Anesthesiology 2002; 96:125–33

Sevoflurane before or after Ischemia Improves Contractile and Metabolic Function while Reducing Myoplasmic Ca^{2+} Loading in Intact Hearts

Srinivasan G. Varadarajan, M.D., Jianzhong An, M.D.,† Enis Novalija, M.D.,‡ David F. Stowe, M.D., Ph.D.§*

Background: **Ca^{2+} loading occurs during myocardial reperfusion injury. Volatile anesthetics can reduce reperfusion injury. The authors tested whether sevoflurane administered before index ischemia in isolated hearts reduces myoplasmic diastolic and systolic [Ca^{2+}] and improves function more so than when sevoflurane is administered on reperfusion.**

Methods: **Four groups of guinea pig hearts were perfused with crystalloid solution (55 mmHg, 37°C): (1) no treatment before 30 min global ischemia and 60 min reperfusion (CON); (2) 3.5 vol% sevoflurane administered for 10 min before ischemia (SBI); (3) 3.5 vol% sevoflurane administered for 10 min after ischemia (SAI); and (4) 3.5 vol% sevoflurane administered for 10 min before and after ischemia (SBAI). Phasic myoplasmic diastolic and systolic [Ca^{2+}] were measured in the left ventricular free wall with the fluorescence probe indo-1.**

Results: **Ischemia increased diastolic [Ca^{2+}] and diastolic left ventricular pressure (LVP). In CON hearts, initial reperfusion greatly increased diastolic [Ca^{2+}] and systolic [Ca^{2+}] and reduced contractility (systolic–diastolic LVP, $dLVP/dt_{max}$), relaxation (diastolic LVP, $dLVP/dt_{min}$), myocardial oxygen consumption (Mvo_2), and cardiac efficiency. SBI, SAI, and SBAI each reduced ventricular fibrillation, attenuated increases in systolic and systolic–diastolic [Ca^{2+}], improved contractile and relaxation indices, and increased coronary flow, percent oxygen extraction, Mvo_2, and cardiac efficiency during 60 min reperfusion compared with CON. SBI was more protective than SAI, and SBAI was generally more protective than SAI.**

Conclusions: **Sevoflurane improves postischemic cardiac function while reducing Ca^{2+} loading when it is administered before or after ischemia, but protection is better when it is administered before ischemia. Reduced Ca^{2+} loading on reperfusion is likely a result of the anesthetic protective effect.**

REPERFUSION after cardiac ischemia can cause reversible mechanical dysfunction (stunning) or cell death (infarction) depending on the duration and magnitude of ischemia.[1] Myoplasmic Ca^{2+} overload during ischemia, and particularly on reperfusion, contributes to the injury.[2–6] Ca^{2+} loading is due largely to intracellular acidosis and a large transmembrane pH gradient that promotes Na^+–H^+ exchange and, in turn, reverse-mode Na^+–Ca^+ exchange at the onset of reperfusion.[2–4] Impaired adenosine triphosphate synthesis on reperfusion results from cytoskeletal damage by reactive oxygen (O_2) species and mitochondrial Ca^{2+} overload.[7–9] These effects can cause hypercontracture and incomplete relaxation of myofibrils or cell death.[1,9]

It has long been known that administering a volatile anesthetic before ischemia, or before and after hypoxia or ischemia, improves cardiac function and reduces the occurrence of dysrhythmias on reperfusion.[10–14] This was thought to arise from a metabolic sparing effect or from inhibition of Ca^{2+} influx by the anesthetic. It was discovered earlier that hearts can be protected by "preconditioning" before ischemia, *i.e.*, by brief pulses of ischemia before prolonged ischemia.[15] Protection was assessed by reduced infarct size, attenuated mechanical dysfunction, or limited ultrastructural abnormality. More recently, anesthetics have also been shown to precondition hearts against ischemia.[16–21] We have shown that sevoflurane preconditioning mimics ischemic preconditioning by improving vascular, mechanical, and metabolic function and induced endothelial nitric oxide release in guinea pig hearts.[17,18] Because these effects were blocked by glibenclamide, the ATP-sensitive potassium channel (K_{ATP}) opening has an important role in mediating the protective effect.[18] We also reported recently that anesthetic preconditioning, like ischemic preconditioning, reduces Ca^{2+} loading and improving function on reperfusion[21]; this indicates an important link between cardiac function and Ca^{2+} homeostasis during both ischemic and anesthetic preconditioning.

We questioned whether treatment with a volatile anesthetic before ischemia protects hearts better than treatment on initial reperfusion. We proposed that protection obtained by the former treatment was triggered or mediated by depressed cardiac metabolism and effected by reduced myoplasmic Ca^{2+} loading on reperfusion; protection afforded by the latter treatment was due to a direct metabolic sparing effect mediated only on reperfusion that reduced Ca^{2+} loading. To test this, we administered sevoflurane before and/or after global ischemia in isolated hearts and examined the changes in

* Assistant Professor of Anesthesiology, † Research Scientist, Anesthesiology, ‡ Postdoctoral Fellow, Anesthesiology, Anesthesiology Research Laboratories, Department of Anesthesiology, The Medical College of Wisconsin. § Professor of Anesthesiology and Physiology, Anesthesiology Research Laboratories, Departments of Anesthesiology and Physiology, Cardiovascular Research Center, The Medical College of Wisconsin, and Research Service, VA Medical Center.

Received from the Anesthesiology Research Laboratories, the Departments of Anesthesiology and Physiology, and the Cardiovascular Research Center, The Medical College of Wisconsin, Milwaukee, Wisconsin, and the Research Service, VA Medical Center, Milwaukee, Wisconsin. Submitted for publication March 16, 2001. Accepted for publication August 8, 2001. Supported in part by grant Nos. R01-HL58691 and R01-5T32 GM-08377 from the National Institutes of Health, Bethesda, Maryland; grant No. 0020503Z from the American Heart Association, Dallas, Texas; and Abbott Laboratories, Abbott Park, Illinois. Presented in part at the annual meetings of the American Society of Anesthesiologists, Dallas, Texas, October 9–13, 1999, and the International Anesthesia Research Society, Honolulu, Hawaii, March 10–14, 2000.

Address correspondence to Dr. Stowe: M4280, 8701 Watertown Plank Road, Medical College of Wisconsin, Milwaukee Regional Medical Center, Milwaukee, Wisconsin 53226. Address electronic mail to: dfstowe@mcw.edu.

mechanical, metabolic, and vascular function associated with myoplasmic systolic and diastolic [Ca^{2+}] on reperfusion.

Materials and Methods

Langendorff Heart Preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health No. 85–23, revised 1996). Approval was obtained previously from the Medical College of Wisconsin animal studies committee (Milwaukee, WI). Portions of our methods have been described in detail previously.[5,6,21–25] Sixty guinea pig hearts were isolated and perfused with a modified Krebs-Ringer's solution[6,21,24,25] and perfused at an aortic root perfusion pressure of 55 mmHg at 37.3 ± 0.1°C. Left ventricular pressure (LVP) was measured isovolumetrically, and right atrial and ventricular electrograms were recorded to monitor spontaneous heart rate (HR) and atrioventricular conduction time.[21–25] Coronary flow (CF) was measured at the aorta, and coronary sinus venous partial pressure of oxygen (Po_2) tension was measured continuously online with an O_2 Clark-type electrode. Percent O_2 extraction was calculated as $100 \cdot (Po_2a - Po_2v)/Po_2a$, myocardial O_2 consumption (Mvo_2) was calculated as $CF/g \cdot (Po_2a - Po_2v) \cdot 24$ μl O_2/ml at 760 mmHg, and cardiac work efficiency was calculated as systolic–diastolic LVP · HR/Mvo_2.

Measurement of Cytosolic and Noncytosolic Free Ca^{2+} in Intact Hearts

Loading Fluorescent Probe Indo-1 and Recording Ca^{2+} Transients. We have published this methodology in detail.[5,6,21,22,24,25] Experiments were performed in a light-blocking Faraday cage. The heart was partially immobilized by hanging it from the aortic cannula, the pulmonary artery catheter, and the left ventricular balloon catheter. The heart was immersed in a bath. The distal end of a trifurcated fiber silica fiberoptic cable (optical surface area 3.85 mm^2) was placed against the left ventricular epicardial surface through a hole in the bath. A rubber O ring was placed between the ferrule and the heart to reduce cardiac motion at the contact point of the fiberoptic tip. Background autofluorescence was determined for each heart after initial perfusion and equilibration at 37°C. Thereafter, hearts were loaded with indo-1 acetoxymethyl ester (AM) at room temperature (25 ± 0.6°C) for 20–30 min with 165 ml recirculated Krebs-Ringer's solution containing 6 μM indo-1 AM (Sigma Chemical, St. Louis, MO). Indo-1 AM was initially dissolved in 1 ml dimethyl sulfoxide containing 16% (weight/volume) Pluronic I-127 (Sigma Chemical) and was diluted to 165 ml with modified Krebs-Ringer's solution. Loading was stopped when the F_{385} intensity was increased 10-fold. Residual indo-1 AM was washed out by perfusing the heart with standard perfusate for at least another 20 min, and then each heart was rewarmed to 37.2 ± 0.1°C before initiating the study. The perfusate contained probenecid (100 μM) to retard leakage of indo-1. Loading and washout of indo-1 reduces LVP approximately 25%, an effect due to the vehicle and to intracellular Ca^{2+} buffering by indo-1 *per se*.[6,22,24]

Fluorescence emissions at F_{385} and F_{456} were recorded using a modified luminescence spectrophotometer (SLM Aminco-Bowman II; Spectronic Instruments, Urbana, IL). The left ventricular region of the heart was excited with light from a xenon arc lamp, and the light was filtered through a 355-nm monochromator with a bandwidth of 8 nm. The beam was focused onto the in-going fibers of the optic bundle. The arc lamp shutter was only opened for 2.5-s recording intervals. Fluorescence emissions were collected by fibers of the remaining two limbs of the cable and filtered by square interference filters (Corion, Franklin, MA) at 385 (390 ± 5) and 456 (460 ± 5) nm.

An advantage of the indo-1 ratiometric procedure is that although both F_{385} and F_{456} decrease over time, the F_{385}/F_{456} ratio remains stable during the experimental period.[6] In companion studies, tissue autofluorescence was measured throughout each experiment in the absence of ischemia (autofluorescence control, n = 8) and before, during, and after ischemia (ischemia control, n = 8). The mean F_{385} and F_{456} background values obtained were then subtracted from the corresponding F_{385} and F_{456} values from the indo-1–treated hearts at the same time point and for the same experimental condition.

Calculation of Cytosolic and Noncytosolic Ca^{2+} Concentrations from F_{385}/F_{456} Transients. The Ca^{2+} transient obtained from the indo-1 fluorescence ratio F_{385}/F_{456} is proportional in a nonlinear manner to [Ca^{2+}]. Calibration curves were derived according to previously published protocols by Brandes *et al.*[26,27] using modifications of a standard equation for fluorescent indicators.[28] Total (tot) intracellular [Ca^{2+}] was calculated from the ${}_{tot}F_{385}/{}_{tot}F_{456}$ ratio (${}_{tot}R$), $R_{max} = S_r/bH$ (for > 100 μM Ca^{2+}), $R_{min} = R_{max} \cdot S_{385}/S_{456}$ (for 0 Ca^{2+}), $S_{385} = I_{385}/I_{385}$ (at min/max Ca^{2+}) = 0.05, $S_{456} = I_{456}/I_{456}$ (at min/max Ca^{2+}) = 2.4, and K_d, according to the equation:

$$ {}_{tot}[Ca^{2+}] = S_{456} \cdot K_d[({}_{tot}R - R_{min})/(R_{max} - {}_{tot}R)], \quad (1) $$

where S = ratio of light intensities (I) at the same wavelength at min and max Ca^{2+}, $S_r = (1 - S_{456})/(1 - S_{385}) = -1.48$, and bH = the average slope (b) of ${}_{tot}F_{385}$ as a function of ${}_{tot}F_{456} = -0.25$.

We calculated the dissociation constant (K_d) of indo-1 using homogenized guinea pig heart protein as 249 ± 8 nM at 37°C.[24] R_{max} was calculated as 5.986, and R_{min} was calculated as 0.059.

Non cytosolic (primarily mitochondrial) $_{mito}[Ca^{2+}]$ was calculated similarly:

$$_{mito}[Ca^{2+}] = S_{456} \cdot K_d[(_{mito}R - R_{min})/(R_{max} - _{mito}R)], \quad (2)$$

where $_{mito}R$ was calculated as the ratio of the noncytosolic fluorescence, $_{mito}F_{385}$, and $_{mito}F_{456}$, respectively. Noncytosolic fluorescence was measured at the *end* of each experiment (185 min) in control (CON; n = 8), sevoflurane before ischemia (SBI; n = 7), sevoflurane before and after ischemia (SBAI; n = 9), and sevoflurane after ischemia (SAI; n = 7) groups after perfusing hearts with 100 μM $MnCl_2$ for 10 min to quench fluorescence derived from the cytosolic (cyto) compartment.[6,27] $_{mito}F_{385}$ and $_{mito}F_{456}$ were calculated at each time point by multiplying the residual mitochondrial fluorescence fractions (f_{385} and f_{456}) by total end-diastolic fluorescence so that:

$$_{mito}R = (f_{385})(\text{end-diastolic } _{tot}F_{385})/(f_{456})(\text{end-diastolic } _{tot}F_{456}). \quad (3)$$

Similar to equations 1 and 2, cytosolic $_{cyto}[Ca^{2+}]$ was calculated as:

$$_{cyto}[Ca^{2+}] = S_{456} \cdot K_d[(_{cyto}R - R_{min})/(R_{max} - _{cyto}R)], \quad (4)$$

where $_{cyto}R$ was derived from the ratio of the cytosolic fluorescence, $_{cyto}F_{385}$, and $_{cyto}F_{456}$, respectively, calculated at each time point by effectively subtracting mitochondrial compartment Ca^{2+} ($_{mito}[Ca^{2+}]$) from $_{tot}[Ca^{2+}]$ and multiplying the remainder by total end-diastolic fluorescence (as in equation 3) so that:

$$_{cyto}R = (_{tot}F_{385} - (f_{385})(\text{end-diastolic } _{tot}F_{385}))/(_{tot}F_{456} - (f_{456})(\text{end-diastolic } _{tot}F_{456})). \quad (5)$$

Nonstimulated endothelium does not contribute significantly to $_{tot}[Ca^{2+}]$.[6,22,27]

Protocol

There were four primary groups subjected to ischemia reperfusion; each experiment lasted 195 min beginning after 30 min equilibration. An untreated time control group (n = 9) was not subjected to ischemia; these data are not plotted. The four ischemia groups underwent 30 min of fluorescent dye loading, 20 min of washout, and 45 min of equilibration; this was followed by 30 min of global, no-flow ischemia, 60 min of reperfusion, and 10 min of Mn^{2+} quenching. During ischemia, hearts were immersed in a bath of Krebs-Ringer's solution at 37.3 ± 0.3°C. The SBI group had 85 min of perfusion followed by a 10-min exposure to 3.5 vol% sevoflurane immediately before 30 min of ischemia. The SAI group had 95 min of perfusion, 30 min of ischemia, and 60 min reperfusion, with exposure to 3.5 vol% sevoflurane only during the first 10 min of reperfusion. Hearts of the SBAI group were exposed to 3.5 vol% sevoflurane for 10 min just before 30 min ischemia and also immediately during the first 10 min of reperfusion. If ventricular fibrillation occurred, a 0.25-ml bolus of lidocaine (250 μg) was administered immediately *via* the aortic cannula. All hearts reverted to sinus rhythm, and data were collected after stabilization of LVP. Sevoflurane was bubbled into the perfusate using an agent-specific vaporizer placed in the oxygen–carbon dioxide gas mixture line. Coronary effluent was collected anaerobically from the aortic cannula to measure sevoflurane concentration by gas chromatography.[12–14,17,18] Inflow concentrations (in mM) were 0.62 ± 0.04 for SBI, 0.66 ± 0.04 for SAI, and 0.63 ± 0.05 and 0.67 ± 0.03 for SBAI, which combined was equivalent to 3.34 ± 0.28 vol% atmospheres for a minimal alveolar concentration (MAC of sevoflurane = 2.4%) of approximately 1.52 ± 0.2%. Sevoflurane was not detectable in the effluent during the initial equilibration period, the onset of the reperfusion period (CON and SBI groups), or from 15 to 60 min of reperfusion (all groups).

Data Presentation and Interpretation

Cytosolic Systolic and Diastolic Ca^{2+}. Initial measurements obtained throughout fluorescence dye loading and vehicle washout are not displayed; results shown in graphs begin at 50 min before ischemia. Mechanical, metabolic, and F_{385} and F_{456} measurements were made at 1- to 30-min intervals throughout the study. F_{385}, F_{456}, and F_{385}/F_{456} Ca^{2+} transient signals, LVP, and dLVP/dt were displayed simultaneously on a computer screen and stored digitally using proprietary software (SLM Aminco-Bowman II, series 2, on OS/2 version 3, International Business Machines Corp., Thornwood, NY). After correcting for tissue autofluorescence over time, with or without ischemia and reperfusion, and quenching cytosolic (myoplasmic) compartment Ca^{2+} to reveal noncytosolic compartment Ca^{2+}, the signals were calibrated to nM $[Ca^{2+}]$ using algorithms developed by our group. LVP and raw metabolic data were recorded (MacLab; AD Instruments, Castle Hills, Australia) and, together with the Ca^{2+} transient data, were later analyzed together (Microsoft Excel®; Microsoft Corp., Redmond, WA). Several characteristics of myoplasmic $[Ca^{2+}]$ were analyzed: peak systolic, peak diastolic, and phasic systolic–diastolic $[Ca^{2+}]$, *i.e.*, released or phasic $[Ca^{2+}]$; mitochondrial (noncytosolic) $[Ca^{2+}]$, measured at the end of each experiment, is not phasic. Characteristics of LVP analyzed were systolic, diastolic, and systolic–diastolic LVP, and $dLVP/dt_{max}$ and $dLVP/dt_{min}$.

Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM), and a *P* value (two-tailed) less than 0.05 was considered significant. Within-group data over time at specific times (88,124,128, and 181 min) for a given

variable were compared with a pretreatment control period (at 78 min) by the Duncan comparison of means test whenever univariate analysis of variance fluorescence values for repeated measures were significant ($P < 0.05$; Super ANOVA 1.11® software for Macintosh® from Abacus Concepts, Inc., Berkeley, CA). Among-group data were analyzed at specific time points before (at 78 min) and after (at 88,124, 128, and 181 min) ischemia using multiple analysis of variance (Super ANOVA®). If fluorescence values were significant, *post hoc* comparisons of means were performed using the Student *t* test with Duncan adjustment for multiplicity to differentiate among the four analyzed treatment groups. The incidence of ventricular fibrillation *versus* sinus rhythm per group and the number of ventricular fibrillations per heart per group were determined by Fisher exact test. Differences in ventricular fibrillation duration were determined by unpaired *t* tests. Some among-group statistical notations that are not given in the figures are given in the text. Within-group comparisons on reperfusion *versus* before ischemia are given only in the text.

Results

In the time control group, no measured mechanical or metabolic variable changed over 180 min perfusion; systolic [Ca^{2+}] and diastolic [Ca^{2+}] also did not change. Variables unchanged (not significant) at 60 min of reperfusion from 50 min before ischemia and averaged for all ischemia groups, were HR, 254 ± 3 to 251 ± 3 beats/min, and atrioventricular conduction time, 76 ± 2 to 77 ± 2 ms. Venous pH values for CON, SBI, SBAI, and SAI groups, respectively, were not different before ischemia, 7.16 ± 0.03, 7.12 ± 0.03, 7.12 ± 0.04, and 7.11 ± 0.05, and were not different at 60 min of reperfusion, 7.09 ± 0.05, 7.08 ± 0.06, 7.09 ± 0.04, and 7.08 ± 0.05. Ventricular fibrillation was the most notable dysrhythmia observed on reperfusion. The percentage of hearts that fibrillated during reperfusion was 100% for CON, 72% for SBI , 92% for SBAI, and 89% for SAI ($P > 0.05$). The median number of fibrillations per heart was 3.0 for CON, 1.0 for SBI, 1.0 for SBAI, and 1.0 for SAI; this number was lower ($P < 0.05$) in SBI, SAI, and SBAI groups than in the CON group.

Figures 1A–6B display the associated temporal changes in cytosolic [Ca^{2+}] and cardiac function at 25 time points beginning 45 min before the onset of 30-min ischemia and 60-min reperfusion. Systolic [Ca^{2+}] decreased slowly over 10 min during ischemia and then increased abruptly twofold during initial reperfusion in the CON group (fig. 1A). The increase in systolic [Ca^{2+}] on 1 min reperfusion was much less in SBI, SAI, and SBAI groups and returned to control levels after 5 min in each treated group but only after 15 min of reperfusion in CON. Systolic LVP decreased faster than systolic [Ca^{2+}] during early ischemia (fig. 1B) and began to increase after 25 min of ischemia. Systolic LVP increased on initial reperfusion to the control level in SBI and SBAI groups, but not in SAI and CON groups; moreover, systolic LVP remained higher in the SBI group than in the SAI group for the first 30 min of reperfusion. Diastolic [Ca^{2+}] increased progressively twofold during ischemia and up to fourfold during initial reperfusion in each group (fig. 2A); diastolic [Ca^{2+}] remained increased up to 30 min in CON but returned faster toward controls in sevoflurane-treated groups. Diastolic LVP (fig. 2B) increased in each group during the last 5 min of ischemia. During reperfusion, diastolic LVP increased less in SBI, SBAI, and SAI groups compared with CON and remained above the

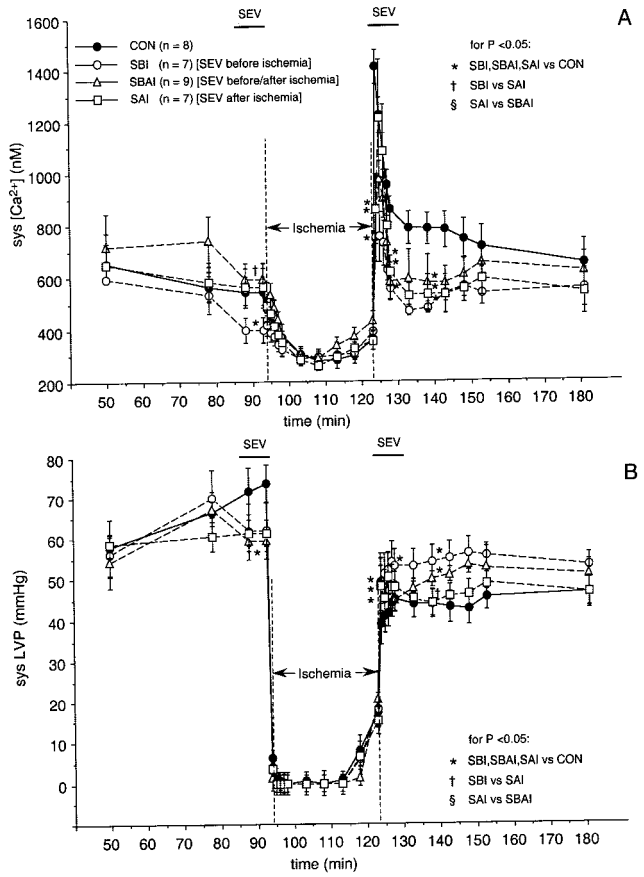

Fig. 1. Time course of systolic (sys) [Ca^{2+}] (*A*) and systolic left ventricular pressure (LVP) (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that systolic [Ca^{2+}] and systolic LVP decreased moderately during sevoflurane (SEV) exposure in SBI and SBAI groups and markedly in all groups during ischemia. Systolic [Ca^{2+}] increased maximally on initial reperfusion, whereas systolic LVP remained reduced throughout reperfusion in CON and SAI groups but recovered in SBI and SBAI groups. CON = control, no treatment before 30 min global ischemia and 60 min reperfusion (n = 8); SBI = 3.5 vol% sevoflurane administered 10 min before ischemia (n = 7); SAI = 3.5 vol% sevoflurane administered 10 min after ischemia (n = 7); SBAI = 3.5 vol% sevoflurane administered 10 min before and after ischemia (n = 9). Comparisons among and within groups were determined at specific time points before ischemia (at 78 and 88 min) and on reperfusion (at 124, 128, and 181 min).

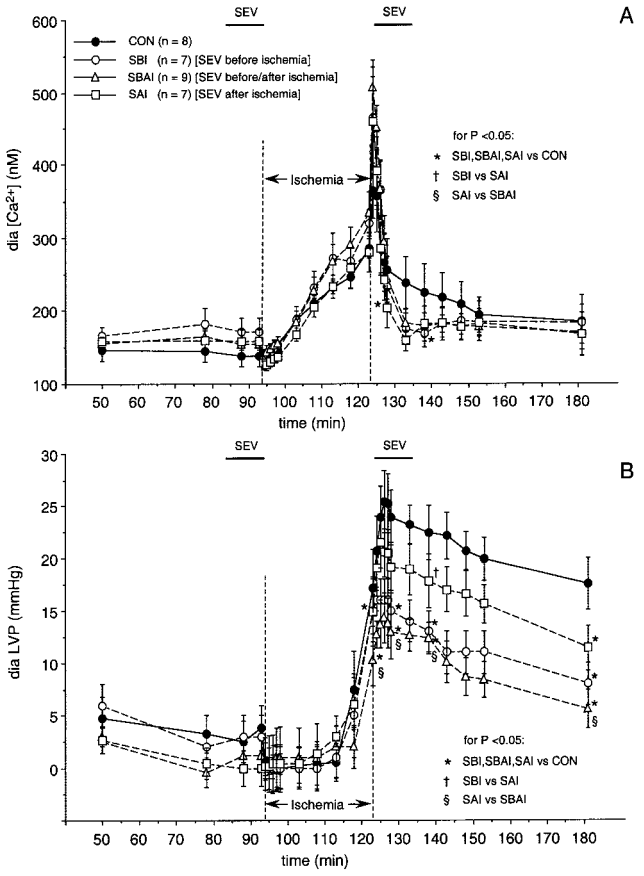

Fig. 2. Time course of diastolic (dia) $[Ca^{2+}]$ (*A*) and diastolic left ventricular pressure (LVP) (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that diastolic $[Ca^{2+}]$ increased gradually and similarly by twofold in all groups during ischemia and increased additionally during reperfusion. Diastolic LVP increased similarly during late ischemia and increased markedly during reperfusion in all groups. Diastolic LVP was lower in the SAI group than in the CON group throughout reperfusion and was lowest in SBI and SBAI groups. See figure 1 for details on groups and statistics. SEV = sevoflurane.

control level only in CON and SAI groups at 60 min of reperfusion.

Systolic–diastolic $[Ca^{2+}]$, *i.e.*, phasic $[Ca^{2+}]$, (fig. 3A) decreased slowly over 10 min in all groups during initial ischemia, after which the transients disappeared until reperfusion. Systolic–diastolic $[Ca^{2+}]$ increased abruptly approximately twofold during the first minute of reperfusion in CON but less so in sevoflurane-treated groups and remained higher during reperfusion in CON compared with the SBI and SAI groups. Unlike phasic $[Ca^{2+}]$, developed systolic–diastolic LVP (fig. 3B) decreased abruptly to near zero at the onset of ischemia. At 5 min of reperfusion, systolic–diastolic LVP was decreased by 66% in CON, whereas it was reduced approximately 41% averaged for all sevoflurane-treated groups. Between 20 and 60 min of reperfusion, systolic–diastolic LVP was higher in SBI and SBAI than in CON and SAI groups and higher in SAI than in CON.

Contractility, $dLVP/dt_{max}$ (fig. 4A), was decreased moderately during SBI and SBAI treatments and approached zero in each group during ischemia; $dLVP/dt_{max}$ was decreased by 70% on initial reperfusion in CON and by approximately 34% averaged for the sevoflurane-treated groups. $dLVP/dt_{max}$ remained depressed at 60 min reperfusion in the CON and SAI groups, but not in the SBI and SBAI groups. Relaxation, $dLVP/dt_{min}$ (fig. 4B), exhibited trends for each group similar to those of $dLVP/dt_{max}$.

Coronary flow (fig. 5A) was not altered by sevoflurane in SBI and SBAI groups before ischemia. Coronary flow at 60 min of reperfusion remained lower than before ischemia in each group but was higher in each sevoflurane-treated group. Mvo_2 (fig. 5B) was indeterminate during ischemia and was lower in all groups but the SBI group on initial reperfusion and was higher in each sevoflurane group after 20 min of reperfusion compared with the CON group. Among the sevoflurane groups,

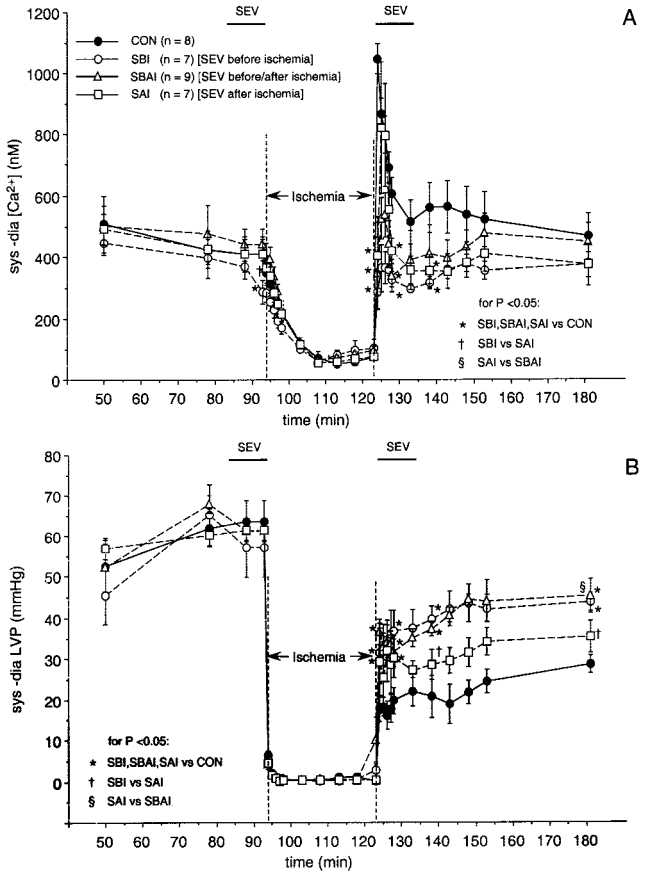

Fig. 3. Time course of phasic (systolic–diastolic [sys-dia]) $[Ca^{2+}]$ (*A*) and systolic–diastolic left ventricular pressure (LVP) (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that systolic–diastolic $[Ca^{2+}]$ decreased during ischemia in all groups and increased approximately twofold during initial reperfusion in the CON group, but that the increase was smaller in each sevoflurane (SEV) group. Phasic LVP was higher in each sevoflurane group on reperfusion and was especially higher in the SBI and SBAI groups up to 60 min of reperfusion. See figure 1 for details on groups and statistics.

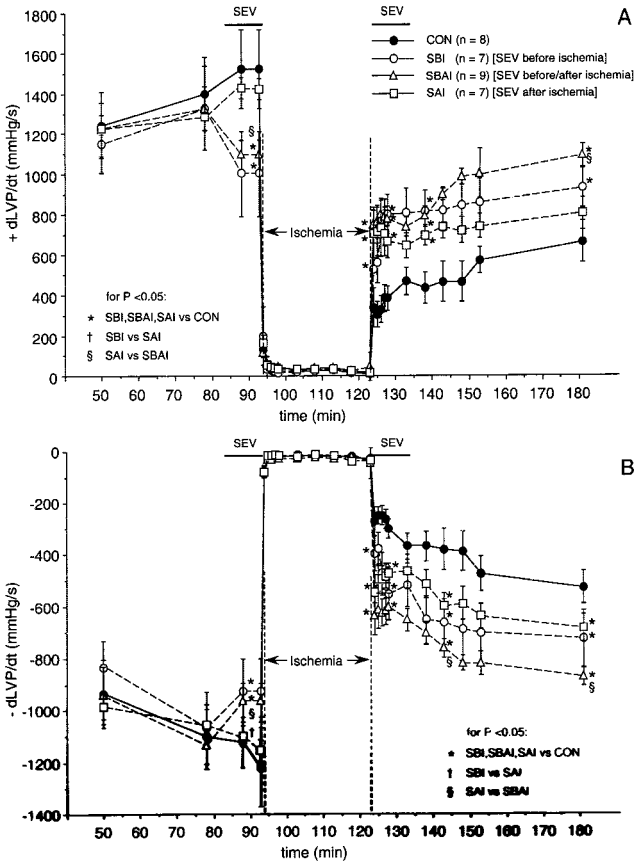

Fig. 4. Time course of $dLVP/dt_{max}$ (*A*) and $dLVP/dt_{min}$ (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that sevoflurane (SEV) treatment in SBI and SBAI groups slightly decreased $dLVP/dt_{max}$ and $dLVP/dt_{min}$ such that $dLVP/dt_{max}$ and $dLVP/dt_{min}$ were moderately decreased during reperfusion but that these changes were smaller in each sevoflurane group compared with the CON group. See figure 1 for details on groups and statistics.

Mvo_2 was highest on final reperfusion in the SBI and SBAI groups and lowest in the SAI group.

Cardiac work efficiency (fig. 6A) was not altered by sevoflurane in SBI and SBAI groups before ischemia among the groups. On reperfusion, cardiac efficiency was higher in all sevoflurane groups and especially in the SBI and SBAI group; it remained decreased at 60 min of reperfusion only in CON. Percent O_2 extraction (fig. 6B) was reduced by sevoflurane in SBI and SBAI groups before ischemia; on reperfusion, it was lower in all sevoflurane-treated groups compared with CON.

Discussion

We examined cardioprotective effects of a volatile anesthetic, sevoflurane, administered for 10 min immediately before global ischemia, for 10 min on reperfusion immediately after ischemia, and both before and after ischemia to ascertain whether the timing of exposure to the anesthetic influences the extent of protection. At the same time, we measured whether diastolic and systolic myocyte $[Ca^{2+}]$ during ischemia and reperfusion are modulated by the protective effect of the anesthetic when administered before or after ischemia. As in previous studies using other volatile anesthetics, we observed that sevoflurane treatment afforded cardioprotection by reducing dysrhythmias and improving mechanical and metabolic function. Concomitantly, we observed that improved function with each sevoflurane treatment was associated with reduced systolic and phasic Ca^{2+} loading on reperfusion.

It was interesting that sevoflurane administered before ischemia was more protective than sevoflurane administered on reperfusion. One might expect that because it is during the reperfusion period that the major damage to cells occurs, administering a depressant agent during reperfusion would be more protective that when administered before ischemia. Sevoflurane administered before ischemia exerted minimal cardiac effects except for small decreases in contractility ($dLVP/dt_{max}$), relaxation ($dLVP/dt_{min}$), and percent O_2 extraction. However, this small metabolic sparing effect before ischemia could trigger mechanisms that lead to cardioprotection on

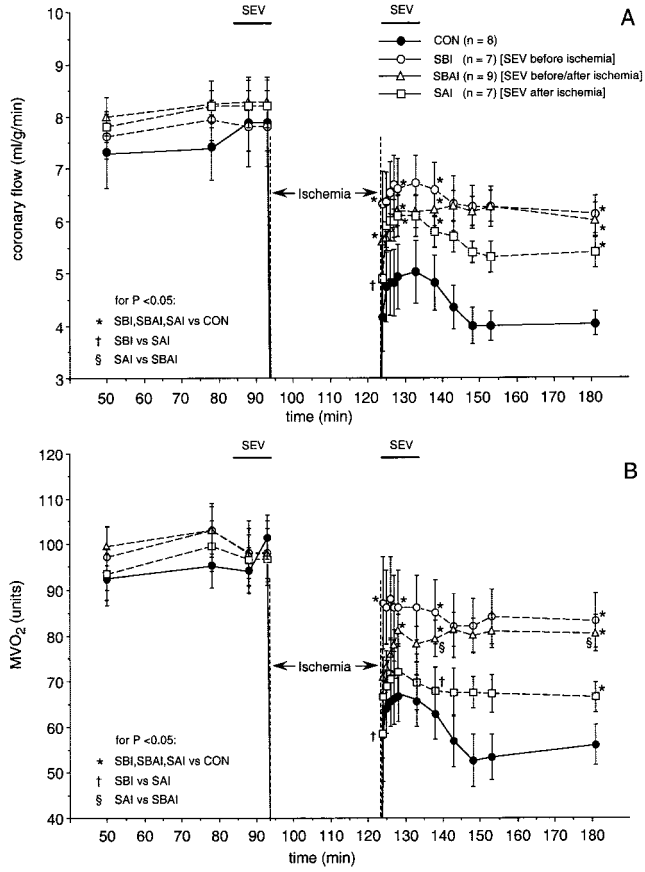

Fig. 5. Time course of coronary flow (*A*) and myocardial oxygen consumption (Mvo_2; $\mu l\ O_2 \cdot g^{-1} \cdot min^{-1}$) (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that coronary flow and Mvo_2 were higher in each sevoflurane (SEV) treatment group throughout reperfusion compared with the CON group and were higher in the SBI and SBAI groups than in the SAI group. See figure 1 for details on groups and statistics.

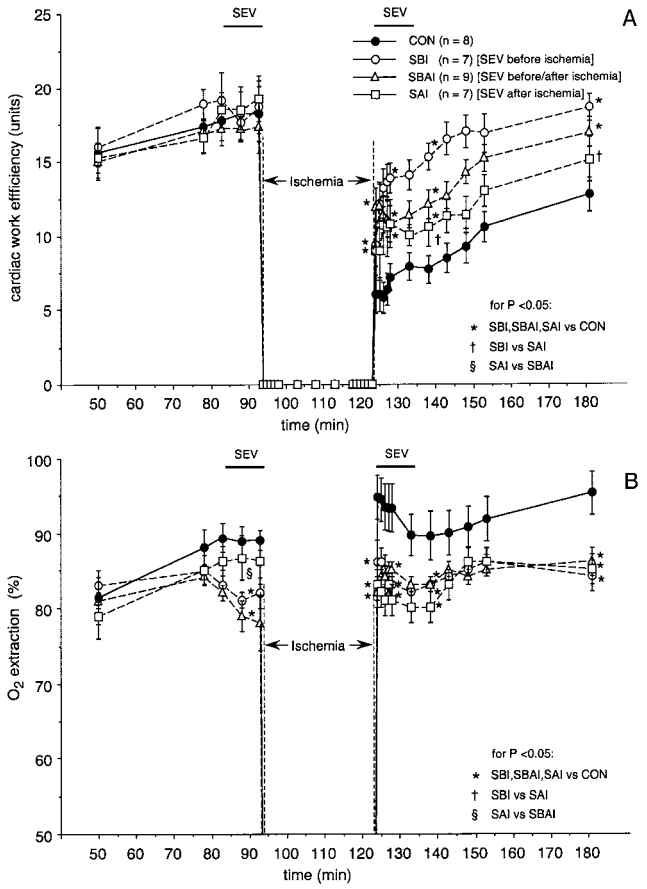

Fig. 6. Time course of cardiac work efficiency (mmHg · beats · g^{-1} · 0.1 μl O_2^{-1}) (*A*) and percent oxygen (O_2) extraction (*B*) before, during, and after global ischemia in CON, SBI, SBAI, and SAI groups. Note that cardiac work efficiency was higher in each sevoflurane (SEV) treatment group throughout reperfusion compared with the CON group and was highest in the SBI group. Percent oxygen extraction was higher in the CON group compared with each sevoflurane treatment group throughout reperfusion. See figure 1 for details on groups and statistics.

reperfusion. Indeed, the degree of protection was similar to that found previously for sevoflurane preconditioning.[17,18,21] Although there was no memory phase after anesthetic exposure involved in these experiments, *i.e.*, no washout, as in our previous studies,[17,18,21] it is likely that the anesthetic activates protective cellular pathways regardless of whether ischemia subsequently occurs. We speculate that the small sevoflurane-induced decrease in metabolic rate, without a change in O_2 delivery, metabolically triggers a protective mechanism mediated by a change in mitochondrial energetics, *e.g.*, a small decrease in mitochondrial Ca^{2+}, an increase in reduced nicotinamide adenine dinucleotide (NADH), or a small release of O_2-derived free radicals.[29] Because we could not statistically differentiate effects of the three sevoflurane treatments on reducing Ca^{2+} loading during ischemia and reperfusion, we suggest that the reduction in Ca^{2+} loading is a consequence rather than a cause of improved function after sevoflurane treatment.

The immediate period of reperfusion sets the stage for Ca^{2+} overload[2–7] and a large release of reactive O_2[30,31] species that leads to mitochondrial and cellular injury and demise. In a preliminary study[31] using a protocol identical to the SBI group, we found that sevoflurane pretreatment reduced peroxynitrite fluorescence in the effluent by 77%. Because peroxynitrite is the product of two free radicals, superoxide and nitric oxide, the reduced release of one or both of these species after ischemia is likely associated with the reduced Ca^{2+} overloading we observed. This suggests that sevoflurane pretreatment triggers a sequence of events to protect the heart by reducing free radical damage and Ca^{2+} loading. It is noteworthy that not only was sevoflurane administered before ischemia more protective than when administered on initial reperfusion, but there was also no additive protective effect when sevoflurane was administered both before and after ischemia. Also, postischemic sevoflurane treatment did not seem to suppress metabolism or function compared with sevoflurane pretreatment. This suggests that sevoflurane initiates a maximal protective effect before ischemia that overrides a lesser protective effect elicited by sevoflurane on reperfusion. A major effector of both preischemic and postischemic sevoflurane was reduced Ca^{2+} loading; combining these protocols also did not additively reduce Ca^{2+} loading. As in anesthetic preconditioning, the more favorable outcome was garnered if the anesthetic was administered before ischemia.

Role of Cations in Ischemia and Reperfusion Injury

Postischemic reperfusion injury results in large part from mitochondrial[6–8] and myoplasmic Ca^{2+} overloading.[2–6,21,25] If the increase in [Ca^{2+}] is prolonged, a cascade of events is initiated, which ultimately results in lethal injury.[1,15] We have recently detailed the time course of changes in contractility and relaxation associated with altered [Na^+], myoplasmic and mitochondrial [Ca^{2+}], and [NADH] during reperfusion injury in the intact heart.[6] It was apparent that contractile performance becomes dissociated from myoplasmic Ca^{2+}, particularly during early reperfusion, in that higher Ca^{2+} concentrations are associated with reduced contractile force and impaired relaxation. Moreover, the increased [Na^+] and mitochondrial [Ca^{2+}] during and after ischemia probably underlies contractile dysfunction. Because Ca^{2+} loading was temporally associated with Na^+ loading during early reperfusion[6,25] and because [Na^+] and [Ca^{2+}] were reduced after blocking Na^+–H^+ exchange,[25] our studies strongly support the notion that Na^+–H^+ exchange and reverse Na^+–Ca^+ exchange are major cationic factors that underlie reperfusion injury.

Anesthetic-induced Cardiac Protection

It is not understood how volatile anesthetics protect the myocardium from reperfusion damage, but the over-

all mechanism is likely multifactorial. We reported previously that halothane and isoflurane improve function and metabolism on reperfusion and reduce dysrhythmia development when administered 10 min before and after hypoxia[12] or graded or complete global ischemia[13,14] in isolated guinea pig hearts. Many other studies have supported the cardiac protective effects of anesthetics.[10,11,16–21,23] We showed that halothane protects against dysrhythmias and improves mechanical, metabolic, and vascular endothelial function when administered during low-flow perfusion for 1 day at 3°C.[23] Isoflurane administered for 45 min before and during 15 min of coronary occlusion in dogs enhanced recovery of regional myocardial contractile function after 5 h of reperfusion.[32] Because this effect was partially blocked by glibenclamide, a role for isoflurane to enhance K_{ATP} channel activation during ischemia and reperfusion was suggested.

Preconditioning by isoflurane,[16,19,33] enflurane,[16] and halothane[16] reduced infarct size in rabbit hearts; the protection was similar to that of ischemic preconditioning. In dogs, isoflurane administered alone or during four 5-min occlusions before 60 min of regional myocardial ischemia reduced infarct size.[20] Because the protective effect was reversed by glibenclamide, this again supported a role for K_{ATP} channel opening. Anesthetics may activate A_1 receptors or increase the sensitivity of adenosine A_1 receptors to attenuated adenosine release.[34] In turn, anesthetic-induced increases in protein kinase C activity may underlie reduced infarct size[33] and improved contractility[35] after ischemia.

We reported that anesthetic preconditioning (APC) with sevoflurane is as effective as ischemic preconditioning (IPC) in improving not only mechanical function, but also cardiac rhythm, perfusion, metabolic function, and basal- and nitric oxide–mediated coronary flow.[18] Moreover, the vascular and myocardial protective effects of APC by sevoflurane and IPC were antagonized by glibenclamide, suggesting a common final mechanism *via* activation of K_{ATP} channels. In a recent study, we showed that equivalent improvements in metabolic and contractile functions after IPC and APC by sevoflurane were accompanied by equivalent reductions in Ca^{2+} loading on reperfusion after global ischemia.[21] If K_{ATP} channel antagonism also reverses the reduced Ca^{2+} loading effects of IPC and APC, this would suggest that cardioprotection is afforded, at least in part, by reduced Ca^{2+} loading when K_{ATP} channels are open. Interestingly, both APC and IPC reduce cytosolic Ca^{2+} loading and protect hearts *via* K_{ATP} channel opening. It is widely believed that IPC activates protein kinase C and tyrosine kinase pathways that in turn promote phosphorylation of sarcolemmal and mitochondrial K_{ATP} channels.[36–38] It seems clear that APC and IPC lead to protective effects *via* a common final mechanism that opens K_{ATP} channels and reduces cytosolic Ca^{2+} loading.

In contrast to APC or anesthetic pretreatment, anesthetic treatment on reperfusion was not quite as protective. It is possible that anesthetic pretreatment triggers a powerful protective effect, as good as IPC,[21] and that anesthetic posttreatment is insufficient to reduce the metabolic rate with the onset of reperfusion. Indeed, sevoflurane administered immediately on reperfusion did not seem to reduce function or metabolism compared with the control group. However, because Ca^{2+} loading during reperfusion was reduced by sevoflurane administered during reperfusion, the improvement in Ca^{38} homeostasis and function is likely linked through an anesthetic-induced effect.

Volatile anesthetics are often selected for patients with coronary artery disease who are at risk for ischemia and infarction during cardiac and noncardiac surgery. Temporary ischemia is often induced during cardiac surgery and angioplasty.[39] A volatile anesthetic may be a safe and efficacious method to protect the heart during cardiac and noncardiac procedures in patients with coronary artery disease.[40] In this study, we investigated possible differences in protective mechanisms when the anesthetic was administered before *versus* after ischemia and showed that similarly reduced Ca^{2+} loading contributes to, or results from, both treatment protocols. Further research will seek to determine whether improved cardiac function and Ca^{2+} homeostasis on reperfusion after anesthetic pretreatment results from factors mediated by intracellular signaling pathways that lead to early activation of K_{ATP} channel opening during the treatment. Future studies will also be directed to determine whether improved function and Ca^{2+} homeostasis after anesthetic posttreatment is a consequence of enhanced K_{ATP} channel opening on reperfusion.

Possible Limitations

(1) Our studies in the guinea pig may not be easily compared with those of other species, especially the rat. Ischemia in rats causes a marked contracture beginning early during ischemia that is accompanied by a marked increase in diastolic [Ca^{2+}] (Stowe *et al.*, laboratory observations, July 1999); on early reperfusion, diastolic LVP remains increased, and diastolic [Ca^{2+}] does not increase further. (2) It is possible but unlikely that a portion of the 150-nM increase in diastolic [Ca^{2+}] that we found in each group on initial reperfusion was derived from washout of indo-1–bound Ca^{2+} rather than from cytosolic Ca^{2+}. Subepicardial left ventricular tissue at the location of the fiberoptic probe was not infarcted but was likely stunned. Because it is phasic, systolic–diastolic [Ca^{2+}] must represent cytosolic [Ca^{2+}]. (3) It is not possible to separate mitochondrial compartment Ca^{2+} from other compartments, *e.g.*, nuclear or Ca^{2+} from other cells, *i.e.*, endothelial, vascular, or nerve. The flux of Ca^{2+} through each of these compartments is likely very slow. These compartments likely contribute little to

the total rapid phasic signal of the myoplasm. We estimated average noncytosolic Ca^{2+} from $MnCl_2$ quenching at the end of each experiment, and Mn^{2+} may leak into this compartment over time. We assumed that the residual fluorescence recorded after quenching cytosolic fluorescence arises predominantly from mitochondrial Ca^{2+}, but this may be an overestimate. (4) Our study required a crystalloid perfusate devoid of cells with a high O_2 tension, so our findings may not exactly mimic those in blood-perfused hearts. (5) Our study was limited to one concentration of one anesthetic.

The authors thank Amadou Camara, Ph.D., Qun Chen, M.D., Ming-Tao Jiang, Ph.D., James Heisner, B.S., and Anita Tredeau (Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI), and Samhita Shahane Rhodes, M.S. (Department of Biomedical Engineering, Marquette University, Milwaukee, WI), for their valuable contributions to this study.

References

1. Ferrari R, Hearse D: Myocardial stunning. Dial Cardiovasc Med 1996; 1:3–72
2. Tani M, Neely JR: Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts: Possible involvement of H^+-Na^+ and Na^+-Ca^{2+} exchange. Circ Res 1989; 65:1045–56
3. Allard MF, Flint JD, English JC, Henning SL, Salamanca MC, Kamimura CT, English DR: Calcium overload during reperfusion is accelerated in isolated hypertrophied rat hearts. J Mol Cell Cardiol 1994; 26:1551–63
4. Karmazyn M: Role of sodium-hydrogen exchange in mediating myocardial ischemia and reperfusion injury: Mechanism and therapeutic implications, The Na/H Exchanger. Edited by Fliegel L. Heidelberg, Springer, 1996, pp 189–215
5. Stowe DF, Varadarajan SG, An JZ, Smart SC: Reduced cytosolic Ca^{2+} loading and improved cardiac function after cardioplegic cold storage of guinea pig isolated hearts. Circulation 2000; 102:1172–7
6. Varadarajan SG, An JZ, Novalija E, Smart SC, Stowe DF: Changes in $[Na^+]_i$, compartmental $[Ca^{2+}]$, and NADH with dysfunction after global ischemia in intact hearts. Am J Physiol 2001; 280:H280–93
7. Miyamae M, Camacho SA, Weiner MW, Figueredo VM: Attenuation of postischemic reperfusion injury is related to prevention of $[Ca^{2+}]_m$ overload in rat hearts. Am J Physiol 1996; 271:H2145–53
8. Benzi RH, Lerch R: Dissociation between contractile function and oxidative metabolism in postischemic myocardium: Attenuation by ruthenium red administered during reperfusion. Circ Res 1992; 71:567–76
9. Kusuoka H, Marban E: Cellular mechanisms of myocardial stunning. Annu Rev Physiol 1992; 54:243–56
10. Hoka S, Bosnjak ZJ, Kampine JP: Halothane inhibits calcium accumulation following myocardial ischemia and calcium paradox in guinea pig hearts. ANESTHESIOLOGY 1987; 67:197–202
11. Warltier DC, Al-Wathiqui MH, Kampine JP, Schmeling WT: Recovery of contractile function of stunned myocardium in chronically instrumented dogs is enhanced by halothane and isoflurane. ANESTHESIOLOGY 1988; 69:552–65
12. Marijic J, Stowe DF, Turner LA, Kampine JP, Bosnjak ZJ: Differential protective effects of halothane and isoflurane against hypoxic and reoxygenation injury in the isolated guinea pig heart. ANESTHESIOLOGY 1990; 73:976–83
13. Buljubasic N, Marijic J, Stowe DF, Kampine JP, Bosnjak ZJ: Halothane reduces dysrhythmias and improves contractile function after global hypoperfusion in isolated hearts. Anesth Analg 1992; 74:384–94
14. Buljubasic N, Stowe DF, Marijic J, Roerig DL, Kampine JP, Bosnjak ZJ: Halothane reduces release of adenosine, inosine, and lactate with ischemia and reperfusion in isolated hearts. Anesth Analg 1993; 76:54–62
15. Murry CE, Jennings RH, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36
16. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. ANESTHESIOLOGY 1997; 86:699–709
17. Novalija E, Stowe DF: Prior preconditioning by ischemia or sevoflurane improves cardiac work per oxygen use in isolated guinea pig hearts after global ischemia. Adv Exp Med Biol 1998; 454:533–42
18. Novalija E, Fujita S, Kampine JP, Stowe DF: Sevoflurane mimics ischemic preconditioning effects on coronary flow and nitric oxide release in isolated hearts. ANESTHESIOLOGY 1999; 91:701–12
19. Cason BA, Gamperi AK, Slocum RE, Hickey RF: Anesthetic-induced preconditioning. ANESTHESIOLOGY 1997; 87:1182–90
20. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ: Isoflurane mimics ischemic preconditioning *via* activation of K_{ATP} channels. ANESTHESIOLOGY 1997; 87:361–70
21. An JZ, Varadarajan GS, Smart SC, Stowe DF: Ischemic and anesthetic preconditioning reduce cytosolic $[Ca^{2+}]$ and improve Ca^{2+} responses in intact hearts. Am J Physiol (Heart Circ) 2001; 281:H1508–23
22. Stowe DF, Fujita S, An JZ, Paulsen RA, Varadarajan SG, Smart SC: Modulation of myocardial function and Ca^{2+} sensitivity by moderate hypothermia in guinea pig isolated hearts. Am J Physiol 1999; 277:H2321–32
23. Stowe DF, Habazettl H, Graf BM, Kampine JP, Bosnjak ZJ: One-day hypothermic preservation of isolated hearts with halothane improves cardiac function better than low calcium. ANESTHESIOLOGY 1995; 83:1065–77
24. Fujita S, Smart SC, Stowe DF: Enhanced myofilament Ca^{2+} sensitivity by delta-2 opioid agonist deltorphin in intact guinea pig hearts. J Mol Cell Cardiol 2000; 32:1647–59
25. An JZ, Varadarajan SG, Camara A, Chen Q, Novalija E, Gross GJ, Stowe DF: Inhibiting Na^+/H^+ exchange before ischemia or at reperfusion reduces $[Na^+]_i$ and $Ca^{2+}]_i$ overload and improves function in intact hearts. Am J Physiol (Heart Circ) 2001; 281:H2398–409
26. Brandes R, Figueredo VM, Camacho SA, Baker AJ, Weiner MW: Quantitation of cytosolic $[Ca^{2+}]$ in whole perfused rat hearts using Indo-1 fluorometry. Biophys J 1993; 65:1973–82
27. Brandes R, Figueredo VM, Camacho SA, Baker AJ, Weiner MW: Investigation of factors affecting fluorometric quantitation of cytosolic $[Ca^{2+}]$ in perfused hearts. Biophys J 1993; 65:1983–93
28. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440–50
29. Vanden Hoek TL, Becker LB, Shao Z-H, Li C-Q, Schumaker PT: Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. Circ Res 2000; 86:541–8
30. Maddaford TG, Pierce GN: Myocardial dysfunction is associated with activation of Na^+/H^+ exchange immediately during reperfusion. Am J Physiol 1997; 273:H2232–9
31. Novalija E, An JZ, Camara AK, Varadarajan SG, Stowe DF: Sevoflurane pretreatment improves function and reduces formation of peroxynitrite after global ischemia in isolated hearts (abstract). ANESTHESIOLOGY 2000; 93:A679
32. Kersten JR, Lowe D, Hettrick DA, Pagel PS, Gross GJ, Warltier DC: Glyburide, a K_{ATP} channel antagonist, attenuates the cardioprotective effects of isoflurane in stunned myocardium. Anesth Analg 1996; 83:27–33
33. Ismaeil MS, Tkachenko I, Gamperl AK, Hickey RF, Cason BA: Mechanisms of isoflurane-induced myocardial preconditioning in rabbits. ANESTHESIOLOGY 1999; 90:812–21
34. Kersten JR, Orth KG, Pagel PS, Mei DA, Gross GJ, Warltier DC: Role of adenosine in isoflurane-induced cardioprotection. ANESTHESIOLOGY 1997; 86:1128–39
35. Toller WG, Montgomery MW, Pagel PS, Hettrick DA, Warltier DC, Kersten JR: Isoflurane-enhanced recovery of canine stunned myocardium: Role for protein kinase C? ANESTHESIOLOGY 1999; 91:713–22
36. Liu Y, Ytrehus K, Downey JM: Evidence that translocation of protein kinase C is a key event during ischemic preconditioning of rabbit myocardium. J Mol Cell Cardiol 1994; 26:661–8
37. Maulik N, Yoshida T, Zu YL, Sato M, Banerjee A, Das DK: Ischemic preconditioning triggers tyrosine kinase signaling: A potential role for MAPKAP kinase 2. Am J Physiol 1998; 275:H1857–64
38. Gross GJ, Fryer RM: Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. Cir Res 1999; 84:973–9
39. Cribier A, Korsatz L, Koning R, Rath P, Gamra H, Stix G, Merchant S, Chan C, Letac B: Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: A prospective study. J Am Coll Cardiol 1992; 20:578–86
40. Belhomme D, Peynet J, Louzy M, Launay JM, Kitakaze M, Menasche P: Evidence for preconditioning by isoflurane in coronary artery bypass graft surgery. Circulation 1999; 100(suppl II):II-340–4