levels in chronically remodeled postinfarct myocardium.

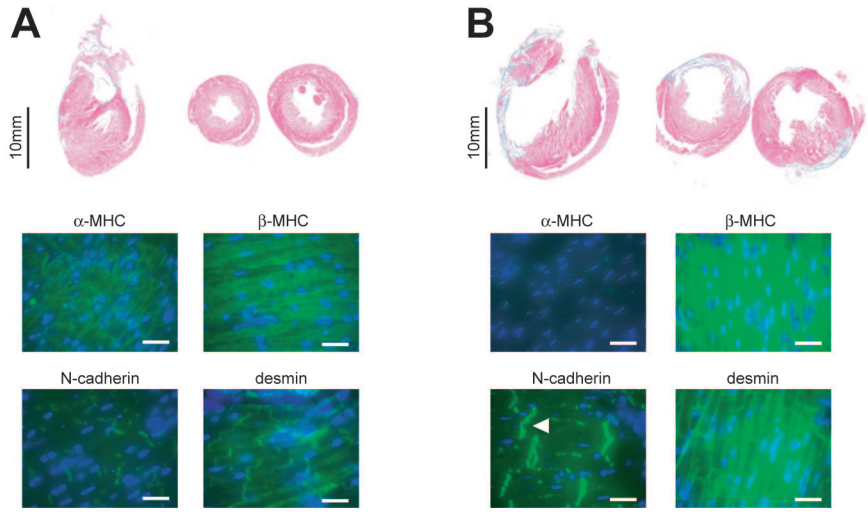

Fig. 4. Histochemistry of remodeled myocardium. Masson's trichrome staining and immunohistochemical stainings for α- and β-myosin heavy chain (α- and β-MHC), N-cadherin, and desmin were used to visualize macroscopic and ultrastructural changes in remodeled hearts. Representative sham-operated hearts 6 weeks after operation are shown on the *left* (*A*), and postinfarct remodeled hearts 6 weeks after ligation are shown on the *right* (*B*). Note the spherical shape of the remodeled hearts with infarct wall thinning and ventricular dilatation in contrast to the ellipsoid shape of the sham-operated hearts. Transverse sections were prepared from tissue taken at the midpapillary level. The fluorescence intensity was increased in the remodeled myocardium for β-MHC, N-cadherin, and desmin but markedly reduced for α-MHC. N-cadherin was accumulated at intercalated disks (*arrowhead*) in remodeled myocardium. *Bar* represents distance of 50 μm.

Table 2. Hemodynamics

			Reperfusion		
	Baseline	Preocclusion Value‡	15 min	30 min	90 min
LVDP, mmHg					
CTRL	69 ± 7	71 ± 8	70 ± 8	72 ± 10	73 ± 10
ISCH	68 ± 6	73 ± 9	6 ± 3*†	17 ± 4*†	16 ± 3*†
ISCH + LY	65 ± 8	70 ± 12	7 ± 2*†	15 ± 3*†	17 ± 4*†
PostC	67 ± 5	69 ± 8	21 ± 4*†	53 ± 7*†	56 ± 4*†
PostC + LY	70 ± 9	72 ± 5	5 ± 2*†	16 ± 4*†	15 ± 4*†
DMSO	65 ± 10	68 ± 4	7 ± 3*†	18 ± 5*†	14 ± 3*†
LVEDP, mmHg					
CTRL	9 ± 2	8 ± 1	9 ± 2	10 ± 1	9 ± 1
ISCH	8 ± 2	9 ± 2	42 ± 9*†	47 ± 7*†	39 ± 8*†
ISCH + LY	9 ± 1	10 ± 3	40 ± 8*†	45 ± 5*†	39 ± 6*†
PostC	8 ± 1	9 ± 2	19 ± 5*†	19 ± 1*†	16 ± 2*†
PostC + LY	10 ± 2	8 ± 1	41 ± 6*†	41 ± 8*†	39 ± 7*†
DMSO	10 ± 1	10 ± 3	42 ± 8*†	41 ± 3*†	40 ± 5*†
+dp/dt, mmHg/s					
CTRL	2,060 ± 200	2,100 ± 159	2,080 ± 220	2,070 ± 190	1,880 ± 210
ISCH	1,900 ± 300	2,076 ± 200	160 ± 100*†	570 ± 320*†	720 ± 200*†
ISCH + LY	1,867 ± 240	1,900 ± 230	130 ± 50*†	550 ± 290*†	800 ± 250*†
PostC	2,160 ± 181	2,190 ± 200	460 ± 100*†	1,260 ± 200*†	1,588 ± 75*†
PostC + LY	1,936 ± 307	2,076 ± 280	1,650 ± 100*†	600 ± 300*†	720 ± 250*†
DMSO	1,960 ± 305	2,070 ± 282	130 ± 100*†	550 ± 310*†	700 ± 240*†
−dp/dt, mmHg/s					
CTRL	1,540 ± 114	1,400 ± 234	1,454 ± 160	1,400 ± 100	1,580 ± 130
ISCH	1,600 ± 310	1,520 ± 400	80 ± 30*†	224 ± 123*†	264 ± 112*†
ISCH + LY	1,650 ± 400	1,440 ± 170	80 ± 25*†	180 ± 95*†	260 ± 200*†
PostC	1,670 ± 300	1,480 ± 200	350 ± 120*†	800 ± 120*†	1,070 ± 205*†
PostC + LY	1,600 ± 400	1,700 ± 400	85 ± 40*†	214 ± 100*†	274 ± 100*†
DMSO	1,400 ± 210	1,580 ± 420	62 ± 40*†	234 ± 120*†	294 ± 111*†
CF, ml/min					
CTRL	13.0 ± 1.3	13.4 ± 2.3	12.0 ± 1.4	12.6 ± 1.1	12.5 ± 1.5
ISCH	12.0 ± 1.5	11.6 ± 1.5	2.8 ± 1.6*†	3.4 ± 1.1*†	3.8 ± 0.4*†
ISCH + LY	12.8 ± 0.8	12.0 ± 1.8	2.4 ± 1.1*†	2.6 ± 1.5*†	3.6 ± 1.6*†
PostC	13.4 ± 2.0	12.2 ± 2.0	9.8 ± 1.3*†	10.6 ± 1.1*†	11.2 ± 0.8*†
PostC + LY	13.6 ± 1.7	12.8 ± 0.8	2.2 ± 0.8*†	3.4 ± 1.2*†	2.6 ± 0.8*†
DMSO	12.8 ± 1.0	12.4 ± 2.1	2.4 ± 1.1*†	2.8 ± 1.3*†	3.0 ± 1.8*†
HR, beats/min					
CTRL	283 ± 6	306 ± 19	300 ± 23	285 ± 20	300 ± 18
ISCH	300 ± 25	292 ± 16	180 ± 60*†	192 ± 27*†	204 ± 21*†
ISCH + LY	296 ± 11	294 ± 15	134 ± 50*†	150 ± 33*†	196 ± 30*†
PostC	296 ± 20	296 ± 11	192 ± 18*†	246 ± 12*†	243 ± 11*†
PostC + LY	282 ± 15	302 ± 21	136 ± 33*†	196 ± 20*†	204 ± 13*†
DMSO	300 ± 27	291 ± 20	138 ± 30*†	190 ± 27*†	210 ± 12*†

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

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

+dp/dt = inotropy; −dp/dt = lusitropy; CF = coronary flow; CTRL = control (time-matched perfusion of remodeled hearts); DMSO = dimethyl sulfoxide (< 0.1%); HR = heart rate; ISCH = test ischemia without postconditioning; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; LY = LY294002; PostC = isoflurane postconditioning.

Protection by Isoflurane Postconditioning Depends on PI3K-PKB/Akt Signaling Pathway in the Remodeled Myocardium

To determine whether isoflurane postconditioning would also provide protection in the diseased remodeled myocardium, chronically infarcted hearts were exposed to 40 min of ischemia. Anesthetic postconditioning with isoflurane (1.5 MAC) administered for 15 min immediately at the onset of reperfusion significantly improved functional recovery and decreased infarct size when compared with unprotected remodeled hearts (table 2 and fig. 5). Protection by isoflurane postconditioning was completely abolished by coadministration of the PI3K inhibitor LY294002. LY294002 (or dimethyl sulfoxide) alone administered during reperfusion did not further deteriorate postischemic recovery, nor did it affect infarct size (table 1 and fig. 5A). To independently corroborate the results of infarct size determinations by triphenyltetrazolium, LDH release was measured in the perfusate during reperfusion. LDH release was significantly reduced by isoflurane postconditioning, and this protection was abolished by LY294002 administration

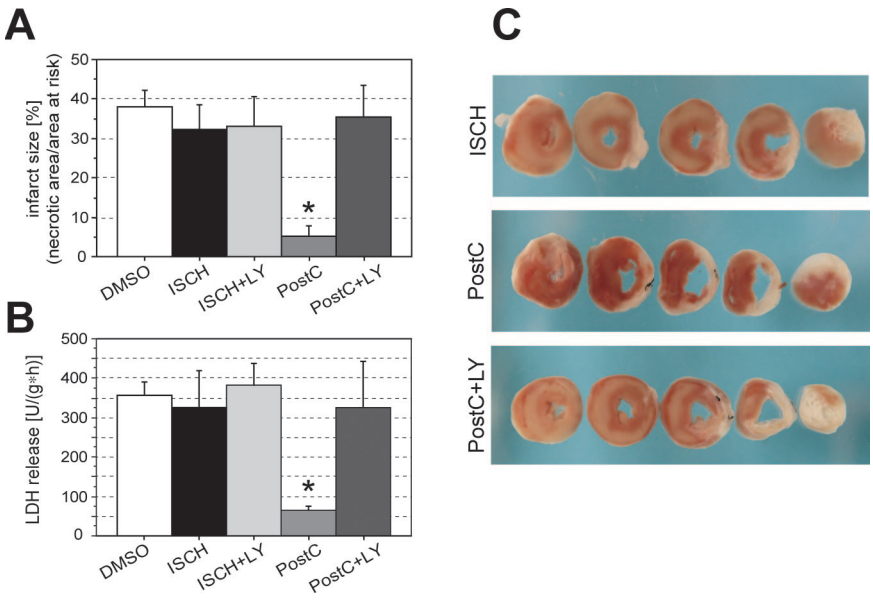

Fig. 5. Infarct size was determined using 1% triphenyltetrazolium chloride staining, as described in the Materials and Methods section. Areas infarcted *in vivo* by coronary artery ligation were excluded from infarct size determination (*A*). The scarred chronic infarct (*white*) resulting from coronary ligation was clearly distinguished from fresh infarcts (*salmon pink*). Release of lactate dehydrogenase (LDH) during reperfusion (*B*) served as an independent method to estimate infarct size (see Materials and Methods). Transverse sections of representative experiments (*C*). DMSO = dimethyl sulfoxide (< 0.1%; used to dissolve LY294002); ISCH = unprotected remodeled hearts exposed to ischemia–reperfusion alone; LY = LY294002 (15 μM); PostC = anesthetic postconditioning. Data are given as mean ± SD (n = 5 in each group). * $P < 0.05$ *versus* ISCH.

(fig. 5B). These results provide strong evidence that protection by isoflurane postconditioning is preserved and operative in this model of postinfarct myocardium.

Isoflurane Postconditioning Activates PKB/Akt, Which Subsequently Phosphorylates the Downstream Targets GSK3β, eNOS, and p70S6K in a PI3K-dependent Manner

It has been well demonstrated that GSK3β is a major downstream target of PKB/Akt and that phosphorylation of the N-terminal Ser-9 residue by PKB/Akt leads to inhibition of GSK3β.[7] Similar to our previous results in healthy myocardium, we here 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. 6A and B). However, when isoflurane was administered during the early reperfusion phase, a significant additional increase in phosphorylation of PKB/Akt and GSK3β was observed (figs. 6A and B). Both ischemia-reperfusion–induced and isoflurane-induced phosphorylation of PKB/Akt and GSK3β were strongly suppressed by LY294002, a specific inhibitor of PI3K. In contrast, eNOS and p70S6K, downstream targets of PKB/Akt, were only marginally activated by ischemia–reperfusion alone, but strikingly enhanced by isoflurane administration and completely abolished by LY294002 (figs. 6C and D). LY294002 (or dimethyl sulfoxide) alone administered during reperfusion did not alter phosphorylation of the investigated enzymes when compared with ischemic control (data not shown). Taken together, these experiments show for the first time a PI3K-dependent activation of three important highly protective downstream targets of PKB/Akt including GSK3β, eNOS, and p70S6K in response to isoflurane postconditioning. The data further provide evidence that PKB/Akt signaling is functional in remodeled myocardium.

Profile of Potential RISKs Does Not Reveal Additional Kinases Involved in the Protection by Isoflurane Postconditioning in This Postinfarct Rat Heart Model

In sharp contrast to PKB/Akt and its downstream signaling targets, ischemia-reperfusion–induced activation of ERK1/2 and p38 mitogen-activated protein kinase was not altered by isoflurane. Also, no change was detected in the PKC-ε phosphorylation status of the various experimental protocols, implying that the role of these kinases may be of limited importance in mediating the protection by isoflurane postconditioning (fig. 6E). The data clearly underscore the unique role of PKB/Akt signaling in the protection of the remodeled myocardium by isoflurane postconditioning.

Discussion

Here, we show for the first time that isoflurane postconditioning retains its protection against ischemia in the severely diseased myocardium. Our experimental model of a postinfarct remodeled myocardium exhibited macroscopic and ultrastructural changes consistent with marked architectural rearrangements of the myocardium, which were accompanied with characteristic alterations at the gene and protein expression level. Despite this profound remodeling process, the diseased hearts were still receptive to functional and structural protection by anesthetic postconditioning. Of note, infarct size reductions by isoflurane postconditioning, as measured by triphenyltetrazolium staining, were corroborated by a reduced release of the necrosis marker LDH into the perfusate during reperfusion. Furthermore, the preserved protection in the postinfarct remodeled hearts completely depended on activation of PI3K and was commensurate with enhanced phosphorylation of the

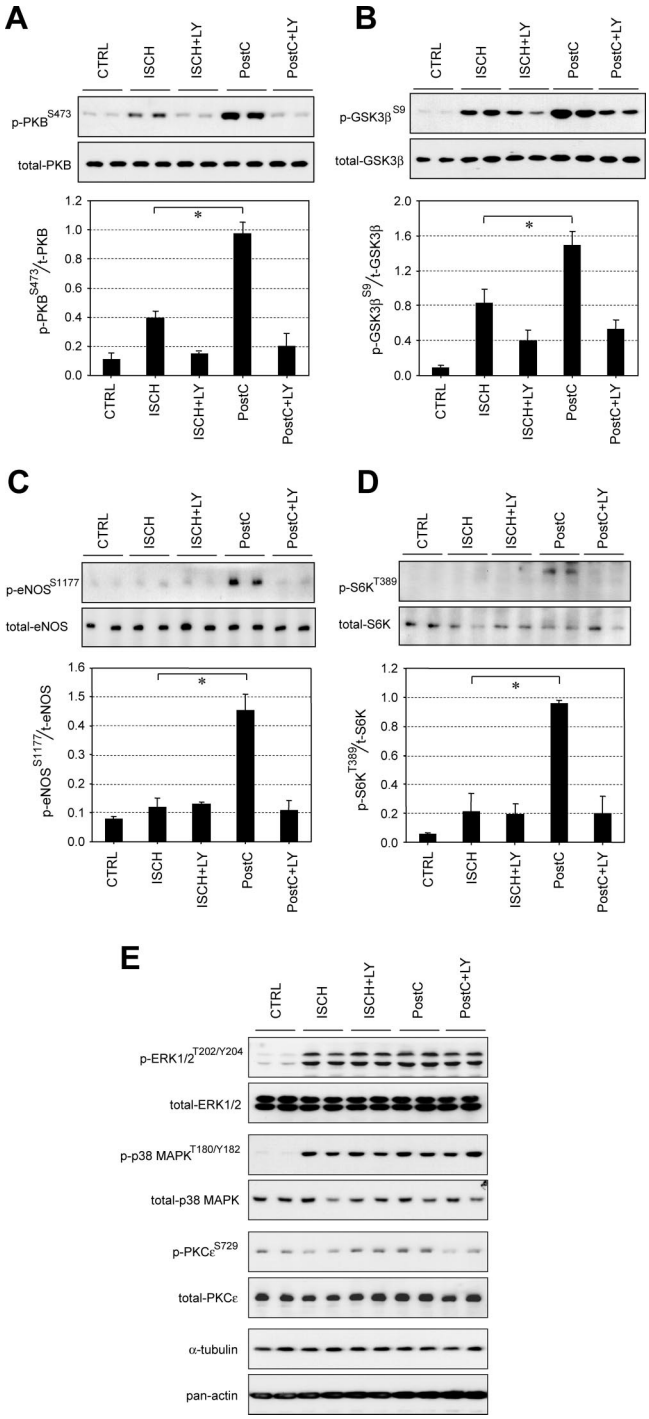

Fig. 6. Western blots analysis. Phosphorylation status of protein kinase B/Akt (PKB, 60 kd, phosphorylation at Ser-473) (*A*), glycogen synthase kinase 3β (GSK3β, 46 kd, phosphorylation at Ser-9) (*B*), endothelial nitric oxide (eNOS, 140 kd, phosphorylation at Ser-1177) (*C*), ribosomal S6 kinase (p70S6K, 70/85 kd, phosphorylation at Thr-389) (*D*), extracellular signal–regulated kinase 1/2 (ERK1/2, 44 and 42 kd, phosphorylation at Thr-202/Tyr-204), p38 mitogen-activated protein kinase (p38 MAPK, 43 kd, phosphorylation at Thr-180/Tyr-182), and protein kinase C ε (PKC-ε, 95 kd, phosphorylation at Ser-729) (*E*) were analyzed with specific phospho-antibodies. CTRL = time-matched perfusion; ISCH = unprotected remodeled hearts exposed to ischemia/reperfusion alone; LY = LY 294002 (15 μM); PostC = anesthetic postconditioning. Data are given as mean ± SD (n = 5 in each group). * $P < 0.05$ PostC *versus* ISCH.

prosurvival PKB/Akt and its protective downstream signaling targets GSK3β, eNOS, and p70S6K. This confirms and further extends previous work[6–8,19] showing the unique role of PKB/Akt signaling in the cellular protection by volatile anesthetics not only in the healthy but also in the diseased heart. Our observation of a functional anesthetic postconditioning mechanism in severely diseased myocardium is of great clinical importance, because an increasing number of elderly patients with hypertension, coronary artery disease, or congestive heart failure and concomitant myocardial remodeling must undergo high-risk cardiac or noncardiac surgery.

Postconditioning: Old Concept Behind a Novel Term?

Postconditioning was first established in 2003 by Vinten-Johansen's group.[20,21] It is a mechanical process whereby brief cycles of alternating ischemia and reperfusion right at the onset of reperfusion markedly decrease ischemic heart damage. Beneficial effects by gradual reperfusion or administration of postconditioning-mimicking agents at the time of reperfusion ("pharmacologic postconditioning") including volatile anesthetics[22] have been known since the 1980s (for review, see Vinten-Johansen *et al.*[23]). However, postconditioning is not simply a passive form of gentle controlled or chemically modified reperfusion, but rather an active biologic process recruiting prosurvival signaling pathways.[12] Yang *et al.*[24] reported a 70% reduction in infarct size using six cycles of 10 s reperfusion–10 s ischemia starting at the beginning of reperfusion in buffer-perfused rabbit hearts, a magnitude of protection similar to the one previously reported for ischemic[25] and anesthetic[26,27] preconditioning. The current study now shows that administration of 1.5 MAC isoflurane during the first 15 min of reperfusion can achieve comparable protection in remodeled hearts.

Cardioprotection in Remodeled Myocardium

Loss of functional myocardium imposes deleterious biomechanical and neurohumoral stress on the residual tissue, leading to compensatory hypertrophy but also additional myocyte death and slippage. This vicious circle, also called remodeling, ultimately results in ventricular dilatation and pump failure. Remodeling is characterized by activation of the fetal gene program and is accompanied by misexpression and rearrangement of myofibrillar, cytoskeletal, and extracellular proteins.[28] Our postinfarct hearts clearly exhibited marked remodeling, with α-skeletal-actin, β-MHC, and desmin being up-regulated. Increased micromechanical stress at cell–cell contacts could be directly visualized by accumulation of N-cadherin at adherens junctions.[29] In accordance with our study, previous experimental data showed that 6 weeks after a large infarction, contractile

dysfunction and hypertrophy consistently evolve in rats.[30] In the clinical setting, angina before myocardial infarction, a clinical correlate to ischemic preconditioning, protects against left ventricular remodelling.[31] Recently, we reported that anesthetic preconditioning but not postconditioning effectively suppressed the early activation of the deleterious remodeling program after ischemia.[27] Vice versa, remodeling may disrupt signaling pathways and abolish innate protective cellular strategies such as preconditioning and postconditioning, and render the myocardium refractory to protection and more susceptible to ischemia. Results from muscle slices of human right atrial appendices of patients with failing hearts indicate that failing myocardium is less amenable to protection by ischemic preconditioning.[11] In fact, loss of preconditioning and postconditioning may be involved in the poor prognosis of patients with postinfarct ventricular remodeling.[32] In line with this observation are findings from a study investigating the effects of preconditioning in postinfarct rabbit hearts, demonstrating complete failure of ischemic but not diazoxide-induced preconditioning to protect the remodeled heart.[33] Refractoriness of the remodeled myocardium to preconditioning was explained by interruption of signal transduction between G-protein and PKC-ε.[34] Other conditions such as hyperglycemia[35] and aging[36] were also found to diminish anesthetic preconditioning. On the other hand, functional preconditioning was reported in severely atherosclerotic ApoE/LDLr$^{-/-}$ knockout mice and several rat heart models of hypertrophied myocardium (for review, see Zaugg *et al.*[37]). Our study now extends these observations and fills a gap showing protection by isoflurane postconditioning in postinfarct remodeled hearts. Whether postconditioning is more effective than preconditioning in the remodeled heart remains to be investigated in future studies.

Signaling in Postconditioning: Current Controversies

Both preconditioning and postconditioning activate RISKs.[38] In ischemic preconditioning, Hausenloy *et al.*[12] previously reported that inhibition of PKB/Akt and ERK1/2 during the first 15 min of reperfusion completely abolished protection. In ischemic postconditioning, Tsang *et al.*[5] and Yang *et al.*[4] recently uncovered the importance of PKB/Akt but also ERK1/2 signaling. Similarly, da Silva *et al.*[39] showed the pivotal role of ERK1/2 during reperfusion in isoflurane preconditioning, while three recent studies unequivocally demonstrate the dependence of isoflurane postconditioning on activation of the PI3K-PKB/Akt pathway.[6–8] However, the relative importance of PKB/Akt and ERK1/2 in mediating the protection of postconditioning is still controversial. A study in isolated perfused rabbit hearts of regional ischemia reports that activation of ERK1/2 but not PKB/Akt is required.[40] Conversely, another study investigating pharmacologic postconditioning by bradykinin suggests that PKB/Akt is upstream of ERK1/2.[41] The authors also found that bradykinin administered at reperfusion caused only a brisk transient increase of ERK1/2 in half of the hearts. These obviously divergent observations may depend on species, the site of tissue sampling (transmural *vs.* epicardial/endocardial), or both but certainly require additional investigation. Consistent with previous reports showing a complex multiphase time course of PKB/Akt and ERK1/2 activation in hypertrophied, ischemic, and failing hearts,[16–18] we could not observe activation of these kinases 6 weeks after coronary ligation when compared with sham-operated hearts.

In addition to RISKs, preconditioning and postconditioning share other signaling components, such as the triggers adenosine and opioids,[8] reactive oxygen species, nitric oxide, and the end-effectors adenine trinucleotide phosphate–dependent potassium channels[42,43] and the mitochondrial permeability transition pore.[7,44] We recently demonstrated that anesthetic postconditioning prevents opening of the mitochondrial permeability transition pore *via* inhibition of GSK3β.[7] The current study now also shows the phosphorylation of the PKB/Akt downstream targets eNOS and p70S6K in anesthetic postconditioning, confirming that most of the critical signaling entities are shared between ischemic and anesthetic postconditioning.[5] Activation of eNOS improves endothelial function, whereas activation of p70S6K facilitates protein synthesis. Taken together, activation of survival signaling by anesthetic postconditioning is preserved in postinfarct remodeled hearts and successfully marshaled by PKB/Akt.

Clinical Implications

Reduction of infarct size is clinically important because it directly correlates with survival. We have previously shown that brief administration of sevoflurane on the fully established cardiopulmonary bypass before induction of cardioplegia significantly improves postoperative cardiac function[45] as well as long-term cardiovascular outcome[46] in patients undergoing coronary artery bypass graft surgery. The concept of postconditioning now shows that reperfusion *per se* is a major factor contributing to ischemic damage and opens the clinically attractive possibility to successfully treat "ischemic" damage at the time of reperfusion. A recent landmark study investigated ischemic postconditioning in patients undergoing coronary angioplasty and stenting for acute myocardial infarction.[47] In this study, creatine kinase release was markedly decreased by 36% in patients treated with four episodes of 1-min balloon inflations starting within 1 min of reflow. Similarly, De Hert *et al.*[48] showed beneficial cardiac effects of anesthetic postconditioning in patients undergoing on-pump coronary artery bypass grafting. Interestingly, in this study the combination of

anesthetic preconditioning and postconditioning was most protective in accordance with a recent *in vivo* animal study[43] and the molecular findings by Lucchinetti *et al.*[27] demonstrating differential but potentially synergistic protective gene expression patterns after anesthetic preconditioning and postconditioning. Because ischemic postconditioning by repetitive clamping or balloon inflation may lead to embolization or endothelial damage, pharmacologic postconditioning by volatile anesthetics should be preferred in the clinical setting. Anesthetic postconditioning of remodeled human hearts may particularly hold promise in nonsurgical coronary artery interventions.

Limitations of the Study and Specific Comments

The following remarks should be added. (1) Although ERK1/2 and PKC-ε were not activated by isoflurane postconditioning in our study, we can not rule out some role of these kinases in mediating the protection by isoflurane, because phosphorylation was determined in whole tissue extracts, which does not consider the possible accumulation of phosphorylation at specific subcellular targets. In fact, ERK1/2 has been reported to form signaling modules with mitochondrial PKC-ε,[49] which confers cardioprotection through inhibition of the mitochondrial permeability transition pore. Also, there may be a complex time course of activation of RISKs, which we could not follow up by determining phosphorylation at a single time point. (2) We have noted increased phosphorylation of eNOS and p70S6K in a PI3K-dependent manner. Therefore, further studies are required using specific blockers to test whether these changes are only epiphenomena or active components of the protection. (3) Future studies should evaluate the optimal dosing of volatile anesthetics to obtain maximal protection during reperfusion. With this regard, it is interesting to note that Obal *et al.*[26] obtained maximal protection after only a 2-min period of sevoflurane application immediately at the onset of reperfusion. Any prolongation of sevoflurane administration rather decreased the protection. (4) As always pertinent to animal experiments, no direct extrapolation into the clinical setting should be made.

Conclusions

Using a highly controlled experimental model of postinfarct rat hearts, we were able to show that recruitment of PKB/Akt signaling at early reperfusion is a universal cardioprotective strategy, which is functional not only in healthy but also in the remodeled myocardium. This is the first demonstration to show that pharmacologic postconditioning by isoflurane retains its profound protection in severely diseased myocardium.

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