

Choice of Anesthetic Combination Determines Ca²⁺ Leak after Ischemia-Reperfusion Injury in the Working Rat Heart

Favorable versus Adverse Combinations

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ABSTRACT

Background: There is a lack of studies investigating cardioprotection by common combinations of anesthetics. However, because a general anesthetic consists of a mixture of drugs with potentially interfering effects on signaling and cytoprotection, the most favorable combination should be used.

Methods: Working rat hearts were exposed to 20 min of ischemia and 30 min of reperfusion. Periischemic sevoflurane (2 vol-%), propofol (10 μ M), or remifentanyl (3 nM) (single treatments) and the three combinations thereof (combination treatments) were assessed for their ability to improve postischemic left ventricular work and to prevent intracellular Ca²⁺ leak and overload. Beat-to-beat oscillations

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What We Already Know about This Topic

- Volatile anesthetics, propofol, and opioids may elicit cardioprotection in hearts exposed to ischemia-reperfusion injury

What This Article Tells Us That Is New

- The choice of anesthetic combination determines the post-ischemic sarcoplasmic Ca²⁺ leak and intracellular Ca²⁺ overload after ischemia-reperfusion injury in the working rat heart

in intracellular [Ca²⁺] were measured using indo-1 AM. Phosphorylation of calcium/calmodulin-dependent protein kinase II δ , ryanodine receptor-2, and phospholamban was determined.

Results: The single treatments with sevoflurane or remifentanyl were highly protective with respect to functional recovery and Ca²⁺ overload, but propofol, even at high concentrations, did not show similar protection. Sevoflurane combined with propofol completely lost its protection in the presence of low sedative propofol concentrations (≥ 1 μ M), whereas remifentanyl combined with propofol (10 μ M) retained its protection. Propofol antagonism of sevoflurane protection was concentration-dependent and mimicked by the reactive oxygen species scavenger *N*-2-mercapto-propionyl-glycine. Addition of propofol to sevoflurane activated calcium/calmodulin-dependent protein kinase type II δ and hyperphosphorylated the ryanodine receptor-2, consistent with causing a postischemic Ca²⁺ leak from the sarcoplasmic reticulum. Remifentanyl did not enhance sevoflurane protection.

Conclusions: The choice of anesthetic combination determines the postischemic Ca²⁺ leak and intracellular overload. The results from these experiments will help to design studies for optimizing perioperative cardioprotection in high-risk surgical patients.

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META-analyses on the antiischemic cardioprotective properties of general anesthetic agents in high-risk surgical patients remain elusive. Even for volatile anesthetics, a highly promising class of cardioprotective drugs, the currently available evidence is still controversial.¹ Some studies show impressive postischemic functional improvement and favorable changes in biomarkers associated with cardioprotection,^{2,3} whereas other studies could only find mediocre or no signs of protection.⁴ Nonetheless, the American Heart Association recommends in its current Guidelines for Perioperative Management of High-Risk Surgical Patients Undergoing Noncardiac Surgery the preferential use of volatile anesthetics in these patients.⁵

In sharp contrast to these disparate clinical findings, there is overwhelming evidence of cardioprotection in the experimental setting, not only by volatile anesthetics but also by other anesthetics^{6,7} such as propofol⁸ and opioid receptor agonists.⁹ We and others have previously speculated that this discrepancy between experimental and clinical observations might be explained, in part, by the fact that diseased diabetic and/or infarct-remodeled hearts of patients may be less amenable to pharmacologic protection against ischemia–reperfusion (IR) injury.¹⁰ Another possible explanation could be potential antagonistic interactions between individual anesthetics when used in combination resulting in a loss of cardioprotection. In fact, it is well known that some frequently used perioperative drugs enhance (nitroglycerin, statins), whereas others abolish (sulfonylureas, cyclooxygenase-2 inhibitors, β -adrenoceptor antagonists) the most effective endogenous mechanism of cardioprotection, namely preconditioning.¹¹ One recent study in rabbit hearts demonstrated inhibitory effects of propofol on desflurane-induced preconditioning,¹² whereas another study showed that morphine could enhance cardioprotection by isoflurane preconditioning.¹³ However, so far no study has systematically explored the interaction between common clinically used anesthetics on cardioprotection. The question of whether and how different combinations of anesthetics might antagonize or potentiate their effects on cardioprotection is essential for the clinician, because a general anesthetic procedure consists of a mixture of drugs, and the most favorable cardioprotective combination of anesthetics should be preferentially used in at-risk patients. To gain insight into such interactions, we designed a study using a standardized experimental model, *i.e.*, the working rat heart, in which the net effects of three combinations of anesthetics, namely sevoflurane, propofol, and remifentanyl, were examined on the recovery of postischemic left ventricular work. In addition, to obtain a mechanistic understanding of potential interactions, we used this experimental model to measure drug-induced alterations in Ca^{2+} overload and sarcoplasmic Ca^{2+} leak. We hypothesized that certain combinations would show antagonism, whereas others might show synergy in cardioprotection.

Materials and Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the experimental protocol used in this investigation was approved by the University of Alberta Animal Policy and Welfare Committee (Edmonton, Alberta, Canada).

Working Heart Perfusion

One hundred eleven male Sprague-Dawley rats (350–400 g) were anesthetized with pentobarbital (150 mg/kg, intraperitoneally). Hearts were rapidly removed and perfused initially in a nonworking Langendorff mode with Krebs-Henseleit solution for 15 min. The working mode perfusion was established (11.5 mmHg preload, 80 mmHg afterload, 5 Hz) with a recirculating perfusate of 100 ml (37°C, pH 7.4) gassed with 95% O_2 /5% CO_2 mixture that consisted of a modified Krebs-Henseleit solution containing (mM): KCl (4.7), NaCl (118), KH_2PO_4 (1.2), MgSO_4 (1.2), CaCl_2 (2.5), NaHCO_3 (25), glucose (11) palmitate (1.2, prebound to 3% bovine serum albumin) and insulin 100 mU/l. Hearts were paced at 5 Hz.

Measurements of Left Ventricular Work

Cardiac output (ml/min) and aortic flow (ml/min) were measured using ultrasonic flow probes (Transonic T206, Transonic Systems Inc., Ithaca, NY) placed in the left atrial inflow and the aortic outflow lines.¹⁴ Left ventricular work (ml/min \cdot mmHg) was calculated as left ventricular work = cardiac output \cdot (aortic systolic pressure - preload). Coronary flow (ml/min) was calculated as the difference between cardiac output and aortic flow.

Measurement of Beat-to-Beat Intracellular $[\text{Ca}^{2+}]_i$

The measurements of intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) were performed as described previously.¹⁵ The hearts were loaded with 5 μM fluorescent Ca^{2+} indicator indo-1 AM (TEFLabs, Austin, TX) dissolved in dimethyl sulfoxide (final concentration less than 0.25%) for 25 min during the working mode perfusion. After a washout period of 5 min in the Langendorff mode, the working mode was reestablished with fresh modified Krebs-Henseleit solution using a second perfusion circuit and the hearts were assigned to the treatment groups. Indo-1 fluorescence was measured from a small area (approximately 0.3 cm^2) of the epicardial surface of the left ventricle free wall using a spectrofluorometer (Photon Technology International, London, Ontario, Canada) (see Supplemental Digital Content 1, <http://links.lww.com/ALN/A814>). Signals were acquired at 500 Hz, and the ratio of indo-1 fluorescence emitted at 405 nm and 485 nm (F405/F485 ratio) was calculated to provide an index of $[\text{Ca}^{2+}]_i$. Diastolic $[\text{Ca}^{2+}]_i$, systolic $[\text{Ca}^{2+}]_i$, and Ca^{2+} transient amplitude were determined.

Experimental Protocols

After 15 min of perfusion in the working mode, all hearts were subjected to 20 min of 37°C zero-flow ischemia and 30

min of reperfusion (see Supplemental Digital Content 1, <http://links.lww.com/ALN/A814>). All treatments were initiated 15 min before ischemia and continued until the end of reperfusion. The duration of 20 min of global ischemia induced marked left ventricular dysfunction. Longer periods of global ischemia would have resulted in no functional recovery at reperfusion, which would have biased measurements of $[Ca^{2+}]_i$. Hearts were assigned to one of the following 15 groups: (1) time-matched perfusion without treatment (IR) (n = 8), (2) sevoflurane (Abbott Canada, Saint-Laurent, Québec, Canada) (SEVO+IR) 2 vol.-% (n = 8), (3) propofol 10 μ M (n = 6) (PROP+IR) in the formulation of Diprivan® 1% (AstraZeneca Canada, Mississauga, Ontario, Canada), (4) remifentanyl (Abbott Canada) 3 nM (REMI+IR) (n = 9), (5) N-2-mercaptpropionyl-glycine (MPG, Sigma-Aldrich, Oakville, Ontario, Canada) 10 μ M (MPG+IR) (n = 5), (6) sevoflurane 2 vol.-% plus propofol 10 μ M (SEVO/PROP+IR) (n = 9), (7) remifentanyl 3 nM plus propofol 10 μ M (REMI/PROP+IR) (n = 7), (8) sevoflurane 2 vol.-% plus remifentanyl 3 nM (SEVO/REMI+IR) (n = 9), (9) sevoflurane 2 vol.-% plus MPG 10 μ M (SEVO/MPG+IR) (n = 5), (10) sevoflurane 2 vol.-% plus Intralipid 10% (Sigma-Aldrich) (SEVO/INTRA+IR; corresponding to Intralipid control of propofol 100 μ M) (n = 6), (11) remifentanyl 3 nM plus MPG 10 μ M (REMI/MPG+IR) (n = 6). Additional groups (12–14) were studied to determine the concentration-response relationship for the sevoflurane 2 vol.-% - propofol interaction (0.1, 1, and 100 μ M) (n = 7 for each concentration) and (15) to test whether Intralipid alone affects functional recovery or Ca^{2+} overload after IR (n = 6). Hearts with time-matched aerobic perfusion served to determine baseline phosphoprotein levels (n = 6). At the end of the perfusions, all hearts were immediately frozen in liquid nitrogen with Wollenberger clamps and stored at $-80^{\circ}C$ for subsequent molecular analyses.

Immunoblotting of Calcium/Calmodulin-dependent Protein Kinase Type II δ , Phospholamban, and Ryanodine Receptor-2

Tissue homogenate was used for immunoblotting of calcium/calmodulin-dependent protein kinase type II δ (CaMKII δ) in 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and for phospholamban in 10% SDS-PAGE. The following primary antibodies were used: rabbit anti-CaMKII δ (1:500) (Abcam, Cambridge, MA), rabbit antiThr286-CaMKII δ (1:1000) (Abcam), anti-phospholamban (1:500) (Abcam), antiThr17-phospholamban, and antiSer16-phospholamban (1:300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For ryanodine receptor-2 (RyR2) immunoblots, sarcoplasmic reticulum fractions were prepared. Frozen heart tissue was homogenized in ice-cold buffer containing 10 mM Hepes (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.3 M sucrose, and 0.05% Nonidet

P-40 and centrifuged at 500 g for 10 min at $4^{\circ}C$. The resulting supernatant was centrifuged at 100,000 g for 45 min and the obtained pellet was suspended in buffer containing 50 mM Tris (pH 7.4), 2 mM EDTA, 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, and 5 mM 1,4-dithiothreitol supplemented with protease and phosphatase inhibitor cocktail mix (Sigma-Aldrich). There were 60 μ g of protein subjected to electrophoresis on a 5% gel and the following primary antibodies anti-RyR2 (1:1000) (Abcam) and antiSer2814-RyR2 (1:1000) (Badrilla Ltd., Leeds, United Kingdom) were used. Protein concentration was measured (Bradford assay) to ensure that equal amounts of protein were subjected to electrophoresis. Immunoreactivity was visualized by horseradish peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (ECL) (PerkinElmer, Woodbridge, Ontario, Canada). The intensity of the bands was quantified using ImageJ** software. Phosphorylation was normalized to the expression of the corresponding total protein. Actin (I-19; Santa Cruz) and/or α -tubulin (clone B-5-1-2; Sigma-Aldrich) were used as loading controls.

Statistical Analysis

Values are given as mean (SD) or median (interquartile range) except for figures 1 and 2 where standard errors of the mean (SEM) are given to avoid confusion with multiple overlapping lines. With an expected difference of 50% between group means, 20% SD of the means, significant levels of $\alpha = 0.05$ and $\beta = 0.8$, a sample size of five hearts per group was necessary.¹⁵ Time-dependent measurements were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by multiple comparison procedures (Student-Newman-Keuls method). The significance of differences in hemodynamic and calcium measurements among groups was determined by unpaired two-tailed Student *t* test (two groups) or by one-way ANOVA followed by the Holm-Sidak method or by Kruskal-Wallis one-way ANOVA on ranks followed by the Dunn method for *post hoc* analysis, depending on the underlying data distribution. Differences are considered significant if $P < 0.05$. Statistical analyses were performed using Sigmaplot Version 11.0 (Systat Software, Inc., Chicago, IL).

Results

Single Treatments—Sevoflurane, Remifentanyl, and Propofol Exert Differential Effects on Functional Recovery and Ca^{2+} Overload in Working Rat Hearts Exposed to IR

Periischemic sevoflurane (2 vol.-%) and remifentanyl (3 nM) treatment similarly improved recovery of left ventricular work during reperfusion when compared with untreated hearts (fig. 1). These beneficial actions were accompanied by higher peak systolic pressures, stroke volumes, and coronary flows (see Supplemental Digital Content 2, <http://links.lww.com/ALN/A815>). Interestingly, the amplitudes of the Ca^{2+} transients in hearts exposed to remifentanyl (3 nM) were lower, but left ventricular

** National Institutes of Health, Bethesda, Maryland. Available at: <http://rsbweb.nih.gov/ij/>. Accessed December 1, 2008.

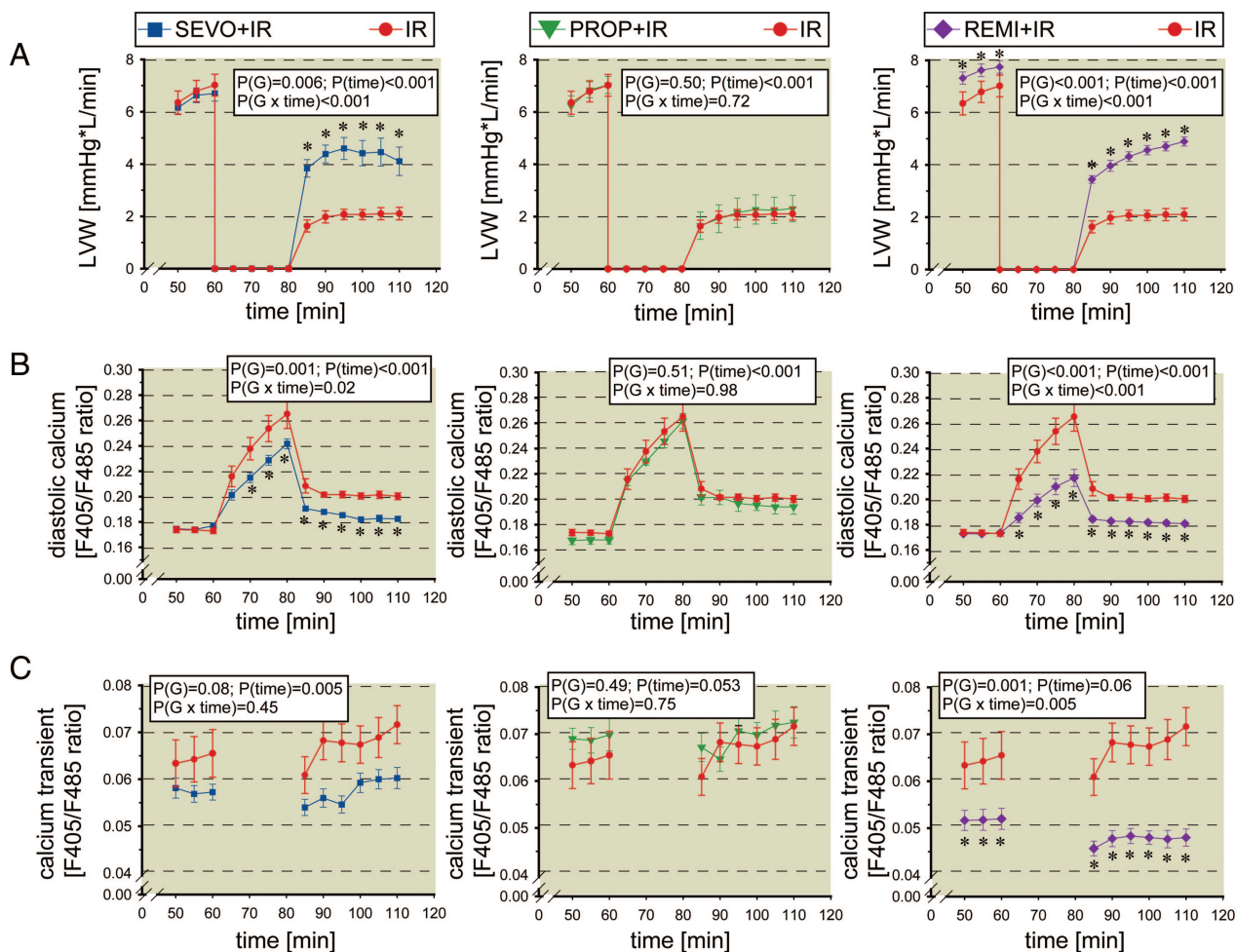


Fig. 1. Single treatments. (A) Left ventricular work (LVW). (B) Left ventricular diastolic $[Ca^{2+}]_i$. (C) Ca^{2+} transient. IR, hearts exposed to ischemia–reperfusion without treatment; SEVO+IR, periischemic treatment with sevoflurane 2 vol.-%; PROP+IR, periischemic treatment with propofol 10 μ M; REMI+IR, periischemic treatment with remifentanyl 3 nM; G, group; G \times time, group-time interaction. * $P < 0.05$ compared with IR. Data are mean \pm SEM.

work was higher than in untreated hearts; the lack of positive correlation suggests that remifentanyl prevented ischemia-induced Ca^{2+} desensitization. Remifentanyl and sevoflurane comparably reduced postischemic diastolic $[Ca^{2+}]_i$, a sensitive marker of ischemic damage. Peak ischemic $[Ca^{2+}]_i$ was lower in remifentanyl-treated than sevoflurane-treated hearts ($P = 0.008$). Consistent with previous results,¹⁵ treatment with propofol 10 μ M did not improve functional recovery or decrease postischemic Ca^{2+} overload. Intralipid alone or in combination with sevoflurane did not affect functional recovery or Ca^{2+} overload (see Supplemental Digital Content 3, <http://links.lww.com/ALN/A816>).

Combination Treatments—Propofol Antagonizes Sevoflurane but Not Remifentanyl-mediated Cardioprotection in a Concentration-dependent Manner

Concomitant administration of propofol 10 μ M with sevoflurane 2 vol.-% did not reduce left ventricular work under aerobic conditions, but completely abolished sevoflurane-mediated improvement in the recovery of post-

ischemic left ventricular work (fig. 2). Peak ischemic $[Ca^{2+}]_i$ and postischemic Ca^{2+} transient amplitudes were not altered by the combination treatment when compared with sevoflurane alone. However, the addition of propofol to sevoflurane prevented the beneficial effects of sevoflurane on postischemic diastolic $[Ca^{2+}]_i$ and values were increased to levels measured in untreated hearts. In contrast, administration of propofol 10 μ M with remifentanyl 3 nM did not interfere with remifentanyl-induced cardioprotection. Examination of the concentration-response relationship for propofol-mediated inhibition of sevoflurane-mediated protection indicates that 1 μ M propofol reduced functional recovery elicited by sevoflurane by 33% ($P = 0.004$) (fig. 3A) and increased diastolic $[Ca^{2+}]_i$ by 6% compared with protected hearts (fig. 3, B and C). Only the lowest concentration of propofol (0.1 μ M) did not diminish sevoflurane-induced protection. Our demonstration that the different combinations of anesthetics exhibit disparate interactions is likely due to distinct mechanisms of cardioprotection.

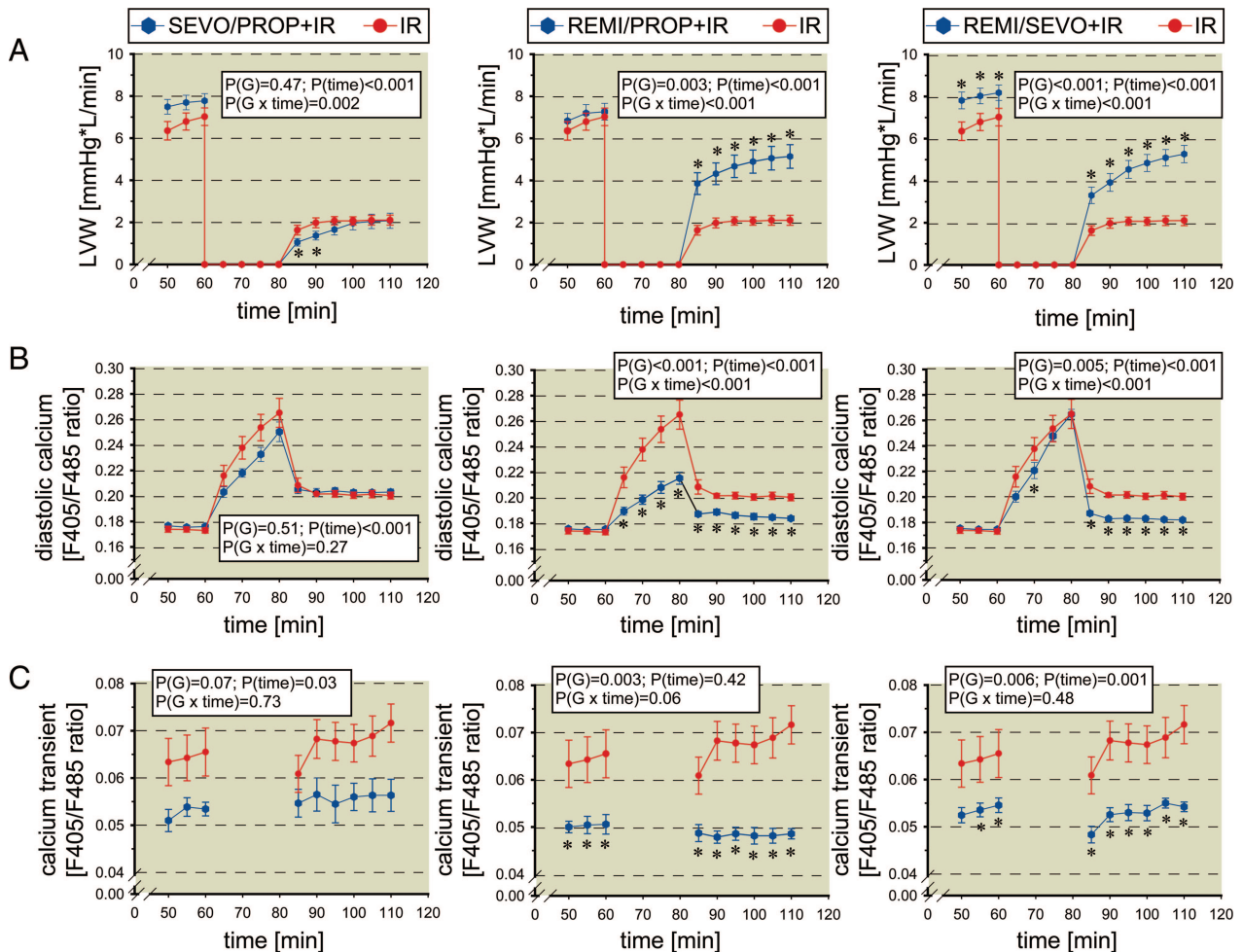


Fig. 2. Combination treatments. (A) Left ventricular work (LVW). (B) Left ventricular diastolic $[\text{Ca}^{2+}]_i$. (C) Ca^{2+} transient. IR, hearts exposed to ischemia-reperfusion without treatment; SEVO/PROP+IR, periischemic sevoflurane (2 vol.-%) plus propofol (10 μM) treatment; REMI/PROP+IR, periischemic remifentanyl (3 nM) plus propofol (10 μM) treatment; REMI/SEVO+IR, periischemic remifentanyl (3 nM) plus sevoflurane (2 vol.-%) treatment; G, group; G \times time, group-time interaction. * $P < 0.05$ compared with IR. Data are mean \pm SEM.

To demonstrate the ability of MPG, a known scavenger of reactive oxygen species (ROS) similar to propofol, to mimic the antagonistic actions of propofol, MPG 10 μM was concomitantly administered to sevoflurane 2 vol.-%. Although MPG 10 μM alone did not affect postischemic functional recovery or Ca^{2+} overload compared with unprotected hearts, MPG completely abolished sevoflurane-mediated protection similar to propofol (fig. 4). This confirms previous experiments showing the essential role of ROS in sevoflurane preconditioning.^{16,17} Finally, when remifentanyl 3 nM was added to sevoflurane 2 vol.-%, functional recovery was similar to sevoflurane alone or to remifentanyl alone. Unexpectedly, this combination markedly increased peak ischemic $[\text{Ca}^{2+}]_i$ ($P < 0.001$) and Ca^{2+} transient ($P = 0.02$) compared with remifentanyl alone, but did not increase postischemic Ca^{2+} overload (fig. 2B). Coadministration of MPG to remifentanyl did not block remifentanyl-induced cardioprotection (see Supplemental Digital Content 4, <http://links.lww.com/ALN/A817>).

Choice of Anesthetic Combination Determines Sarcoplasmic Reticulum Ca^{2+} Leak after IR Injury in the Working Rat Heart

Because CaMKII δ activity is known to play a causal role in postischemic Ca^{2+} overload and Ca^{2+} leak from the sarcoplasmic reticulum,^{18,19} CaMKII δ phosphorylation at Thr286 was determined in aerobic hearts as well as in postischemic hearts that were either untreated or that were exposed to single and combination treatments. These experiments show that CaMKII δ phosphorylation was very low in aerobic hearts, but was significantly increased in untreated postischemic hearts. In hearts exposed to cardioprotective single or combination treatments, CaMKII δ phosphorylation was inhibited, whereas unprotected hearts exhibited markedly higher phosphorylation levels (fig. 5). Similarly, examination of ratios of phospho-RYR2 to total RYR2 indicates that RYR2, a downstream target of CaMKII δ , was significantly more phosphorylated in the nonprotective combination treatment, sevoflurane plus propofol, as opposed to

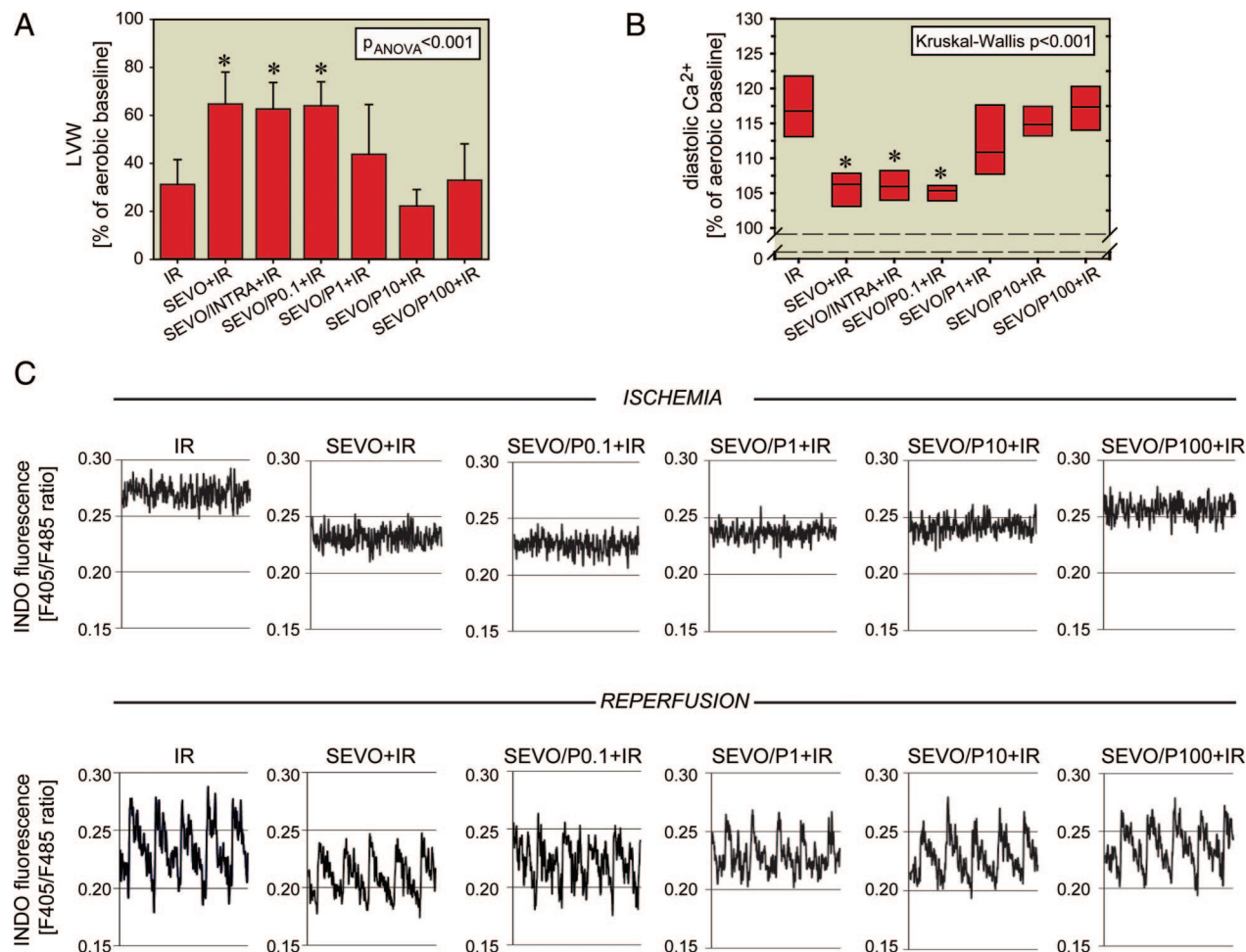


Fig. 3. Concentration-response curve of propofol-mediated inhibition of sevoflurane protection. (A) Left ventricular work (LVW). (B) Diastolic $[Ca^{2+}]_i$. (C) Representative $[Ca^{2+}]_i$ traces of 1-s duration. INDO, Ca^{2+} indicator indo-1 AM; IR, ischemia–reperfusion alone; SEVO+IR, periischemic sevoflurane treatment (2 vol.-%); SEVO/P0.1+IR, periischemic sevoflurane (2 vol.-%) plus propofol (0.1 μ M) treatment; SEVO/P1+IR, periischemic sevoflurane (2 vol.-%) plus propofol (1 μ M) treatment; SEVO/P10+IR, periischemic sevoflurane (2 vol.-%) plus propofol (10 μ M) treatment; SEVO/P100+IR, periischemic sevoflurane (2 vol.-%) plus propofol (100 μ M) treatment. * $P < 0.05$ compared with IR. Data are mean \pm SD (A) and median (25th, 75th percentile) (B).

the protective combo-treatments, sevoflurane plus remifentanyl, or propofol plus remifentanyl (fig. 6). Phosphorylation of phospholamban at position Thr17, which is selectively mediated by CaMKII δ , and at position Ser16, which is selectively mediated by protein kinase A, was not different between unprotected and protected hearts (see Supplemental Digital Content 5, <http://links.lww.com/ALN/A818>) implying that the postischemic sarcoplasmic Ca^{2+} leak in this model is not caused by phospholamban phosphorylation but is due to selective phosphorylation of RyR2 by activated CaMKII δ .

Discussion

Our study systematically investigated the interactions of commonly applied classes of anesthetics on cardioprotection. For this purpose, the isolated working rat heart model perfused with glucose and palmitate as metabolic substrates was used to measure left ventricular work and beat-to-beat oscil-

lations in intracellular Ca^{2+} during IR injury. The anesthetics or their combinations were administered before, during, and after the ischemic insult and therefore mimic clinical protocols. We report the following salient findings. First, when the anesthetics were administered as single treatments remifentanyl or sevoflurane, but not propofol, markedly improved the recovery of left ventricular mechanical function and decreased diastolic Ca^{2+} overload. This is consistent with previous findings showing no protection with propofol at concentrations as high as 100 μ M.^{12,15} Second, in the combination treatments propofol had no effect on remifentanyl-induced cardioprotection, but antagonized sevoflurane-induced cardioprotection in a concentration-dependent manner. Antagonism was clearly demonstrable at low sedative concentrations of propofol (1 μ M). Conversely, the combination of the two protective drugs, sevoflurane (2 vol.-%) and remifentanyl (3 nM), preserved protection but did not show additive or synergistic interactions. Finally, our

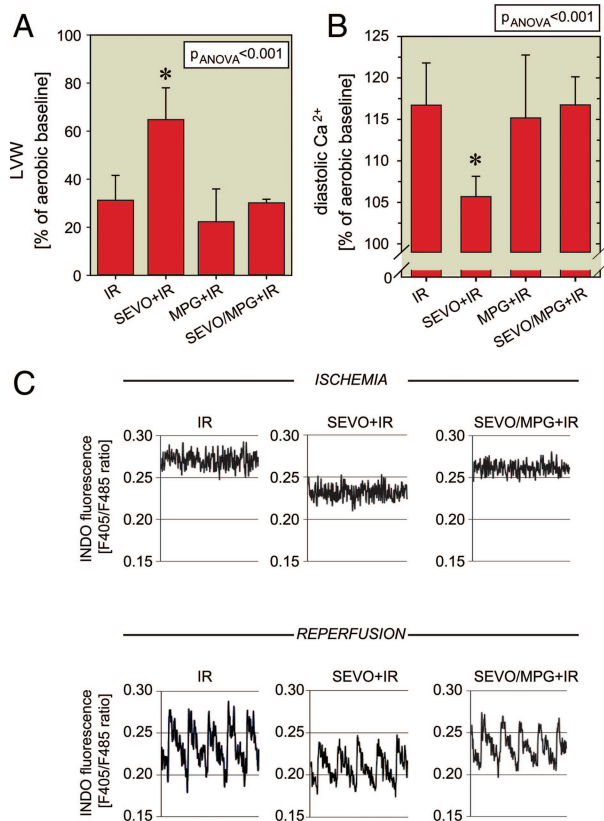


Fig. 4. Mimicking inhibitory propofol-effects with *N*-2-mercaptopyropionyl-glycine (MPG). (A) Left ventricular work (LVW). (B) Diastolic $[Ca^{2+}]_i$. (C) Representative $[Ca^{2+}]_i$ traces of 1-s duration. INDO, Ca^{2+} indicator indo-1 AM; IR, ischemia–reperfusion alone; SEVO+IR, periischemic sevoflurane treatment (2 vol.-%); SEVO/MPG+IR, periischemic sevoflurane (2 vol.-%) plus MPG (10 μ M) treatment. * $P < 0.05$ compared with IR. Data are mean \pm SD.

experiments demonstrate that posts ischemic diastolic Ca^{2+} overload in the myocardium exposed to the various anesthetic combinations correlates closely with CaMKII δ activation and Ca^{2+} leak from the sarcoplasmic reticulum as reflected by hyperphosphorylation of the RyR2. The clinical consequences of such interactions, particularly the antagonistic action of low concentrations of propofol on sevoflurane-induced protection, need careful evaluation.

Postischemic Ca^{2+} Handling in Anesthetic Combinations

Ca^{2+} is vital for modulating cellular functions, serves as activator of the contractile apparatus, and regulates the release and uptake of Ca^{2+} from the sarcoplasmic reticulum.²⁰ At higher intracellular concentrations, however, Ca^{2+} is highly toxic and impairs left ventricular mechanical function and causes irreversible cell injury and death. It is therefore a sensitive and reliable marker of cellular dysfunction during and after IR injury. In quiescent cardiomyocytes, cytosolic Ca^{2+} concentrations reach approximately $1-2 \times 10^{-7}$ M, which is 10,000-fold lower compared with the extracellular space. Elevation of average intracellular Ca^{2+} concentration en-

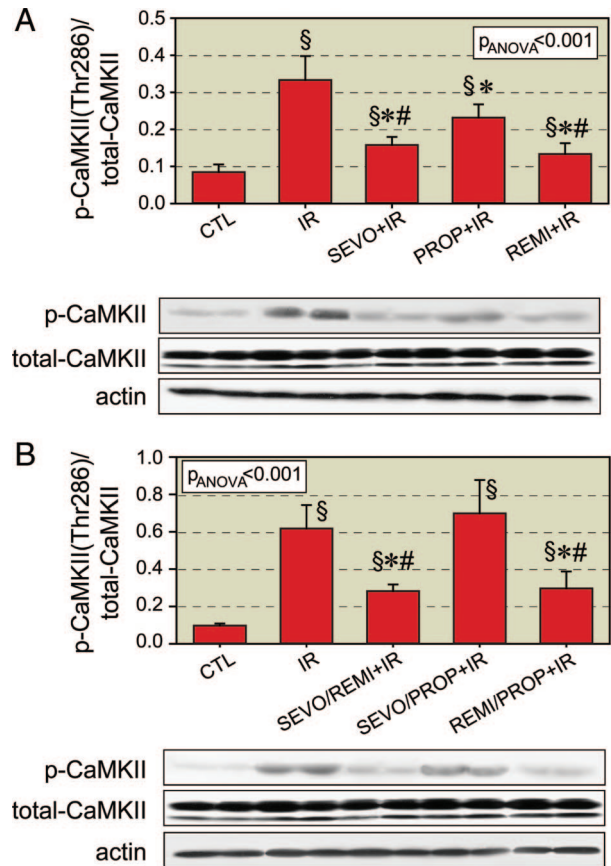


Fig. 5. CaMKII δ (56 kDa) phosphorylation in single (A) and combination treatments (B). CaMKII δ activity was estimated from the ratio of p-CaMKII δ and total CaMKII δ . Actin (42 kDa) was used as loading control. CTL, time-matched aerobic control; IR, ischemia–reperfusion alone; SEVO+IR, periischemic sevoflurane treatment (2 vol.-%); PROP+IR, periischemic propofol treatment (10 μ M); REMI+IR, periischemic remifentanyl treatment (3 nM); SEVO/PROP+IR, periischemic sevoflurane (2 vol.-%) plus propofol (10 μ M) treatment; REMI/PROP+IR, periischemic remifentanyl (3 nM) plus propofol (10 μ M) treatment; REMI/SEVO+IR, periischemic remifentanyl (3 nM) plus sevoflurane (2 vol.-%) treatment. * $P < 0.05$ compared with IR. # $P < 0.05$ compared with PROP+IR or SEVO/PROP+IR. \S $P < 0.05$ compared with CTL. Data are mean \pm SD.

hances binding of Ca^{2+} to calmodulin, the intracellular Ca^{2+} sensor, which subsequently interacts with more than 100 proteins, one of which is CaMKII δ , the predominant isoform of CaMKII expressed in the heart.²⁰ The Ca^{2+} -calmodulin complex promotes CaMKII δ autophosphorylation at Thr286, which in turn enhances its affinity to Ca^{2+} -calmodulin ensuing additional autophosphorylation at Thr305 and Thr306. Phosphorylated CaMKII δ critically affects the release of Ca^{2+} from the sarcoplasmic reticulum *via* phosphorylation of RyR2 at Ser2814. CaMKII δ further regulates sarcoplasmic Ca^{2+} uptake by phospholamban phosphorylation at Thr17, diminishing its inhibitory effects on sarcoplasmic reticulum Ca^{2+} ATPase, cardiac isoform (SERCA2a), which pumps Ca^{2+} from the cytoplasm back

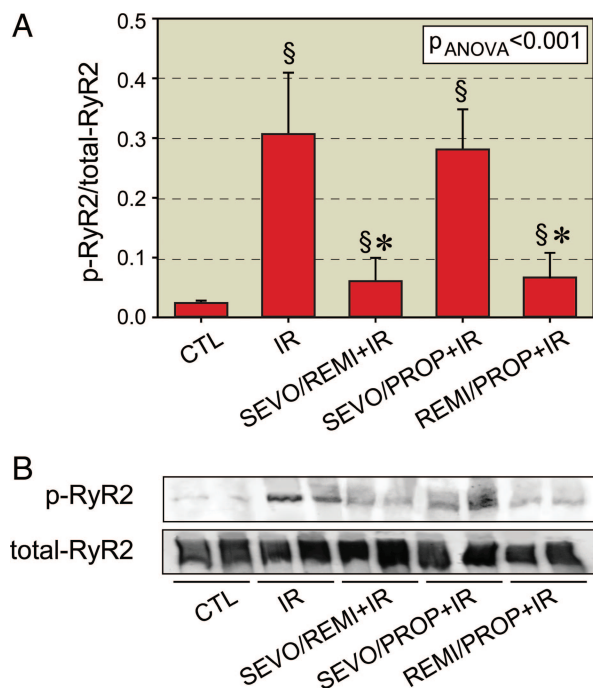


Fig. 6. Ryanodine receptor-2 (RyR2; 560 kDa) phosphorylation given as ratio between p-RyR2 to total RyR2 for combo treatments (A) and representative immunoblots (B). CTL, time-matched aerobic control; IR, ischemia-reperfusion alone; SEVO/PROP+IR, periischemic sevoflurane (2 vol.-%) plus propofol (10 μ M) treatment; REMI/PROP+IR, periischemic remifentanyl (3 nM) plus propofol (10 μ M) treatment; REMI/SEVO+IR, periischemic remifentanyl (3 nM) plus sevoflurane (2 vol.-%) treatment. * $P < 0.05$ compared with IR. § $P < 0.05$ compared with CTL. Data are mean \pm SD.

into the sarcoplasmic reticulum against a 10,000-fold concentration gradient.²⁰ Thus, measurements of the phosphorylation status of CaMKII δ and its downstream targets RyR2 and phospholamban serve as useful indices of the mechanisms that regulate Ca^{2+} leak and Ca^{2+} sequestration, respectively. Our finding that CaMKII δ phosphorylation was increased during reperfusion suggests that the reduced left ventricular work and enhanced intracellular Ca^{2+} overload occurred in response to CaMKII δ -mediated facilitation of Ca^{2+} release from the sarcoplasmic reticulum by the RyR2. In support, increased CaMKII δ activity has been reported in failing human hearts²¹ and rabbit hearts after coronary artery ligation.²² Multiple studies have also shown a close relation between CaMKII δ activity and apoptosis.^{23,24} Moreover, CaMKII δ activation *per se* exerts direct detrimental effects. Transgenic overexpression of CaMKII δ causes hyperphosphorylation of RyR2 with subsequent diastolic Ca^{2+} leakage from the sarcoplasmic reticulum, leading to arrhythmias or heart failure.²⁵ In contrast, inhibition of CaMKII δ , by transgenic overexpression of an inhibitory peptide, abolishes cardiac deterioration after infarction.²⁶ Despite the differential CaMKII δ activation in protective *versus* nonprotective anesthetic combinations in our experiments, phospholamban phosphorylation was not different among the treatments.

This is similar to previous observations in rat cardiomyocytes where selective hyperphosphorylation of the RyR2 at Ser2814 without simultaneous alterations in phospholamban phosphorylation was shown.²⁷ Collectively, CaMKII δ activation with subsequent hyperphosphorylation of RyR2 reflects a common feature of impaired postischemic Ca^{2+} handling in hearts exposed to nonprotective anesthetic combinations. On the other hand, protective anesthetic combinations prevent hyperphosphorylation of RyR2 and thereby enable improved Ca^{2+} homeostasis and enhanced functional recovery after IR.

Molecular Pathways in Anesthetic Combinations

Sevoflurane- and opioid-induced cardioprotection share many signaling components such as PKC, mitochondrial K_{ATP} channels, and ROS.^{17,28} While ROS may be essential in sevoflurane-induced protection,^{16,17} other mechanisms, such as δ - and κ -opioid or *N*-methyl-D-aspartate receptor activation may be more important in remifentanyl protection.^{9,29} It is noteworthy that Ca^{2+} transient amplitudes in remifentanyl-treated hearts were lower than in sevoflurane-treated hearts even during preischemic aerobic conditions. This points to additional differences in the mechanism of action of these two agents on Ca^{2+} homeostasis and Ca^{2+} sensitivity, and suggests that their cardioprotective mechanisms may be distinct. This dissociation between Ca^{2+} transient amplitude and cardiac contractility, indicative of enhanced Ca^{2+} sensitization, was published previously based on studies in isolated rat cardiomyocytes—a model where contractility was not measured,³⁰ and isolated perfused rat hearts,³¹ where remifentanyl had marked positive inotropic action. Together, these data indicate that there is an increase in Ca^{2+} sensitization in the presence of remifentanyl. This is consistent with less Ca^{2+} loading and may contribute to its cardioprotective effectiveness. The participation of ROS in sevoflurane-induced but not remifentanyl-induced protection is supported by the observation that the ROS scavenger MPG, with properties similar to propofol, markedly reduces the salutary effects of sevoflurane but not remifentanyl. This mechanism also explains the lack of inhibitory effects of the ROS scavenger propofol on remifentanyl-induced protection. Although a previous study showed inhibitory effects of propofol on permeability transition opening in mitochondria during reperfusion,⁸ which is likely due to the scavenging effects of propofol, we and others were unable to demonstrate any protection by propofol, even at high concentrations.^{12,15}

According to the commonly accepted threshold theory of preconditioning, which implies that a certain degree of stimulation is required to reach the level where a cell or organ is able to activate its endogenous protection program, it would be conceivable that the application of two well-defined protection stimuli should induce a more consistent and reliable overall cell protection. Indeed, morphine enhances isoflurane preconditioning and postconditioning in hearts sub-

jected to coronary artery ligation *in vivo*.^{13,32} However, in our experiments, we did not observe any synergistic or additive protection in hearts treated with a combination of sevoflurane (2 vol.-%) and remifentanyl (3 nM). It is possible that the maximum preconditioning trigger stimulus has been already reached with 2 vol.-% sevoflurane alone or 3 nM remifentanyl alone, making the protection elicited by the second drug redundant. In addition, we cannot exclude that a more sustained ischemic injury might uncover additive cardioprotective effectiveness in hearts exposed to a sevoflurane-remifentanyl combination. Nonetheless, 2 vol.-% sevoflurane and 3 nM remifentanyl are relatively low concentrations compared with those previously used to elicit effective cardioprotection.^{3,9} In addition, the extent of improvement in functional recovery and attenuation of Ca²⁺ overload in our working rat heart model by sevoflurane or remifentanyl still left room for further improvements.

Clinical Implications

The potential for interactions between different protection strategies including pharmacologic agents and/or ischemic conditioning has been largely neglected in experimental and more so clinical studies in the past. However, the question of whether and how different anesthetic agents might interact to either antagonize or potentiate the beneficial effects of ischemia- or drug-induced cardioprotection is of great importance for the clinician, because a general anesthetic procedure consists of a mixture of drugs, and the most favorable cardioprotective combination of anesthetics should be preferentially used in at-risk patients. Our results from this study in rat hearts imply that the outcome in cardioprotection for combined therapies is highly context-sensitive and cannot be inferred or extrapolated from the assessment of single cardioprotective therapies. Indeed, our results suggest that the lack of standardization in the use of anesthetics (choice of combination, timing, and concentrations) may have contributed to ambiguous and contradictory results arising from both basic and clinical studies concerning anesthetic-induced cardioprotection, as well as preconditioning or postconditioning.

In conclusion, this study shows that the choice of anesthetic combination determines the functional recovery and postischemic Ca²⁺ homeostasis in working rat hearts. We anticipate that results from these experiments may encourage the careful design of clinical studies so that perioperative cardioprotection can be optimized.

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