

# Differential Role of Calcium/Calmodulin-dependent Protein Kinase II in Desflurane-induced Preconditioning and Cardioprotection by Metoprolol

## Metoprolol Blocks Desflurane-induced Preconditioning

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**Background:** Anesthetic preconditioning is mediated by  $\beta$ -adrenergic signaling. This study tested the hypotheses that desflurane-induced preconditioning is dose-dependently blocked by metoprolol and mediated by calcium/calmodulin-dependent protein kinase II (CaMK II).

**Methods:** Pentobarbital-anesthetized New Zealand White rabbits were instrumented for measurement of systemic hemodynamics and subjected to 30 min of coronary artery occlusion followed by 3 h of reperfusion. Rabbits were assigned to receive vehicle (control), 0.2, 1.0, 1.75, or 2.5 mg/kg metoprolol for 30 min, or the CaMK II inhibitor KN-93 in the absence or presence of 1.0 minimum alveolar concentration desflurane. Protein expression of CaMK II, phospholamban, and phospho-phospholamban was measured by Western blotting. Myocardial infarct size and area at risk were measured with triphenyltetrazolium staining and patent blue, respectively.

**Results:** Baseline hemodynamics were not different among groups. Infarct size was  $60 \pm 3\%$  in control and significantly ( $P < 0.05$ ) decreased to  $33 \pm 2\%$  by desflurane. The CaMK II inhibitor KN-93 did not affect infarct size ( $55 \pm 4\%$ ) but blocked desflurane-induced preconditioning ( $57 \pm 3\%$ ). Metoprolol at 0.2 and 1.0 mg/kg had no effect on infarct size ( $55 \pm 3\%$  and  $53 \pm 3\%$ ), whereas metoprolol at 1.75 and 2.5 mg/kg reduced infarct size to  $48 \pm 4\%$  and  $39 \pm 5\%$ , respectively. Desflurane-induced preconditioning was attenuated by metoprolol at 0.2 mg/kg, leading to an infarct size of  $46 \pm 5\%$ , and was completely abolished by metoprolol at 1.0, 1.75, and 2.5 mg/kg, resulting in infarct sizes of  $51 \pm 3\%$ ,  $52 \pm 3\%$ , and  $55 \pm 3\%$ , respectively.

**Conclusions:** Desflurane-induced preconditioning is dose-dependently blocked by metoprolol and mediated by CaMK II.

DESPITE all efforts to reduce cardiac adverse events, perioperative myocardial ischemia and infarction remain life-threatening complications to patients at high cardiac risk undergoing noncardiac surgery.<sup>1</sup> Perioperative  $\beta$ -adrenoceptor blocker prophylaxis is recommended by American College of Cardiologists–American Heart Association guidelines in patients with cardiac high risk,<sup>2</sup> and volatile anesthetic-induced preconditioning is consid-

ered to be a novel and promising strategy to reduce sequelae of ischemic injury.<sup>3</sup> A combination of  $\beta$ -blocker prophylaxis and anesthetic-induced preconditioning as distinct cardioprotective strategies might exert additional beneficial effects. However, we and others have recently reported that anesthetic-induced preconditioning is mediated by the  $\beta_1$ -adrenergic pathway in the rabbit heart *in vivo*.<sup>4,5</sup> As part of the  $\beta_1$ -adrenergic pathway, calcium/calmodulin-dependent protein kinase II (CaMK II) is a serine/threonine kinase that is involved in intracellular  $\text{Ca}^{2+}$  handling by phosphorylating various  $\text{Ca}^{2+}$  handling proteins, including phospholamban and ryanodine receptors.<sup>6</sup> Furthermore, CaMK II has been demonstrated to mediate ischemic preconditioning.<sup>7,8</sup> However, the role of CaMK II in anesthetic-induced preconditioning is unclear. Therefore, in the current study, we tested the hypothesis that metoprolol, a clinically widely used  $\beta$ -blocker, dose-dependently blocks desflurane-induced preconditioning and evaluated the role of CaMK II in desflurane-induced preconditioning and in  $\beta$ -blocker-derived cardioprotection.

## Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the local authorities (Government of Unterfranken, Würzburg, Germany) and conformed to the regulations of the German animal protection law. Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.<sup>9</sup>

### General Preparation

General preparation was performed as previously described.<sup>4</sup> Briefly, male New Zealand White rabbits were anesthetized with sodium pentobarbital (30 mg/kg intravenous bolus, followed by an infusion of 20–30 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  h<sup>-1</sup>) *via* the left marginal auricular vein. Sodium pentobarbital was chosen because of its negligible effects on preconditioning.<sup>10</sup> No opioids or neuromuscular blocking agents were used throughout the investigation. Depth of anesthesia was verified by recurrent

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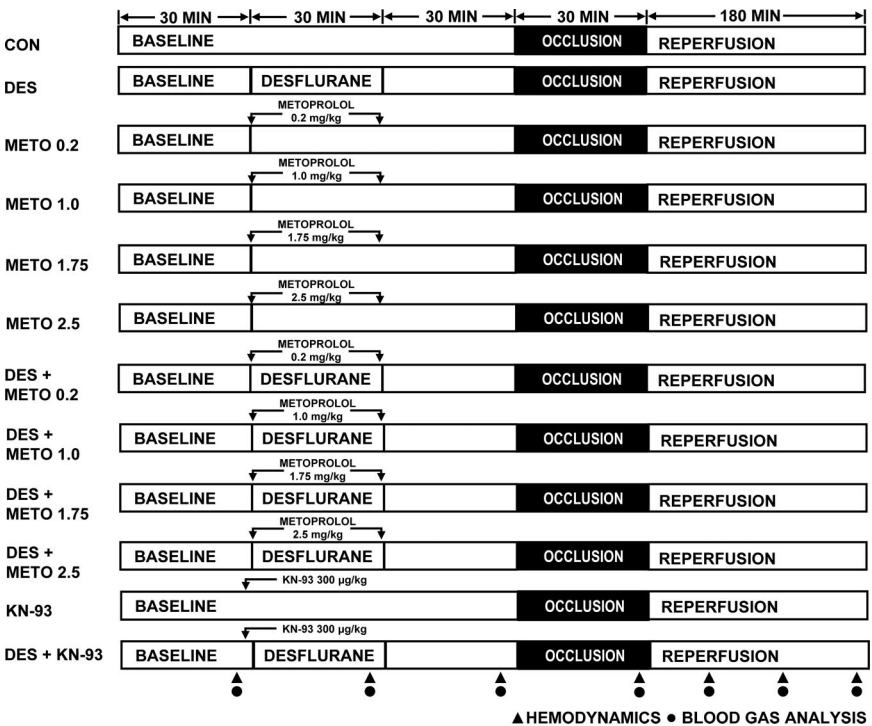
testing of palpebral reflexes and hind paw withdrawal throughout the experiment. After tracheotomy and tracheal cannulation, animals were artificially ventilated (Cicero®; Dräger, Lübeck, Germany) using positive pressure with an air and oxygen mixture (70%/30%). Arterial blood drawn from the auricular artery was analyzed using an ABL 505 blood gas analyzer (Radiometer, Copenhagen, Denmark), and blood gases were maintained within a normal physiologic range by adjusting the respiratory rate or tidal volume. End-tidal concentration of desflurane was measured at the tip of the endotracheal tube by an infrared anesthetic gas analyzer that was calibrated with known standards before and during experimentation. The rabbit minimum alveolar concentration (MAC) of desflurane used in the current investigation was 8.9%.<sup>11</sup> Left ventricular (LV) pressure and the maximum increase of LV pressure (+dP/dt<sub>max</sub>) were measured with a saline-filled PE 50 polyethylene catheter inserted into the left ventricle *via* the right carotid artery. Mean arterial pressure was monitored by insertion of a 2.5-French microtipped catheter (Millar Instruments Inc., Houston, TX) *via* the right femoral artery into the descending aorta. Rectal body temperature was maintained at 38.5° ± 0.5°C<sup>12</sup> by a servo-controlled heating pad (Föhr Instruments, Seeheim, Germany). After a left fourth thoracotomy and pericardiotomy, the left heart was exposed and suspended in a pericardial cradle. A silk ligature (2-0) was placed halfway between the base and the apex of the heart around a prominent branch of the left anterior descending coronary artery to form a snare. By tightening the snare, a coronary artery occlusion was produced, and reperfusion was instituted by

loosening the snare. Each rabbit received 300 U/kg heparin 5 min before coronary artery occlusion for anticoagulation. Coronary artery occlusion was verified by epicardial cyanosis, regional dyskinesia in the ischemic zone, and electrocardiographic changes. Adequate reperfusion was confirmed by epicardial hyperemic response and reversion of electrocardiographic changes. Hemodynamic parameters, body temperature, and electrocardiogram were continuously recorded and analyzed using a personal computer (Hewlett Packard, Palo Alto, CA) and hemodynamic data acquisition and analysis software (Notocord® hem 3.5; Croissy sur Seine, France). Data were digitized at a sampling rate of 1,000 Hz.

Experimental Protocol

The experimental protocol used in this investigation is illustrated in figure 1. Baseline systemic hemodynamics were recorded following a 30-min equilibration period after completion of instrumentation and calibration. All rabbits were subjected to 30 min of coronary artery occlusion followed by 3 h of reperfusion. Rabbits were randomly assigned to one of the study groups by opening a sealed envelope containing information about the study group after completion of the preparation of each animal. After 8 rabbits were randomized to each group and a preliminary data analysis had been performed, 2 more rabbits were randomized to each group to achieve a group size of 10 per group. To investigate the interaction between desflurane and metoprolol, rabbits received either vehicle (0.9% saline [control]), 1.0 MAC desflurane, or metoprolol at 0.2, 1.0, 1.75, or 2.5 mg/kg. Metoprolol at 0.2, 1.0, 1.75, and 2.5 mg/kg was coad-

Fig. 1. Schematic diagram illustrating the experimental protocol. CON = control; DES = 1.0 minimum alveolar concentration (MAC) desflurane; DES + KN-93 = desflurane + KN-93; DES + METO = 1.0 MAC desflurane + 0.2, 1.0, 1.75, or 2.5 mg/kg metoprolol; KN-93 = 300 µg/kg KN-93; METO = 0.2, 1.0, 1.75, or 2.5 mg/kg metoprolol. n = 10 per group, except for KN-93 and DES + KN-93 (n = 6 per group).



ministered in four separate groups with desflurane ( $n = 10$  per group). Desflurane and metoprolol were administered continuously for 30 min and discontinued 30 min before coronary occlusion. In two separate groups, a bolus of KN-93 (300  $\mu\text{g/kg}$ ; Sigma-Aldrich, Munich, Germany) solved in dimethyl sulfoxide, a specific inhibitor of CaMK II, was administered directly into the left ventricle in the presence or absence of desflurane ( $n = 6$  per group).

#### Measurement of Myocardial Infarct Size

Infarct size and area at risk (AAR) were gravimetrically determined according to standard procedures.<sup>13</sup> Briefly, at the end of each experiment, the coronary artery was reoccluded and the AAR was determined by infusion of 2 ml patent blue (0.1 g/ml; Sigma-Aldrich, Taufkirchen, Germany). The rabbits were then killed with a lethal dose of pentobarbital, and the heart was rapidly excised. The heart was cut into five slices from apex to base, and the nonstained red myocardium (AAR) was separated from the nonischemic blue-stained LV normal areas. The samples of ischemic and nonischemic regions were incubated at 37°C for 20 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. After overnight storage in 10% formaldehyde, infarcted (pale) and noninfarcted (brick-red) myocardium within the AAR was carefully separated and weighed. Infarct size was expressed as a percentage of the AAR. Rabbits with an AAR less than 15% of LV mass and those with intractable ventricular fibrillation or LV pump failure were excluded from the study. Infarct size was determined by an investigator blinded to the study protocol.

#### Western Immunoblotting

In a separate set of experiments, the effects of saline (control), 1.0 MAC desflurane, or 2.5 mg/kg metoprolol and KN-93 in the presence or absence of desflurane on CaMK II and phospholamban content and phosphorylation of phospholamban was investigated by Western immunoblotting. An interim analysis was performed after 5 animals per group had been studied. As a result, group size was adjusted to 7 animals per group to rule out a possible type I error ( $n = 7$  per group). Five minutes after cessation of desflurane or metoprolol, the hearts were rapidly excised, and the left ventricle was shock frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further treatment. In the KN-93 group, the hearts were excised at the corresponding time point. The samples were homogenized in ice-cold RIPA buffer (1 $\times$  phosphate-buffered saline, 1% Igepal CA-630, 0.5% sodium deoxycholic acid, 0.1% sodium dodecylsulfate polyacrylamide, 20 mM sodium fluoride, 1 mM sodium orthovanadate, containing a protease-inhibitor cocktail [Roche, Grenzach-Wyhlen, Germany]) and centrifuged at 12,000g. Cytosolic and particulate cell fractions were left unsepa-

rated for further analysis. Proteins were separated on 15% polyacrylamide sodium dodecylsulfate polyacrylamide gels and subsequently transferred electrophoretically on nitrocellulose membranes (Whatman, Maidstone, United Kingdom). After transfer, nonspecific background was blocked using 5% nonfat milk powder in phosphate-buffered saline-Tween 20 (1 h at room temperature). Membranes were then incubated for 1 h at room temperature with the following specific antibodies: mouse antiphospholamban 1:3,000 (Affinity BioReagents, Golden, CO); mouse anti-glyceraldehyde-3-phosphate dehydrogenase 1:3,000 (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti- $\alpha\text{B-crystallin}$  (Assay Designs, Ann Arbor, MI) in 5% nonfat milk in phosphate-buffered saline-Tween 20 or incubated overnight (rabbit anti-p-phospholamban 1:1,000 (Badrilla, Leeds, United Kingdom) and goat anti-CaMK II 1:200 (Santa Cruz Biotechnology) at 4°C, respectively. Horseradish peroxidase-linked antibodies against mouse, rabbit, or goat 1:5,000 (Santa Cruz Biotechnology) were used as secondary antibodies. The protein bands were detected by ECL<sup>®</sup> detection reagent (GE Healthcare, Buckinghamshire, United Kingdom) and visualized using an x-ray film. The films were scanned and the band optical density was analyzed using Scan pack Software 3.0 (Bio-Rad Laboratories, Munich, Germany). Gel loading was normalized to  $\alpha\text{B-crystallin}$  or glyceraldehyde-3-phosphate dehydrogenase expression, respectively.

#### Statistical Analysis

Power analysis revealed a group size of  $n = 8$  to detect a difference in means of 15% with a power of 0.8 at an  $\alpha$  level of 0.05. Statistical analysis of hemodynamic data were performed using an overall 12 (control *vs.* desflurane *vs.* 0.2 mg/kg metoprolol *vs.* 1.0 mg/kg metoprolol *vs.* 1.75 mg/kg metoprolol *vs.* 2.5 mg/kg metoprolol *vs.* 0.2 mg/kg metoprolol + desflurane *vs.* 1.0 mg/kg metoprolol + desflurane *vs.* 1.75 mg/kg metoprolol + desflurane *vs.* 2.5 mg/kg metoprolol + desflurane *vs.* KN-93 *vs.* KN-93 + desflurane)  $\times 7$  (baseline *vs.* preconditioning *vs.* memory *vs.* coronary artery occlusion *vs.* reperfusion 1 *vs.* reperfusion 2 *vs.* reperfusion 3) analysis of variance with repeated measures. In case of significant main effects of interactions, *post hoc* one-way analyses of variance were conducted for each group and for each time. Statistical analysis for body weight, LV, AAR, AAR/LV, infarct size/AAR, and densitometry was performed using one-way analysis of variance (control *vs.* desflurane *vs.* 0.2 mg/kg metoprolol *vs.* 1.0 mg/kg metoprolol *vs.* 1.75 mg/kg metoprolol *vs.* 2.5 mg/kg metoprolol *vs.* 0.2 mg/kg metoprolol + desflurane *vs.* 1.0 mg/kg metoprolol + desflurane *vs.* 1.75 mg/kg metoprolol + desflurane) and *post hoc* Duncan test where appropriate. Statistical analysis of data was performed on a personal computer using SPSS 15.0 software (The Apache Software Foundation, Forest Hill, MD). All data are expressed as mean  $\pm$  SEM.



Table 1. Group Sample Sizes

	Infarct Size	Western Blot
CON	10	7
DES	10	7
METO 0.2	10	—
METO 1.0	10	—
METO 1.75	10	—
METO 2.5	10	7
METO 0.2/DES	10	—
METO 1.0/DES	10	—
METO 1.75/DES	10	—
METO 2.5/DES	10	7
KN-93	6	7
KN-93/DES	6	7

Data are mean  $\pm$  SEM.

CON = control; DES = desflurane; KN-93 = 300  $\mu$ g/kg KN-93; KN-93/DES = KN-93 + desflurane; METO 0.2 = 0.2 mg/kg metoprolol; METO 1.0 = 1.0 mg/kg metoprolol; METO 1.75 = 1.75 mg/kg metoprolol; METO 2.5 = 2.5 mg/kg metoprolol; METO 0.2/DES = 0.2 mg/kg metoprolol + desflurane; METO 1.0/DES = 1.0 mg/kg metoprolol + desflurane; METO 1.75/DES = 1.75 mg/kg metoprolol + desflurane; METO 2.5/DES = 2.5 mg/kg metoprolol + desflurane.

## Results

One hundred thirty rabbits were instrumented to obtain 112 successful experiments (table 1). Three rabbits were excluded because of intractable ventricular fibrillation during the experimental protocol (one control, one 1.0 mg/kg metoprolol, and one 2.5 mg/kg metoprolol), 11 due to LV pump failure (one desflurane, two desflurane + 0.2 mg/kg metoprolol, two desflurane + 2.5 mg/kg metoprolol, two 0.2 mg/kg metoprolol, two 1.0 mg/kg metoprolol, one 1.75 mg/kg metoprolol, and one 2.5 mg/kg metoprolol), and 4 because LV AAR was less than 15% of the LV mass (one desflurane + 1.75 mg/kg metoprolol, one 1.0 mg/kg metoprolol, and two 2.5 mg/kg metoprolol).

### Hemodynamics

There were no differences in hemodynamic parameters between experimental groups at baseline (table 2). Metoprolol given alone had no effect on mean arterial pressure, but decreased heart rate at doses of 1.0 mg/kg and higher. This reduction was sustained throughout the study protocol. Metoprolol had no effect on  $+dP/dt_{\max}$  at any dose given. Desflurane had no effect on heart rate. However, desflurane given alone or coadministered with different doses of metoprolol reduced mean arterial pressure, rate-pressure product, and  $+dP/dt_{\max}$  during its application. After discontinuation of desflurane, mean arterial pressure and  $+dP/dt_{\max}$  returned to baseline values. LV end-diastolic pressure was not affected by any of the study drugs. KN-93, given alone, reduced mean arterial pressure.

### Myocardial Infarct Size

Myocardial infarct size (infarct size/AAR) was  $60 \pm 3\%$  in control experiments. Desflurane at 1.0 MAC for 30

min and discontinued 30 min before coronary artery occlusion reduced infarct size to  $33 \pm 2\%$ . The specific inhibitor of CaMK II, KN-93, had no effect on infarct size that was  $55 \pm 4\%$  ( $n = 6$ ). However, desflurane-induced preconditioning was blocked by KN-93 to an infarct size of  $57 \pm 3\%$  ( $n = 6$ ) (fig. 2). Metoprolol at 0.2 and 1.0 mg/kg did not affect infarct sizes that were  $55 \pm 3\%$  and  $53 \pm 3\%$ , respectively. Metoprolol at 1.75 and 2.5 mg/kg reduced infarct size to  $48 \pm 4\%$  and  $39 \pm 5\%$ . Desflurane-induced preconditioning was dose-dependently blocked by metoprolol at 0.2, 1.0, 1.75, and 2.5 mg/kg to infarct sizes of  $46 \pm 5\%$ ,  $51 \pm 3\%$ ,  $52 \pm 3\%$ , and  $55 \pm 3\%$ , respectively ( $n = 10$  in all groups) (fig. 3).

### CaMK II and Phospholamban Protein Expression

Total CaMK II protein expression (fig. 4) and total phospholamban protein expression (fig. 5A) were not affected by any of the study drugs. Protein kinase A (PKA)-dependent phosphorylation of phospholamban at serine 16 was completely blocked by metoprolol but was left unaffected by desflurane and KN-93 (fig. 5B). Phosphorylation of phospholamban by CaMK II at threonine 17 was blocked by the specific CaMK II inhibitor KN-93 and by metoprolol, given alone or in combination with desflurane (fig. 5C). Desflurane given alone had no impact on the phosphorylation of phospholamban at threonine 17.

## Discussion

The first major finding of this study is that blockade of CaMK II activity by the specific inhibitor KN-93 abolishes desflurane-induced preconditioning.  $Ca^{2+}$  is a pivotal second messenger in myocardial cells. Many of its intracellular actions are mediated by  $Ca^{2+}$ /calmodulin-dependent protein kinases. CaMK II is the predominant isoform of  $Ca^{2+}$ /calmodulin-dependent protein kinases in the heart and modulates voltage-gated L-type  $Ca^{2+}$  channels and  $Ca^{2+}$  release and uptake from the sarcoplasmic reticulum *via* sarcoplasmic reticulum  $Ca^{2+}$  adenosine triphosphatase and ryanodine receptors.<sup>6</sup> Therefore, CaMK II is important for the regulation of intracellular  $Ca^{2+}$  homeostasis. Because cytosolic  $Ca^{2+}$  overload is a major determinant of ischemia and reperfusion (I/R) injury,<sup>14</sup> a reduction of cytosolic  $Ca^{2+}$  levels might beneficially affect the consequences of I/R injury. There is clear evidence that improved  $Ca^{2+}$  handling during reperfusion is one mechanism of cardioprotection of preconditioning, because ischemic preconditioning prevents the depression in sarcoplasmic reticulum  $Ca^{2+}$  uptake and  $Ca^{2+}$  pump adenosine triphosphatase activity triggered by I/R injury.<sup>8</sup> A recent study in isolated rat hearts demonstrated that blockade of CaMK II by KN-93 abrogated the beneficial effects of ischemic preconditioning on the recovery of LV contractile func-

Table 2. Systemic Hemodynamic Parameters

	Base	Precon	Mem	CAO	Reperfusion		
					1 h	2 h	3 h
HR, min <sup>-1</sup>							
CON	268 ± 7	262 ± 7	263 ± 7	263 ± 6	249 ± 6	240 ± 5	234 ± 7
DES	272 ± 9	260 ± 8	264 ± 9	264 ± 7	246 ± 8	230 ± 5*	219 ± 5*
METO 0.2	280 ± 8	245 ± 9*	247 ± 7*	251 ± 11	245 ± 9*	243 ± 11*	237 ± 9*
METO 1.0	276 ± 13	231 ± 10*†	232 ± 9*	238 ± 9*	232 ± 9*	231 ± 9*	229 ± 8*
METO 1.75	273 ± 9	229 ± 4*†	226 ± 5*†	231 ± 8*†	227 ± 5*	225 ± 5*	224 ± 6*
METO 2.5	253 ± 8	209 ± 8*†	208 ± 9*†	210 ± 9*†	210 ± 9*†	200 ± 10*†	203 ± 12*†
METO 0.2/DES	270 ± 11	234 ± 8*	242 ± 9	248 ± 9	239 ± 10	236 ± 11*	230 ± 10*
METO 1.0/DES	268 ± 9	214 ± 7*†	224 ± 8*†	235 ± 8*	225 ± 8*	224 ± 7*	227 ± 9*
METO 1.75/DES	278 ± 10	222 ± 4*†	228 ± 5*†	233 ± 6*	225 ± 4*	219 ± 4*	213 ± 5*
METO 2.5/DES	252 ± 12	199 ± 6*†	209 ± 6†	215 ± 5*†	218 ± 8*†	203 ± 13*†	198 ± 14*†
KN-93	277 ± 7	257 ± 8	247 ± 10	253 ± 9	249 ± 9	246 ± 9	245 ± 10*
KN-93/DES	254 ± 10	256 ± 20	250 ± 10	237 ± 7	243 ± 9	244 ± 9	243 ± 8
MAP, mmHg							
CON	88 ± 5	90 ± 5	90 ± 5	87 ± 5	85 ± 4	80 ± 6	80 ± 5
DES	90 ± 4	55 ± 3*†	84 ± 4	80 ± 5	77 ± 5	71 ± 4*	67 ± 5*
METO 0.2	98 ± 3	94 ± 5	91 ± 5	86 ± 4	84 ± 5	81 ± 5	78 ± 6*
METO 1.0	97 ± 5	94 ± 7	92 ± 7	84 ± 6	82 ± 5	78 ± 5	79 ± 5
METO 1.75	98 ± 5	88 ± 6	88 ± 6	83 ± 6	82 ± 6	80 ± 5*	77 ± 5*
METO 2.5	87 ± 5	84 ± 6	81 ± 6	80 ± 9	77 ± 9	76 ± 8	75 ± 7
METO 0.2/DES	95 ± 5	55 ± 4*†	90 ± 5	87 ± 4	85 ± 5	82 ± 5	79 ± 6
METO 1.0/DES	93 ± 5	55 ± 4*†	90 ± 4	81 ± 4	76 ± 4	77 ± 5	81 ± 4
METO 1.75/DES	96 ± 5	60 ± 3*†	90 ± 6	83 ± 4	81 ± 5	79 ± 5	79 ± 6
METO 2.5/DES	86 ± 5	46 ± 3*†	77 ± 6	75 ± 4	71 ± 4	70 ± 7	67 ± 4*
KN-93	79 ± 4	69 ± 5†	69 ± 4†	65 ± 6†	61 ± 5†	63 ± 4	66 ± 5
KN-93/DES	78 ± 5	56 ± 6†	75 ± 5	72 ± 5	70 ± 3	67 ± 2	65 ± 6
LVEDP, mmHg							
CON	3 ± 1	3 ± 1	3 ± 1	8 ± 2	5 ± 1	6 ± 1	7 ± 1
DES	3 ± 1	2 ± 1	2 ± 1	3 ± 1	3 ± 1	5 ± 2	5 ± 2
METO 0.2	3 ± 1	3 ± 1	4 ± 1	8 ± 1	6 ± 1	5 ± 1	6 ± 2
METO 1.0	4 ± 1	3 ± 1	4 ± 1	9 ± 1	6 ± 1	6 ± 2	7 ± 2
METO 1.75	2 ± 1	1 ± 0	2 ± 0	5 ± 1	3 ± 0	3 ± 0	4 ± 1
METO 2.5	3 ± 1	4 ± 1	3 ± 1	6 ± 1	5 ± 1	8 ± 2	6 ± 2
METO 0.2/DES	3 ± 1	4 ± 1	4 ± 1	6 ± 2	4 ± 1	5 ± 1	6 ± 1
METO 1.0/DES	2 ± 1	2 ± 1	2 ± 1	5 ± 1	2 ± 1	3 ± 1	2 ± 0
METO 1.75/DES	3 ± 1	2 ± 0	2 ± 0	8 ± 1	5 ± 1	5 ± 1	6 ± 1
METO 2.5/DES	3 ± 1	3 ± 1	3 ± 1	7 ± 1	3 ± 1	2 ± 1	2 ± 1
KN-93	8 ± 4	6 ± 3	12 ± 8†	6 ± 4	5 ± 3	5 ± 3	5 ± 3
KN-93/DES	8 ± 3	7 ± 2	6 ± 2	10 ± 2	9 ± 2	10 ± 2	12 ± 2
+dP/dt <sub>max</sub> , mmHg/s							
CON	4,516 ± 395	4,491 ± 339	4,560 ± 396	4,250 ± 402	3,941 ± 369	3,452 ± 459	3,173 ± 440
DES	4,425 ± 670	2,147 ± 163*†	3,730 ± 527	3,440 ± 394	2,956 ± 302	3,434 ± 962	3,098 ± 781
METO 0.2	4,664 ± 583	3,564 ± 434	3,569 ± 581	3,086 ± 318	2,932 ± 284*	2,986 ± 272*	2,680 ± 324*
METO 1.0	3,832 ± 514	3,252 ± 532	3,276 ± 517	2,715 ± 475	2,806 ± 475	2,637 ± 396	2,382 ± 400
METO 1.75	4,995 ± 310	3,442 ± 326	3,677 ± 349	3,304 ± 269*	3,020 ± 248*	2,671 ± 286*	2,368 ± 291*
METO 2.5	4,003 ± 217	3,639 ± 219†	3,565 ± 233	3,238 ± 224	3,036 ± 280	3,100 ± 192	3,206 ± 382
METO 0.2/DES	4,092 ± 660	1,832 ± 256*†	3,688 ± 591	2,969 ± 425	2,924 ± 422	2,620 ± 356	2,500 ± 369*
METO 1.0/DES	4,373 ± 435	1,975 ± 192*†	3,925 ± 406	3,117 ± 378	3,110 ± 543	2,843 ± 348	3,054 ± 366
METO 1.75/DES	4,290 ± 514	2,199 ± 298*†	3,840 ± 492	3,271 ± 365	3,143 ± 403	2,985 ± 388	2,789 ± 346
METO 2.5/DES	3,927 ± 281	2,212 ± 233*†	3,418 ± 387	3,126 ± 314	3,025 ± 280	2,923 ± 253	2,765 ± 197
KN-93	1,876 ± 577†	1,950 ± 630†	2,097 ± 669†	2,125 ± 648†	1,922 ± 541†	1,781 ± 460†	1,760 ± 649
KN-93/DES	2,273 ± 680†	1,656 ± 281†	2,279 ± 646†	2,157 ± 655†	2,163 ± 607†	2,071 ± 546	2,068 ± 477
RPP, HR · MAP							
CON	23.9 ± 1.5	23.9 ± 1.4	23.7 ± 1.4	23.4 ± 1.2	22.1 ± 1.1	20.4 ± 1.4	19.9 ± 1.2
DES	24.6 ± 1.6	14.3 ± 1.1*†	22.3 ± 1.5	21.1 ± 1.3	19.1 ± 1.4	16.4 ± 0.9*	14.5 ± 0.9*
METO 0.2	27.2 ± 1.8	23.2 ± 1.9	22.7 ± 1.8	21.7 ± 1.8*	20.6 ± 1.8*	20.0 ± 1.9*	18.6 ± 1.8*
METO 1.0	26.8 ± 2.0	22.0 ± 2.0	21.6 ± 1.9	20.3 ± 2.0*	19.2 ± 1.8*	18.4 ± 1.7*	18.3 ± 1.6*
METO 1.75	27.1 ± 1.9	20.2 ± 1.5*	20.1 ± 1.4*	19.4 ± 1.6*	18.8 ± 1.6*	18.1 ± 1.4*	17.3 ± 1.2*
METO 2.5	22.3 ± 1.6	17.7 ± 1.7†	17.0 ± 1.8†	16.9 ± 2.3†	16.5 ± 2.3	15.4 ± 2.0*	15.6 ± 2.1*
METO 0.2/DES	25.9 ± 2.1	12.9 ± 1.2*†	21.9 ± 1.9	21.7 ± 1.7	20.7 ± 1.8	19.7 ± 2.0*	18.5 ± 2.1*
METO 1.0/DES	24.9 ± 1.3	11.7 ± 0.9*†	20.3 ± 1.1	19.1 ± 1.3*	17.1 ± 1.0*	17.2 ± 1.3*	18.4 ± 1.2*
METO 1.75/DES	27.1 ± 2.0	13.4 ± 0.8*†	20.5 ± 1.7*	19.2 ± 1.3*	18.1 ± 1.3*	17.4 ± 1.4*	17.0 ± 1.5*
METO 2.5/DES	21.0 ± 1.3	9.1 ± 0.5*†	16.1 ± 1.1†	16.1 ± 0.8†	15.4 ± 1.0†	14.3 ± 1.8*†	13.5 ± 1.4*
KN-93	18.9 ± 1.7	14.4 ± 1.6†	18.9 ± 1.7†	17.0 ± 1.4†	16.8 ± 1.3†	15.9 ± 0.6	16.7 ± 1.2
KN-93/DES	19.1 ± 1.4	17.7 ± 1.4†	17.1 ± 1.2	16.1 ± 1.3†	14.6 ± 0.9	15.6 ± 1.1	16.3 ± 1.5

Data are mean ± SEM. n = 10 per group, except for KN-93 and KN-93/DES (n = 6 per group).

\* Significantly ( $P < 0.05$ ) different from baseline. † Significantly ( $P < 0.05$ ) different from the respective value in control experiments.

Base = baseline values; CAO = coronary artery occlusion; CON = control; DES = desflurane; +dP/dt<sub>max</sub> = maximal rate of increase of left ventricular pressure; HR = heart rate; KN-93 = 300 µg/kg KN-93; KN-93/DES = KN-93 + desflurane; LVEDP = left ventricular end-diastolic pressures; MAP = mean aortic blood pressure; Mem = memory phase without intervention; METO 0.2 = 0.2 mg/kg metoprolol; METO 1.0 = 1.0 mg/kg metoprolol; METO 1.75 = 1.75 mg/kg metoprolol; METO 2.5 = 2.5 mg/kg metoprolol; METO 0.2/DES = 0.2 mg/kg metoprolol + desflurane; METO 1.0/DES = 1.0 mg/kg metoprolol + desflurane; METO 1.75/DES = 1.75 mg/kg metoprolol + desflurane; METO 2.5/DES = 2.5 mg/kg metoprolol + desflurane; Precon = administration of metoprolol or desflurane in the presence or absence of metoprolol; RPP = rate-pressure product.

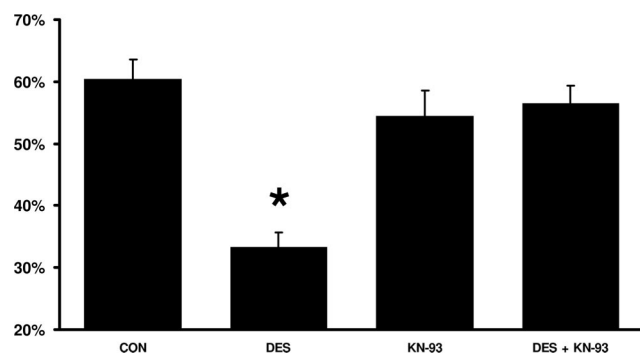


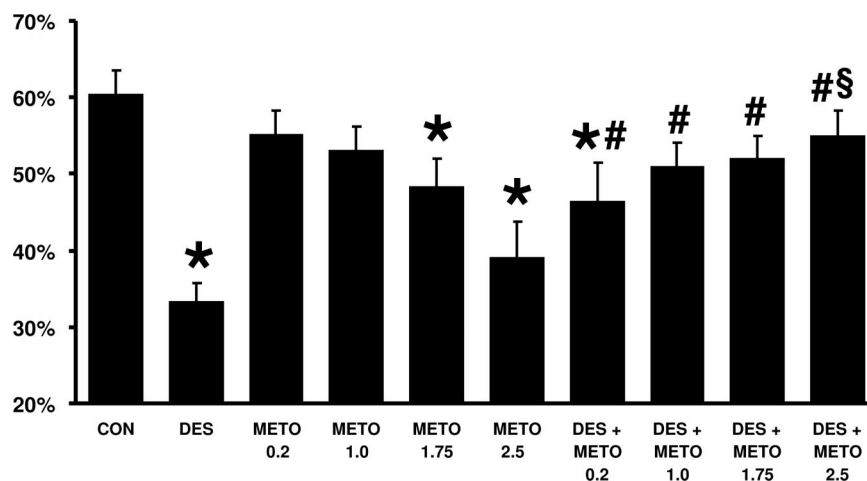
Fig. 2. Myocardial infarct size expressed as a percentage of the area at risk in rabbits receiving 1.0 minimum alveolar concentration (MAC) desflurane and KN-93 alone or in combination. CON = control; DES = 1.0 MAC desflurane; DES + KN-93 = desflurane + KN-93; KN-93 = 300  $\mu$ g/kg KN-93.  $n = 10$  per group, except for KN-93 and DES + KN-93 ( $n = 6$  per group). \* Significantly ( $P < 0.05$ ) different from CON.

tion after simulated ischemia.<sup>7</sup> Like ischemic preconditioning, sevoflurane-induced preconditioning reduces  $Ca^{2+}$  overload and improves ventricular function.<sup>15</sup> The results of the current investigation now demonstrate that desflurane-induced preconditioning is abrogated by specific blockade of CaMK II by KN-93. These findings suggest that CaMK II is part of the signal transduction pathway of desflurane-induced preconditioning. Moreover, the results of the Western blot analysis demonstrated that CaMK II is effectively blocked by KN-93, because CaMK II-dependent phospholamban phosphorylation at threonine 17 was inhibited, whereas PKA-dependent phospholamban phosphorylation at serine 16 was not.<sup>16</sup> In contrast, metoprolol blocked phospholamban phosphorylation at both sites. It is possible that this difference in inhibition of phosphorylation sites of phospholamban accounts for the differences in cardioprotection between desflurane and metoprolol observed in the current study. While CaMK II inhibition and hence phosphorylation of phospholamban at threonine 17 blocks desflurane-induced preconditioning, the effective inhibition of CaMK II and PKA and hence phosphorylation of phospholamban at both sites might be necessary to con-

fer cardioprotection by metoprolol. This contribution is contradictory only at first sight. It should be borne in mind that long and sustained  $\beta$ -adrenergic stimulation, as occurs in chronic heart failure, results in cardiomyocyte apoptosis induced by PKA-independent CaMK II activation and not by the classic cyclic adenosine monophosphate-PKA pathway.<sup>17,18</sup> Furthermore, increased CaMK II activity after myocardial infarction or from excessive  $\beta$ -adrenergic stimulation results in maladaptive remodeling and arrhythmias.<sup>19</sup> This observation can be explained by the finding that during reperfusion phospholamban is mainly phosphorylated by CaMK II.<sup>20</sup> Phospholamban phosphorylation results in increased activity of sarcoplasmic reticulum  $Ca^{2+}$  adenosine triphosphatase with consecutively higher  $Ca^{2+}$  load in and release from the sarcoplasmic reticulum with subsequent cytosolic  $Ca^{2+}$  overload. Because ablation and phosphorylation of phospholamban exert the same effects on sarcoplasmic reticulum  $Ca^{2+}$  adenosine triphosphatase, phospholamban-deficient mice exhibit increased ischemic injury compared with wild-type mice.<sup>21</sup> Therefore, the prevention of phospholamban phosphorylation by metoprolol might contribute to its cardioprotective effects during reperfusion. On the other hand, short-lived  $\beta$ -adrenergic stimulation is known to exert positive effects on I/R injury and to induce preconditioning.<sup>22,23</sup> Therefore, CaMK II is necessary to induce ischemic and anesthetic preconditioning, while sustained and long-termed blockade of CaMK II by  $\beta$ -adrenergic receptor blockers might prevent the deleterious effects of adrenergic stimulation and hence prolonged CaMK II activation.

The second major finding of this study is that metoprolol reduced infarct size only, when the highest doses of metoprolol of 1.75 and 2.5 mg/kg were given before I/R. Metoprolol at 1.0 mg/kg did not confer cardioprotection. In another study using the rabbit myocardial infarction model, metoprolol at a dose of 1.0 mg/kg effectively reduced infarct size.<sup>24</sup> However, in that investigation, metoprolol was not given before ischemia but

Fig. 3. Myocardial infarct size expressed as a percentage of the area at risk in rabbits receiving 1.0 minimum alveolar concentration (MAC) desflurane or metoprolol alone or in combination. CON = control; DES = 1.0 MAC desflurane; DES + METO = 1.0 MAC desflurane + 0.2, 1.0, 1.75, or 2.5 mg/kg metoprolol; METO = 0.2, 1.0, 1.75, or 2.5 mg/kg metoprolol.  $n = 10$  per group. Note that data from CON and DES are the same as in figure 2. \* Significantly ( $P < 0.05$ ) different from CON. # Significantly ( $P < 0.05$ ) different from desflurane. § Significantly ( $P < 0.05$ ) different from METO 2.5.



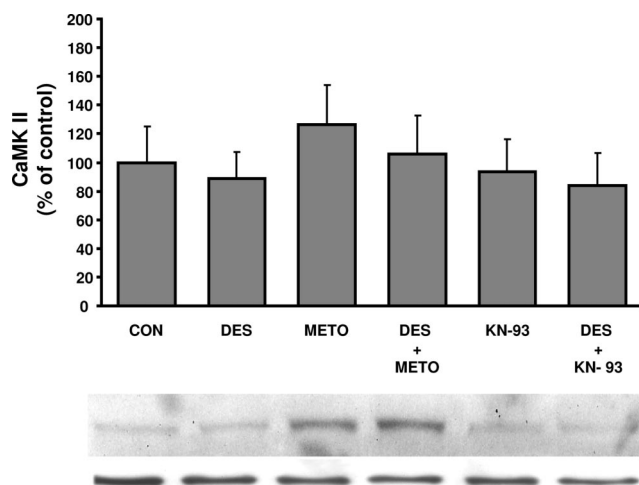


Fig. 4. Calcium/calmodulin-dependent protein kinase II (CaMK II) content after administration of desflurane, metoprolol, or KN-93 alone or in combination with desflurane. Results are presented as representative original immunoblottings and average densitometric results of immunoblotting as percentage of control. Values are mean  $\pm$  SEM. CON = control; DES = 1.0 minimum alveolar concentration (MAC) desflurane; DES + KN-93 = desflurane + KN-93; DES + METO = 1.0 MAC desflurane + 2.5 mg/kg metoprolol; KN-93 = 300  $\mu$ g/kg KN-93; METO = 2.5 mg/kg metoprolol.  $n = 7$  per group.

only during reperfusion. This is likely to result in higher plasma levels during reperfusion, achieved in the current protocol only at doses of metoprolol exceeding 1.0 mg/kg. As a third major finding, the current results further demonstrate that metoprolol dose-dependently attenuated or blocked desflurane-induced preconditioning. This finding is in accord with previously published investigations. Desflurane-induced improvement of the force of contraction after simulated ischemia is abolished by the nonselective  $\beta$ -adrenergic receptor blocker pro-

pranolol.<sup>5</sup> Moreover,  $\beta_1$ -selective receptor blockade by esmolol or inhibition of the downstream PKA by H-89 abolishes desflurane- and sevoflurane-induced preconditioning against myocardial infarction in the rabbit heart *in vivo*.<sup>4</sup> However, whether  $\beta$ -adrenergic blockade interferes with anesthetic-induced preconditioning in the clinical setting has not yet been demonstrated. The mechanism underlying the observation that metoprolol-induced infarct size reduction is not maintained in the presence of desflurane cannot fully be elucidated by the current results. However, cardioprotection by  $\beta$ -blockers aims at the prevention of ischemic episodes that is thought to be conferred by an improved myocardial oxygen supply and demand ratio, mainly induced by heart rate reduction.<sup>25</sup> It is possible that metoprolol-induced infarct size reduction after experimental ischemia depends on alternative mechanisms. This hypothesis is corroborated by the fact that in this and other studies on  $\beta$ -blocker-induced infarct size reduction<sup>26</sup> or restoration of LV function after ischemia,<sup>27</sup> beneficial effects of  $\beta$ -blockers were independent of heart rate reduction. Alternative effects of  $\beta$ -adrenergic receptor blockers include CaMK II inhibition, as demonstrated in this investigation, membrane stabilizing effects,<sup>28,29</sup> inhibition of phospholipase A,<sup>30</sup> and the inhibition of  $\beta$  oxidation of fatty acid accumulation.<sup>31</sup> These alternative effects of metoprolol might be impaired by the effects of desflurane-induced preconditioning. However, further investigations are needed to elicit the exact mechanisms underlying the infarct-size reducing properties of metoprolol and the resulting interaction with desflurane.

The current results should be interpreted within the constraints of several potential limitations. The LV AARs

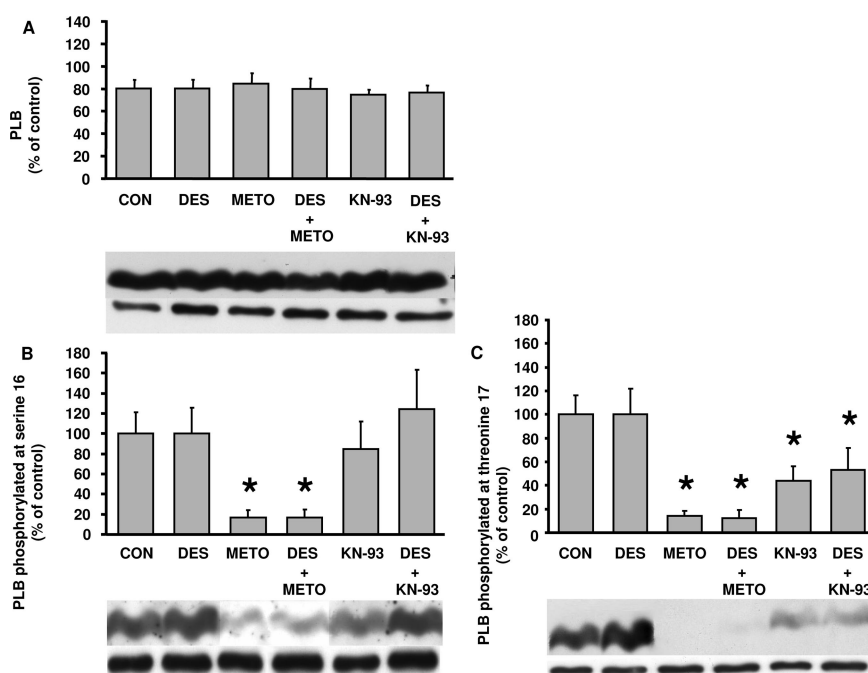


Fig. 5. Phospholamban content (A) and phosphorylation at serine 16 (B) and threonine 17 (C) after administration of desflurane, metoprolol, or KN-93 alone or in combination with desflurane. Results are presented as representative original immunoblottings and average densitometric results of immunoblotting as percentage of control. Values are mean  $\pm$  SEM.  $n = 7$  per group. CON = control; DES = 1.0 minimum alveolar concentration (MAC) desflurane; DES + KN-93 = desflurane + KN-93; DES + METO = 1.0 MAC desflurane + 2.5 mg/kg metoprolol; KN-93 = 300  $\mu$ g/kg KN-93; METO = 2.5 mg/kg metoprolol; PLB = phospholamban. \* Significantly ( $P < 0.05$ ) different from CON.



Table 3. Area at Risk

	Body Weight, kg	LV, g	AAR, g	AAR/LV, %
CON	2.41 ± 0.08	3.26 ± 0.15	1.25 ± 0.12	38 ± 3
DES	2.24 ± 0.10	3.12 ± 0.15	0.99 ± 0.13	32 ± 4
METO 0.2	2.47 ± 0.08	3.45 ± 0.13	1.26 ± 0.16	36 ± 4
METO 1.0	2.50 ± 0.10	3.53 ± 0.18	1.45 ± 0.18	40 ± 4
METO 1.75	2.40 ± 0.06	3.30 ± 0.12	1.10 ± 0.06	34 ± 2
METO 2.5	2.16 ± 0.08	3.02 ± 0.13	1.04 ± 0.16	34 ± 5
METO 0.2/DES	2.44 ± 0.08	3.16 ± 0.14	1.08 ± 0.13	34 ± 3
METO 1.0/DES	2.48 ± 0.11	3.46 ± 0.18	1.45 ± 0.16	41 ± 3
METO 1.75/DES	2.39 ± 0.03	3.28 ± 0.12	1.22 ± 0.11	38 ± 4
METO 2.5/DES	2.25 ± 0.10	3.12 ± 0.16	1.25 ± 0.20	39 ± 6
KN-93	2.53 ± 0.07	3.17 ± 0.15	1.00 ± 0.08	32 ± 2
KN-93/DES	2.59 ± 0.06	3.19 ± 0.26	0.97 ± 0.18	31 ± 6

Data are mean ± SEM. n = 10 per group, except for KN-93 and KN-93/DES (n = 6 per group).

AAR = area at risk; CON = control; DES = desflurane; KN-93 = 300 µg/kg KN-93; KN-93/DES = KN-93 + desflurane; LV = left ventricle; METO 0.2 = 0.2 mg/kg metoprolol; METO 1.0 = 1.0 mg/kg metoprolol; METO 1.75 = 1.75 mg/kg metoprolol; METO 2.5 = 2.5 mg/kg metoprolol; METO 0.2/DES = 0.2 mg/kg metoprolol + desflurane; METO 1.0/DES = 1.0 mg/kg metoprolol + desflurane; METO 1.75/DES = 1.75 mg/kg metoprolol + desflurane; METO 2.5/DES = 2.5 mg/kg metoprolol + desflurane.

for myocardial infarction and coronary collateral blood flow are important determinants of myocardial infarct size. However, AAR was not different among experimental groups (table 3). Coronary collateral blood flow was not determined in this study. However, coronary collateral blood flow is negligible in rabbits.<sup>32</sup> Therefore, neither differences in AAR nor collateral blood flow can be attributed to differences in infarct size. Metoprolol reduced infarct size only at higher doses compared with doses used clinically in humans. The use of these high doses might explain the occurrence of LV pump failure in 10 of the rabbits receiving metoprolol. However, metoprolol at 0.2 mg/kg, a dose comparable to that used in humans, did not reduce infarct size or heart rate throughout ischemia and reperfusion. Heart rate reduction was used as a physiologic marker of effective  $\beta$ -adrenergic blockade, and this was only achieved at high doses. Therefore, as in the study by Feuerstein *et al.*,<sup>24</sup> high doses of metoprolol had to be used to induce the intended effect of reducing heart rate. It has been demonstrated that I/R injury induces compartmentalization of CaMK II during transient ischemia with subsequent redistribution during reperfusion.<sup>33</sup> CaMK II translocation was not tested in our study. However, it is possible that CaMK II translocation might play a role in desflurane-induced preconditioning or cardioprotection by metoprolol. KN-93 is a selective competitive inhibitor of calmodulin binding to CaMK II and CaMK II autophosphorylation and has no inhibitory effect on other protein kinases.<sup>34</sup> However, KN-93 is known to directly inhibit L-type  $\text{Ca}^{2+}$  channels and  $\text{K}^+$  currents.<sup>35</sup> It cannot be excluded that inhibition of these ion fluxes might have influenced the results.

In conclusion, the results of the current study demonstrate that desflurane-induced preconditioning is mediated by CaMK II-dependent phospholamban phosphorylation, whereas metoprolol-induced cardioprotection during reperfusion is mediated by the blockade of PKA- and CaMK II-dependent phosphorylation of phospholamban. Furthermore, metoprolol reduced infarct size only at high doses but dose-dependently abrogated desflurane-induced preconditioning. These results suggest important negative interactions between desflurane-induced preconditioning and  $\beta$ -adrenergic receptor blockade that warrant further investigations on the underlying mechanisms.

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