# Sevoflurane Preconditioning before Moderate Hypothermic Ischemia Protects against Cytosolic [Ca$^{2+}$] Loading and Myocardial Damage in Part via Mitochondrial K$_{ATP}$ Channels

Qun Chen, M.D.,* Amadou K. S. Camara, Ph.D.,† Jianzhong An, M.D.,‡ Enis Novalija, M.D.,§ Matthias L. Riess, M.D.,§ David F. Stowe, M.D., Ph.D.‖

*Background:* Brief sevoflurane exposure and washout (sevoflurane preconditioning [SPC]) before 30-min global ischemia at 37°C is known to improve cardiac function, decrease cytosolic [Ca$^{2+}$] loading, and reduce infarct size on reperfusion. It is not known if anesthetic preconditioning (APC) applies as well to hypothermic ischemia and reperfusion and if K$_{ATP}$ channels are involved. The authors examined in guinea pig isolated hearts the effect of sevoflurane exposure before 4-h global ischemia at 17°C on cardiac function, cytosolic [Ca$^{2+}$] loading, and infarct size. In addition they tested the potential role of the mitochondrial K$_{ATP}$ channel in eliciting the cardioprotection by SPC.

*Methods:* Hearts were randomly assigned to (1) a nontreated hypothermic ischemia group (CON), (2) a group given 3.5 vol% sevoflurane for 15 min with a 15-min washout before hypothermic ischemia (SPC), and (3) an SPC group in which anesthetic exposure was bracketed with 200 $\mu$M 5-hydroxydecanoate (5-HD) from 5 min before until 5 min after sevoflurane (SPC + 5-HD). Cytosolic [Ca$^{2+}$] was measured in the left ventricular (LV) free wall with the intracellularly loaded fluorescence probe indo-1.

*Results:* Initial reperfusion in CON hearts markedly increased systolic and diastolic [Ca$^{2+}$] and reduced contractility (dLVP/dt$_{max}$), relaxation (diastolic LVP, dLVP/dt$_{min}$), myocardial oxygen consumption (Mvo$_2$), and cardiac efficiency. In SPC hearts, cytosolic [Ca$^{2+}$] overloading (especially diastolic [Ca$^{2+}$]) was decreased with increased myocardial [Ca$^{2+}$] influx (d[Ca$^{2+}$]/dt$_{max}$) and efflux (d[Ca$^{2+}$]/dt$_{min}$), improved contractility, relaxation, coronary flow, Mvo$_2$, cardiac efficiency, and decreased infarct size. In SPC + 5HD hearts, the reduction in infarct size was antagonized by 5-HD, but functional return was less affected by 5-HD.

*Conclusions:* Anesthetic preconditioning occurs after long-term hypothermic ischemia, and the infarct size reduction is the result, in part, of mitochondrial K$_{ATP}$ channel opening.

ANESTHETIC preconditioning (APC), *i.e.*, exposure of the heart to a volatile anesthetic followed by its washout,

is as protective as ischemic preconditioning (IPC) in many normothermic ischemia and reperfusion models.[1–7] We have shown that two 2-min exposures to sevoflurane separated with a 5- and 6-min washout before 30-min global ischemia at 37°C improved postischemic contractility, relaxation, and metabolism function.[1,6] This cardioprotection was blocked by glibenclamide, a nonselective K$_{ATP}$ channel blockade,[1] and was demonstrated by improved cardiac function, reduced infarct size, and decreased cytosolic [Ca$^{2+}$] overloading.[6]

Moderate hypothermia protects against ischemia by prolonging the time to stunning or permanent damage.[8–12] However, hypothermia also has the disadvantage that it causes cytosolic [Ca$^{2+}$] loading[8,9,13,14] that leads to reduced diastolic ventricular compliance and contractility on rewarming and reperfusion.[10–12] We reported that halothane and isoflurane administered during 24-h low-flow hypothermic storage at 4°C improved cardiac perfusion and function.[10,11] Moreover, in an IPC protocol, we also reported recently that two brief periods of ischemia and reperfusion before 4-h 17°C global ischemia improved cardiac function and reduced infarct size in association with decreased cytosolic [Ca$^{2+}$] loading during ischemia and reperfusion.[15] Protection against infarction size was blocked partially by glibenclamide and by the K$_{ATP}$ selective inhibitor 5-hydroxydecanoate (5-HD). It has not been known if transient anesthetic exposure with seroflurane (SPC) before 4-h 17°C global ischemia also protects against ischemia reperfusion damage and if this is associated with cytosolic [Ca$^{2+}$] loading and mK$_{ATP}$ opening.

In the present study, we questioned (1) if APC before 4-h cold ischemia at 17°C affords additive cardioprotection with hypothermia, (2) if cardioprotection elicited by APC is associated with the decreased cytosolic [Ca$^{2+}$] loading during hypothermic ischemia and reperfusion, and (3) if cardioprotective effects of APC result from mK$_{ATP}$ opening.

## Methods

### Isolated Heart Preparation and Measurements

The investigation conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH No. 85–23, revised 1996). Previous approval was obtained from the Medical College of Wis-

912

consin Animal Studies Committee. Our preparation and measurements have been described in detail.[1,6,9–11,16–19] Hearts were isolated and prepared by the Langendorff method and perfused at 55 mmHg with Krebs-Ringer's (KR) solution. The perfusate had the following control composition: Na$^+$, 137 mM; K$^+$, 4.5 mM; Mg$^{2+}$, 2.4 mM; Ca$^{2+}$, 2.5 mM; Cl$^-$, 134 mM; HCO$_3^-$, 15.5 mM; H$_2$PO$_4^-$, 1.2 mM; glucose, 11.5 mM; pyruvate, 2 mM; mannitol, 16 mM; EDTA (ethylene-diaminetetraacetic acid), 0.05 mM; probenecid, 0.1 mM; and insulin, 5 U/l. Isovolumetric left ventricular pressure (LVP) and dLVP/dt were measured with a transducer connected to a thin, saline-filled latex balloon inserted into the left ventricle. Balloon volume was adjusted to maintain a diastolic LVP of zero mmHg during the initial baseline period so that any increase in diastolic LVP indicated an increase in LV wall stiffness or diastolic contracture. Coronary inflow (CF) was measured by ultrasound, and coronary effluent Na$^+$, K$^+$, Ca$^{2+}$, Po$_2$, Pco$_2$, and pH were measured off-line with an intermittently self-calibrating analyzer system. Coronary outflow (coronary sinus) O$_2$ tension was also measured continuously on-line with an O$_2$ Clark-type electrode. Myocardial O$_2$ consumption (Mvo$_2$) was calculated as (coronary flow/g) $\times$ (arterial Po$_2$ − venous Po$_2$) $\times$ 24 $\mu$l O$_2$/ml at 760 mmHg, and cardiac work efficiency was calculated as developed LVP $\times$ HR/Mvo$_2$.

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

We have published these methods and calibration techniques in detail.[6,9,15–19] In brief, intracellular Ca$^{2+}$ was measured by spectrophotofluorimetry at the LV free wall using a trifurcated fiberoptic cable to direct the excitation and emission wavelengths. Hearts were loaded with the Ca$^{2+}$ sensitive dye indo-1 AM. Indo-1 fluorescence (F) intensity must be corrected for background fluorescence, which itself is primarily an indicator of cellular NADH. The background F intensities, at each wavelength, were determined before indo-1 loading and subsequently subtracted from the F intensities after indo-1 loading to obtain corrected indo-1 intensities. Because ischemia reperfusion alters NADH tissue levels, which affects F changes, autofluorescence, *i.e.*, without indo-1 loading, was measured in six additional hearts using the same cooling, storage, and warming protocols as used for Ca$^{2+}$ determination. The F values recorded were entered into the equations below to calculate nM [Ca$^{2+}$]. The Ca$^{2+}$ transient obtained from the ratio of F$_{385}$ to F$_{456}$, *i.e.*, at wavelengths of 385 and 456 nm, respectively, is nonlinearly proportional to [Ca$^{2+}$]$_i$. Calibration curves were derived using modifications of the standard equation for fluorescent indicators used by Brandes *et al.*[20] Total intracellular ([Ca$^{2+}$]$_{tot}$) was calculated from (a) the total F$_{385}$ to total F$_{456}$ ratio (R$_{tot}$), (b) R$_{max}$ the ratio of light intensities (I) at the same wavelength (S) ratio for minimum and maximum Ca$^{2+}$

where R$_{max}$ = (S$_r$)/bH of total F$_{385}$ as a function of total F$_{456}$ (for > 100 $\mu$M Ca$^{2+}$), (c) R$_{min}$ = [R$_{max}$ $\times$ S$_{385}$/S$_{456}$ (for 0 [Ca$^{2+}$])], (d) S$_{385}$ = [I$_{385}$/I$_{385}$ (at min/max Ca$^{2+}$) = 0.05], (e) S$_{456}$ = [I$_{456}$/I$_{456}$ (at min/max Ca$^{2+}$) = 2.4, and (f) K$_d$ according to the equation:

$$[Ca^{2+}]_{tot} = S_{456} \times K_d[(R_{tot} - R_{min})/R_{max} - R_{tot})]$$

where S = ratio of light intensities at the same wavelength at min and max Ca$^{2+}$, S$_r$ = (1 − S$_{456}$)/(1 − S$_{385}$) = −1.48, and bH = average slope (b) of $_{tot}$F$_{385}$ as a function of $_{tot}$F$_{456}$ = −0.25. R$_{max}$ was calculated as 6.0, and R$_{min}$ as 0.06. K$_d$ was 249 ± 8 nM at 37°C. Noncytosolic fluorescence (mostly mitochondrial) was measured at the end of each experiment (445 min) after perfusing hearts with 100 $\mu$M MnCl$_2$ for 10 min to quench fluorescence derived from the cytosolic compartment; this does not alter the LVP transients. Details of this subtraction method have been published.[6,9,16–19] In the present study, quenching of cytosolic Ca$^{2+}$ transients gave a baseline noncytosolic [Ca$^{2+}$] of 194 ± 5 nM, and cytosolic Ca$^{2+}$ accounted for about 65% of total Ca$^{2+}$ averaged for all groups. [Ca$^{2+}$] data displayed are cytosolic [Ca$^{2+}$] only. The first derivative of [Ca$^{2+}$], d[Ca$^{2+}$]/dt, was derived on-line and maximal and minimal values determined.

Loss of membrane integrity in infarcted cells on reperfusion could result in the leakage of indo-1 and lower signal intensities; however, because this is a ratiometric determination of [Ca$^{2+}$], both F signals degrade similarly so that the F ratio is relatively unchanged. Raw F signals decrease no more than half the post-loaded signal strength or at least fivefold greater than the unloaded signal strength.[6,9,16–19] The dissociation constant K$_d$ is inversely proportional to temperature. Free indo-1 reduces the fluorescence ratio F$_{385}$/F$_{456}$ in a nearly linear fashion by 0.30, 0.23, and 0.16 per 10°C decrease in temperature. K$_d$ increased 28% at 27°C (305 nM), 44% at 17°C (354 nM), and 67% at 7°C (385 nM).[9,17] The linear relationship (y = mx + b) for temperature and K$_d$ was K$_d$ = −4.6°C + 423.8 (r$^2$ = 0.99).

### Measurement of Infarct Size and Creatine Kinase

The 2,3,5-triphenyltetrazolium chloride (TTC) staining technique was used to determine infarct size after 70-min reperfusion. Fresh TTC was prepared daily in 0.1 M of phosphate buffer adjusted to pH 7.4 and warmed to 37°C for 30 min before incubation of hearts. After each experiment, the heart was weighed and transferred to a microtome for slicing into 6 or 7 transverse sections. The sections were immersed in 1% TTC solution and incubated for 25–30 min at 37°C. TTC stained the noninfarcted myocardium a bright red color that is caused by reduction of TTC by dehydrogenases present in viable tissue. Hearts were labeled and stored in 10% formaldehyde for 48 h for later dissection in blind fashion. The

infarcted and noninfarcted tissues of left and right ventricles were carefully dissected and weighed so that percent infarct size was expressed as percentage of total heart weight.[6,21] Average heart weight was $1.5 \pm 0.1$ g after 70-min reperfusion, and there were no significant differences in total heart weight among the groups.

Cumulative effluent creatine kinase (CK) concentration, a marker of cell demise and membrane integrity, was measured (Creatine Kinase Flex™ reagent cartridge, Dade Behring Dimension, Newark, DE; sensitivity > 10 U/l) by collecting the effluent continuously on reperfusion and spot sampling the collected effluent at 15 min. CK release was corrected for heart weight and total effluent volume, and CK release $(U \cdot g^{-1} \cdot min^{-1})$ = CK concentration (U/1,000 ml) $\times$ effluent volume $(ml \cdot g^{-1} \cdot min^{-1})$.

*Protocol*

Thirty hearts were divided randomly and equally among three groups: nontreated hypothermic ischemia controls (CON), sevoflurane exposed (SPC), and SPC + 5-HD. Initial background (before indo-1 loading) measurements were obtained after 30 min of stabilization. Then each heart was loaded with indo-1 AM for 30 min followed by a 20-min washout of residual dye. Hearts in the CON group were perfused with KR solution for another 35 min (from 80 min to 115 min) until cooling, whereas hearts in SPC and SPC + 5-HD groups were perfused with KR solution bubbled with sevoflurane for 15 min (from 85 min to 100 min) and followed by a 15-min washout with normal KR solution before the onset of cooling (fig. 1). Sevoflurane was bubbled into the perfusate with an agent-specific vaporizer placed in the oxygen–carbon dioxide gas mixture line, and its concentration in KR solution was measured by gas chromatography from samples taken anaerobically from the inflow line. Inflow sevoflurane concentration was $0.49 \pm 0.03$ mM at 37°C; this was equivalent to equilibration with $3.5 \pm 0.22\%$ atm and represents a minimal alveolar concentration of approximately $1.50 \pm 0.2$ vol%. 5-HD (200 $\mu$M), a putative selective $mK_{ATP}$ channel inhibitor, was perfused from 5 min (80 min) before giving sevoflurane until 5 min (105 min) after sevoflurane washout (fig. 1). In preliminary experiments (n = 4), 5-HD given before hypothermic storage did not alter any measured functional, metabolic, or structural variable compared with the CON group (data not shown). Each heart was cooled from 37°C to 17°C for 20 min and subjected to 4-h global ischemia at 17°C by a parallel, refrigerated water circulator. At the onset of reperfusion, hearts were rewarmed to 37°C over 10 min by a heated water circulator (fig. 1). Perfusate and bath were maintained at 37°C before and after hypothermic ischemia. After loading and residual washout of indo-1, recordings were obtained every 1–5 min during normothermia, cooling, and rewarming, and once per hour during hypothermic storage. LVP and dLVP/dt, coronary flow, and
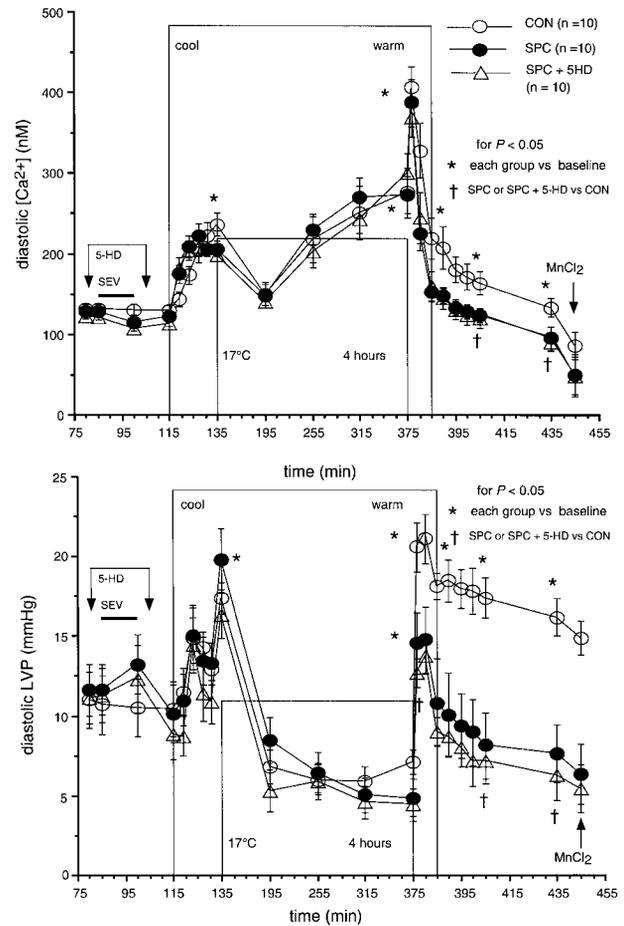


**Fig. 1. Time course of diastolic $[Ca^{2+}]$ and diastolic LVP before, during, and after 4-h ischemia at 17°C in CON, SPC, and SPC + 5-HD groups is shown. Diastolic $[Ca^{2+}]$ increased similarly in each group during cooling and hypothermic ischemia in each group, but was higher during reperfusion in the CON than other groups. Diastolic LVP increased similarly during cooling, but remained markedly elevated throughout reperfusion in the CON group compared with SPC groups.**

coronary sinus oxygen tension ($P_{O_2}$) were measured continuously before and after hypothermic ischemia. $[Ca^{2+}]_i$ and LVP was recorded for every degree change in each temperature from 37°C to 17°C and back to 37°C.

*Statistical Analysis*

All data were expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) for repeated measures (Super Anova 1.11® software for Macintosh® from Abacus Concepts, Inc, Berkeley, CA) was used to assess within-group differences over time at selected time points: 80 min (baseline) *versus* 100 min (sevoflurane at 15 min), 135 min (at 17°C), 375 min (end of 4 h ischemia), and at 377, 385, 405, 435 min (rewarming and reperfusion at 2, 10, 30, and 60 min after cold ischemia). Among-groups data were compared at the same time points as within-group comparison. Two-way ANOVA was used to assess among-group differences at these

**Table 1. Cardiac Effects of 4 h 17°C Ischemia in Control, Sevoflurane Anesthetic Preconditioning, and Sevoflurane Anesthetic Preconditioning Plus 5-Hydroxydecanoate Acid Groups on Systolic $Ca^{2+}$, Systolic Left Ventricular Pressure, Systolic–Diastolic $Ca^{2+}$, and Systolic–Diastolic Left Ventricular Pressure**

| | | | Reperfusion | | | |
|---|---|---|---|---|---|---|
| | Group | Baseline | 2 min | 10 min | 30 min | 60 min |
| Systolic $Ca^{2+}$ (nM) | Control | 300 ± 12 | 485 ± 11* | 325 ± 10 | 335 ± 10 | 330 ± 8 |
| | SPC | 295 ± 10 | 490 ± 12* | 320 ± 14 | 330 ± 11 | 325 ± 10 |
| | SPC + 5-HD | 298 ± 13 | 482 ± 10* | 328 ± 12 | 335 ± 12 | 334 ± 11 |
| Systolic left ventricular pressure (mmHg) | Control | 63 ± 3 | 22 ± 4* | 31 ± 3* | 37 ± 3* | 45 ± 4* |
| | SPC | 62 ± 3 | 24 ± 3* | 32 ± 4* | 39 ± 2* | 47 ± 3* |
| | SPC + 5-HD | 65 ± 4 | 22 ± 3* | 30 ± 3* | 36 ± 4* | 43 ± 4* |
| Systolic–diastolic $Ca^{2+}$ (nM) | Control | 150 ± 12 | 121 ± 11* | 180 ± 12* | 182 ± 13* | 149 ± 12 |
| | SPC | 145 ± 10 | 125 ± 13* | 200 ± 13*† | 210 ± 12*† | 210 ± 12*† |
| | SPC + 5-HD | 147 ± 13 | 119 ± 14* | 182 ± 13* | 183 ± 12* | 165 ± 13 |
| Systolic–diastolic left ventricular pressure (mmHg) | Control | 59 ± 2 | 5 ± 2* | 17 ± 3* | 20 ± 3* | 25 ± 3* |
| | SPC | 58 ± 3 | 6 ± 1* | 28 ± 3*† | 30 ± 4*† | 38 ± 4*† |
| | SPC + 5-HD | 59 ± 3 | 5 ± 2* | 18 ± 3* | 25 ± 3* | 35 ± 3* |

Values are means ± SE; n = 10 for each group. Temperature at 2 min reperfusion was 24 ± 1°C and 37°C at all other time points.

* $P < 0.05$ *versus* baseline within each group.   † $P < 0.05$, sevoflurane anesthetic preconditioning (SPC) *versus* control.

Baseline = perfusion 80 min; 5-HD = 5-hydroxydecanoate acid.

time points. If F values for the analysis of variance were significant, Tukey multiple-comparison *post hoc* test was used to differentiate within- or among-group differences. Differences among means were considered significant when *P* was less than 0.05.

## Results

Table 1 summarizes changes in indices of systolic $[Ca^{2+}]$, systolic LVP, phasic (systolic − diastolic) $[Ca^{2+}]$, and developed LVP in CON, SPC, SPC + 5-HD groups at baseline perfusion, during perfusion of sevoflurane for 15 min, and at 2, 10, and 60 min of reperfusion. Sevoflurane given before hypothermia decreased systolic $[Ca^{2+}]$ and LVP, whereas perfusion of 5-HD did not block the effect of sevoflurane on systolic $[Ca^{2+}]$ and LVP. Systolic $[Ca^{2+}]$ increased and systolic LVP decreased in each group during initial reperfusion, with no significant difference among groups. Systolic $[Ca^{2+}]$ returned to pre-ischemic values, but systolic LVP remained depressed in each group at 60-min reperfusion. Sevoflurane pretreatment also decreased phasic $[Ca^{2+}]$ and developed LVP before cold ischemia. Phasic $[Ca^{2+}]$ and developed LVP decreased in each group compared with baseline values at 2- and 10-min reperfusion. Phasic $[Ca^{2+}]$ and developed LVP were higher in the two sevoflurane-treated groups than that in the CON group, but there were no significant differences between the SPC and SPC + 5-HD groups at 60-min reperfusion.

Figures 1–3 summarize temporal changes in several variables before, during, and after hypothermic ischemia in the CON, SPC, SPC + 5-HD groups. Figure 1 shows that sevoflurane had no effect on diastolic $[Ca^{2+}]$ (fig. 1A) and LVP (fig. 1B) before hypothermia. Diastolic $[Ca^{2+}]$ and LVP gradually increased during cooling, increased abruptly and markedly on initial rewarming, and

then decreased gradually during normothermic reperfusion in each group. Diastolic $[Ca^{2+}]$ and LVP in SPC and SPC + 5-HD groups returned to baseline after 10-min reperfusion, whereas diastolic $[Ca^{2+}]$ and LVP remained elevated throughout reperfusion in the CON group. Figure 2 shows that $d[Ca^{2+}]/dt_{max}$ (fig. 2A) and $dLVP/dt_{max}$ (fig. 2B) were reduced by sevoflurane before cooling; during cooling, $d[Ca^{2+}]/dt_{max}$ increased whereas $dLVP/dt_{max}$ decreased. On reperfusion, $d[Ca^{2+}]/dt_{max}$ and $dLVP/dt_{max}$ were higher in the SPC and SPC + 5-HD groups than in the CON group, although there was no significant differences in these variables between the SPC and SPC + 5-HD groups. Figure 3 shows data for $d[Ca^{2+}]/dt_{min}$ and $dLVP/dt_{min}$. These changes were reversed but qualitatively similar to the findings of figure 2.

Table 2 summarizes changes in indices of coronary flow, heart rate, $Mvo_2$, and cardiac efficiency in CON, SPC, SPC + 5-HD groups before cold ischemia and at 2, 10, 30, and 60 min of reperfusion. Coronary flow was much lower than baseline throughout reperfusion in each group, but it was higher in the SPC group than in the CON group at 10-min reperfusion. Heart rate was slower than baseline during early reperfusion in each group, but it was higher in the SPC and SPC + 5-HD groups than in the CON group at 10-min reperfusion. After that, there were no differences in heart rate among groups. $Mvo_2$ decreased throughout reperfusion in each group, but it was depressed less in the SPC group than in the CON group at 10-min reperfusion. Cardiac efficiency decreased during initial reperfusion in each group, whereas it was higher in the SPC group than in the CON group at 10-min reperfusion. Cardiac efficiency was still lower than baseline in the CON group but not in SPC and SPC + 5-HD groups at 30-min reperfusion, and it returned to baseline in each group at 60-min reperfusion.
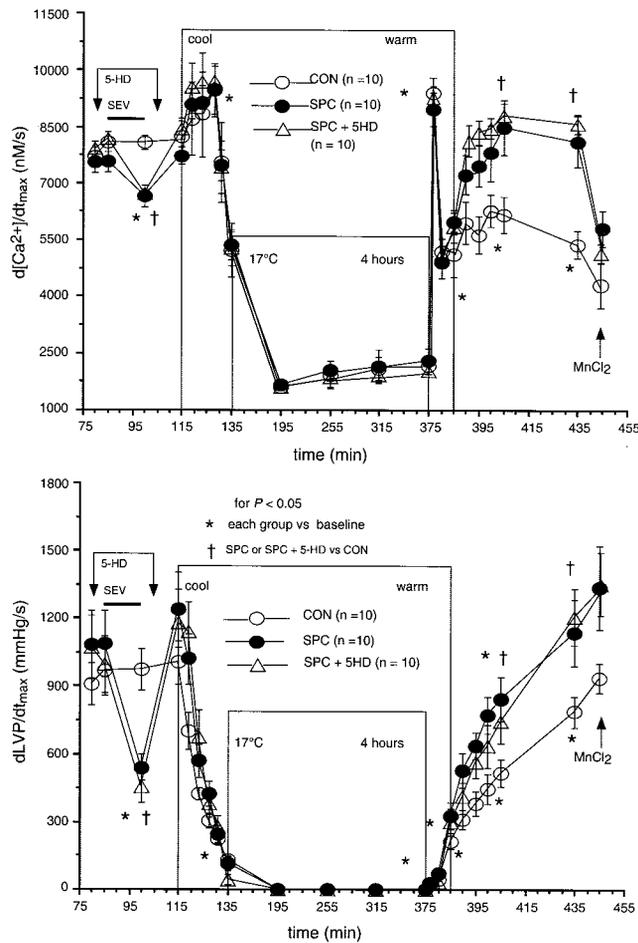
Fig. 2. Time course of $d[Ca^{2+}]/dt_{max}$ and $dLVP/dt_{max}$ before, during, and after 4-h ischemia at 17°C in CON, SPC, and SPC + 5-HD groups. The $d[Ca^{2+}]/dt_{max}$ decreased slightly during exposure to sevoflurane, increased during cooling, and was much lower during hypothermic ischemia in each group. On reperfusion, $d[Ca^{2+}]/dt_{max}$ was greater than baseline in SPC and SPC + 5-HD groups and much greater than in the CON group. The $dLVP/dt_{max}$ decreased during exposure to sevoflurane and was nil during ischemia; on reperfusion, $dLVP/dt_{max}$ gradually increased during reperfusion in each group, but was greater in SPC groups than in the nontreated CON group.
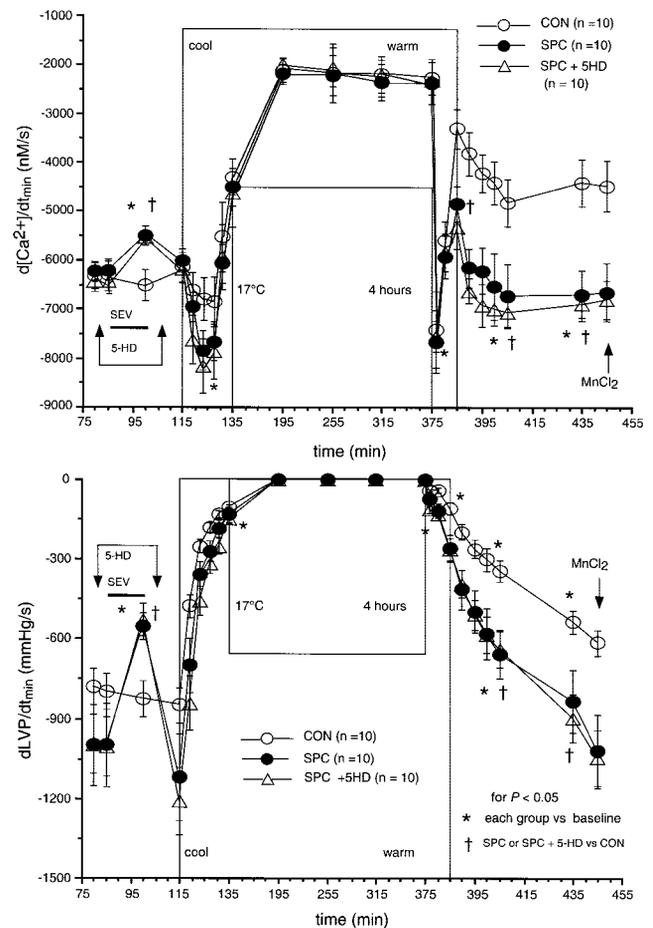


Fig. 3. Time course of $d[Ca^{2+}]/dt_{min}$ and $dLVP/dt_{min}$ before, during, and after 4-h ischemia at 17°C in CON, SPC, and SPC + 5-HD groups. The $d[Ca^{2+}]/dt_{min}$ decreased slightly during exposure to sevoflurane, increased during cooling, and was much lower during hypothermic ischemia in each group. On reperfusion, $d[Ca^{2+}]/dt_{min}$ was greater than baseline in SPC and SPC + 5-HD groups and much greater than in the CON group. The $dLVP/dt_{min}$ decreased during exposure to sevoflurane and was nil during ischemia; on reperfusion, $dLVP/dt_{min}$ gradually increased during reperfusion in each group, but was greater in SPC groups than in the nontreated CON group.

Figure 4 shows that effluent CK release increased in each group on reperfusion, but that it was lower in the SPC group than in CON and SPC + 5-HD groups at 15-min reperfusion. Figure 4 also shows infarct size (% of total heart weight) after 70-min reperfusion. Infarct size in the SPC group was significantly smaller than that in the CON group; moreover, 5-HD reversed the reduction in infarct size provided by SPC.

## Discussion

This study demonstrates for the first time in intact hearts that a 15-min exposure to sevoflurane followed by a 15-min washout period before 4-h global ischemia at 17°C improved cardiac function and perfusion and met-

abolic function compared with no sevoflurane pretreatment. In addition, SPC was noted by a significant reduction in cytosolic $Ca^{2+}$ loading, decreased infarct size, and reduced CK release on reperfusion. Interestingly, the antiinfarct effect and the decrease in CK release after SPC were significantly blocked by 5-HD, a selective $mK_{ATP}$ channel inhibitor, whereas the improvements in mechanical and metabolic function and cytosolic $Ca^{2+}$ handling were not significantly attenuated. This study indicates that APC can be applied to protection against moderately cold ischemia for up to 4 h. A portion of this protection is likely the result of the decrease in cytosolic $Ca^{2+}$ loading associated with APC. But the role of $mK_{ATP}$ channel opening during APC may not be large because blocking these channels had only a small effect on increase of myocardial damage compared with APC alone.

**Table 2. Cardiac Effects of 4 h 17°C Ischemia in Control, Sevoflurane Anesthetic Preconditioning, and Sevoflurane Anesthetic Preconditioning Plus 5-Hydroxydecanoate Acid Groups on Coronary Flow, Heart Rate, Myocardial Oxygen Consumption, and Cardiac Efficiency**

| | | | Reperfusion | | | |
|---|---|---|---|---|---|---|
| | Group | Baseline | RP 2 min | RP 10 min | RP 30 min | RP 60 min |
| Coronary flow (ml · g$^{-1}$ · min$^{-1}$) | Control | 9.3 ± 0.4 | 4.9 ± 0.5* | 6.2 ± 0.4* | 5.0 ± 0.3* | 5.7 ± 0.3* |
| | SPC | 9.6 ± 0.4 | 5.3 ± 0.4* | 7.2 ± 0.3*† | 5.5 ± 0.4* | 6.5 ± 0.4* |
| | SPC + 5-HD | 10.1 ± 0.6 | 4.7 ± 0.5* | 6.9 ± 0.5* | 5.6 ± 0.5* | 6.6 ± 0.5* |
| Heart rate (beats/min) | Control | 241 ± 4 | 9 ± 1* | 147 ± 23* | 233 ± 4 | 244 ± 4 |
| | SPC | 233 ± 6 | 10 ± 1* | 218 ± 8† | 235 ± 8 | 237 ± 7 |
| | SPC + 5-HD | 235 ± 6 | 12 ± 1* | 207 ± 14† | 236 ± 9 | 238 ± 7 |
| MV$O_2$ ($\mu$l · min$^{-1}$ · g$^{-1}$) | Control | 124 ± 4 | 60 ± 6* | 71 ± 4* | 59 ± 3* | 72 ± 5* |
| | SPC | 116 ± 10 | 64 ± 5* | 87 ± 6*† | 66 ± 4* | 83 ± 3*† |
| | SPC + 5-HD | 130 ± 13 | 59 ± 7* | 84 ± 11* | 70 ± 8* | 86 ± 9* |
| Cardiac efficiency | Control | 10.0 ± 0.7 | 0.03 ± 0.01* | 1.4 ± 0.6* | 6.1 ± 0.5* | 9.5 ± 0.5 |
| (mmHg · beat · 0.1 $\mu$l O$_2$ · g$^{-1}$) | SPC | 10.3 ± 1.1 | 0.06 ± 0.02* | 3.5 ± 0.6*† | 9.0 ± 0.9† | 10.7 ± 0.6 |
| | SPC + 5-HD | 8.9 ± 0.8 | 0.06 ± 0.02* | 2.4 ± 0.7* | 8.4 ± 0.5 | 10.7 ± 0.8 |

Values are means ± SE; n = 10 for each group. Temperature at 2 min reperfusion was 24 ± 1°C and 37°C at all other time points.

\* $P$ < 0.05 vs baseline within each group.   † $P$ < 0.05, sevoflurane anesthetic preconditioning (SPC) *versus* control.

Baseline = perfusion 80 min; 5-HD = 5-hydroxydecanoate acid; MV$O_2$ = myocardial oxygen consumption; cardiac efficiency = systolic–diastolic left ventricular pressure · heart rate/MV$O_2$.

### Anesthetic Preconditioning and Hypothermic Ischemia Reperfusion Injury

We reported recently that IPC and APC (seroflurane) before 30-min, 37°C global ischemia of guinea pig isolated hearts significantly improved cardiac function and metabolism and reduced infarct size,[1] and that these protective effects were associated with decreased cytosolic Ca$^{2+}$ loading.[6] In the present study, we showed that SPC can also provide cardioprotection against 4 h of global ischemia at 17°C. In contrast to APC before normothermic ischemia, APC before hypothermic ischemia primarily decreased diastolic Ca$^{2+}$ loading and diastolic LVP and increased dLVP/dt$_{min}$ with little effect on systolic Ca$^{2+}$ loading and LVP. The extent of the APC-

induced reduction in infarct size was smaller after hypothermic ischemia than after normothermic ischemia.[6] Thus, the extent of added protection by APC on long-term hypothermic ischemia is relatively small compared with that of short-term normothermic ischemia. Also, the major beneficial effect of APC on function after hypothermic ischemia was to improve cardiac relaxation in association with reduced diastolic Ca$^{2+}$ loading.

Hypothermia alone is cardioprotective.[9–12,22] We reported that developed LVP recovered to only 38% of the baseline value after 30-min ischemia and 60-min reperfusion at 37°C,[6] whereas in the present study, it returned to more than 50% after 240-min ischemia at 17°C and 60-min reperfusion at 37°C. Because high K$^+$ solutions add to the protection of hypothermia,[9,22] hearts in the present study were perfused only with normal ionic KR solution. IPC was first shown to protect hearts against ischemic reperfusion injury,[23] but IPC is lost when transient ischemia extends over 2 h before the index normothermic ischemia.[24] Although hypothermia extends the cardioprotection of IPC,[25] the degree of protection decreases after the longer period of hypothermic ischemia.[8,14,25] By inference, the protection of APC may wane with the prolonged period of hypothermic ischemia. Diastolic function has been reported to be more sensitive to ischemic damage, so deterioration of diastolic function may occur earlier than systolic dysfunction,[14] which may explain why the primary protective effect of APC in hypothermic ischemia was improved cardiac relaxation function.
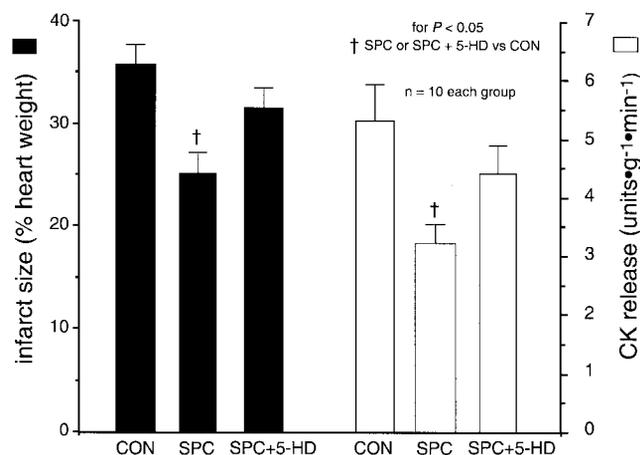


**Fig. 4. Cumulative creatine kinase (CK) release over 15 min of reperfusion after 4-h ischemia at 17°C and infarct size, determined as percent whole heart weight after 70 min of reperfusion, are shown. Anesthetic pretreatment reduced CK release below that of CON, and this effect was partially reversed by 5-HD. Infarct size was smaller after SPC, and this effect was reversed by 5-HD. †P < 0.05, SPC *versus* CON.**

### Anesthetic Preconditioning , Cytosolic [Ca$^{2+}$], and Hypothermic Ischemia Reperfusion Injury

Postischemic reperfusion injury results in large part from cytosolic and mitochondrial Ca$^{2+}$ overload-

ing.[19,26,27] High systolic and diastolic [$Ca^{2+}$] during ischemia and reperfusion at 37°C in intact guinea pig hearts was associated with reduced contractility and impaired relaxation.[6] SPC simultaneously reduced systolic and diastolic $Ca^{2+}$ overloading and improved systolic and diastolic function[6]; similar findings were observed with IPC in the same study. In the present study, we observed that the higher diastolic and lower phasic (systolic–diastolic) [$Ca^{2+}$] after hypothermic ischemia was also associated with depressed cardiac function. Compared with normothermic ischemia, systolic [$Ca^{2+}$] loading occurred only during initial reperfusion and then returned to baseline values during later reperfusion. However, diastolic [$Ca^{2+}$] was markedly increased throughout reperfusion. SPC before hypothermia not only decreased diastolic $Ca^{2+}$ overloading, but it also improved cardiac relaxation. IPC has been shown to significantly reduce diastolic $Ca^{2+}$ loading and to improve diastolic relaxation after 4-h 17°C ischemia.[15] Cardioplegia greatly reduced diastolic $Ca^{2+}$ loading after 4-h 4°C ischemia compared with normal ionic perfusate.[9] Perfusion of paced hearts at 17°C (no ischemia) also caused markedly increased diastolic [$Ca^{2+}$] and impaired relaxation.[17] Taken together, we conclude that diastolic $Ca^{2+}$ loading plays a key role in the development of impaired myocardial relaxation during hypothermic ischemia and normothermic reperfusion and that APC improved cardiac relaxation primary by reducing diastolic $Ca^{2+}$ loading.

Myocardial stunning is characterized by temporary impaired postischemic cardiac function and decreased myofilament $Ca^{2+}$ sensitivity[28]; this decrease may be caused, in part, by altered myofibrillar protein interaction or limited $Ca^{2+}$-activated proteolysis during the early phase of reperfusion.[28,29] Hypothermic perfusion,[17] like normothermic ischemia and reperfusion,[6] decreased myofilament $Ca^{2+}$ sensitivity, whereas IPC and APC before normothermic ischemia afforded an antistunning effect by improving $Ca^{2+}$ sensitization.[6] Increasing $Ca^{2+}$ influx and efflux with positive inotropic drugs can also enhance normal or stunned contractile function.[30,31] In the present study, we showed that the higher $d[Ca^{2+}]/dt_{max}$ (rate of $Ca^{2+}$ influx) and $d[Ca^{2+}]/dt_{min}$ (rate of $Ca^{2+}$ efflux) does not correspondingly transduce into greater $dLVP/dt_{max}$ and $dLVP/dt_{min}$ during rewarming and initial reperfusion because of stunning, *i.e.*, decreased $Ca^{2+}$ responsiveness. After 10-min reperfusion, however, higher $d[Ca^{2+}]/dt_{max}$ and $d[Ca^{2+}]/dt_{min}$ in SPC and SPC + 5-HD groups was associated with improved $dLVP/dt_{max}$ and $dLVP/dt_{min}$. These results suggest that APC improved myocardium contractility and relaxation by better restoring cytosolic $Ca^{2+}$ efflux and influx on reperfusion and that this result was not likely caused by $mK_{ATP}$ channel opening.

L-type $Ca^{2+}$ channel opening triggers a rapid release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) to initiate mechanical contraction.[32] Subsequent activity of $Ca^{2+}$-adenosinetriphosphatase (ATPase) in the SR membrane transports a large fraction of the released $Ca^{2+}$ back into the SR lumen, resulting in a rapid decrease in [$Ca^{2+}$] and muscle relaxation. The resting diastolic [$Ca^{2+}$] is restored *via* SR $Ca^{2+}$ uptake by SR and sarcolemmal $Ca^{2+}$ pumps together with $Ca^{2+}$ extrusion by $Na^{+}$–$Ca^{2+}$ exchange.[32] Hypothermia alone caused cytosolic [$Ca^{2+}$] loading because of dysfunctional SR handling $Ca^{2+}$ in rats.[33] $Na^{+}$, $K^{+}$-ATPase activity of sarcolemmal vesicles was not depressed after 4-h ischemia at 4°C or after 40-min reperfusion at 37°C in rabbit hearts; however, SR $Ca^{2+}$-ATPase activity was markedly lowered after hypothermic ischemia and reperfusion.[34] These results suggested that cytosolic $Ca^{2+}$ loading during hypothermic ischemia and reperfusion resulted primarily from dysfunctional SR $Ca^{2+}$ handling. We observed that hypothermic ischemia alone reduced cellular $Ca^{2+}$ efflux and influx, whereas this was improved with APC. The mechanism for the APC-induced decrease in diastolic $Ca^{2+}$ loading by more rapid myocyte $Ca^{2+}$ handling after hypothermic ischemia is not known, but it does not appear to be initiated by an effect of anesthetic exposure to open $mK_{ATP}$ channels. It is possible, however, that the $K_{ATP}$ channel is an effector of the cardioprotection. Because $Na^{+}$–$H^{+}$ exchange inhibition is cardioprotective,[16,22] it is possible that $K_{ATP}$ channel opening during hypothermic ischemia reperfusion may lead to decreased $Na^{+}$–$H^{+}$ exchange associated with attenuated reverse $Na^{+}$–$Ca^{2+}$ exchange, leading to reduced cytosolic $Ca^{2+}$ loading.

### APC, Mitochondrial $K_{ATP}$ Channel, and Hypothermic Ischemia Reperfusion Injury

5-Hydroxydecanoate, at no greater than 200 $\mu$M, is reported to be a selective $mK_{ATP}$ channel inhibitor with little or no effect on the sarcolemmal (s) $K_{ATP}$ channel.[35,36] It is unclear why the $mK_{ATP}$ channel inhibitor 5-HD reversed the improvement in infarct size provided by APC but did not reverse the improvement in function or [$Ca^{2+}$] loading. Reduced infarct size is not always associated with better functional return during ischemia reperfusion. IPC reduced infarct size but did not improve cardiac function in the isolated mouse hearts after 20-min global ischemia at 37°C.[37] $sK_{ATP}$ and $mK_{ATP}$ channels may have a differential role in reducing infarction and improving cardiac function during ischemia reperfusion. $mK_{ATP}$ channels play an essential role in late preconditioning against infarction but not in late preconditioning against stunning.[35] Reduction of infarct size by adenosine-enhanced IPC was blocked by 5-HD, whereas the better functional return was blocked only by HMR-1883 (a selective $sK_{ATP}$ channel inhibitor) and not by 5-HD or glibenclamide.[36] Selectivity may be species and concentration dependent.

Hypothermia may alter $K_{ATP}$ channel activity. Hypothermia markedly prolonged ventricular action potential

duration, and this effect was reversed by the $K_{ATP}$ channel opener bimakalim.[38] Recently, we showed that 5-HD and glibenclamide blocked the antiinfarction effect but had little effect on inhibiting the improvement of function by IPC after hypothermic ischemia.[15] A better return of function by isoflurane given before normothermic ischemia was partially attenuated by glibenclamide.[39,40] On the other hand, we found that the improved mechanical, metabolic, and vascular endothelial function afforded by isoflurane given during low-flow perfusion for 24 h at 3.8°C was not blocked by glibenclamide perfused throughout the study.[10] Preconditioning by isoflurane, enflurane, halothane,[4] and sevoflurane[6] reduced infarct size in isolated hearts. Further, the antiinfarction effect of isoflurane preconditioning before 30 min of global ischemia at 37°C was blocked by glibenclamide in isolated rabbit hearts.[41] Thus, $K_{ATP}$ channel opening may play a key role in the antiinfarction effect of isoflurane preconditioning. In the present study, we found that the antiinfarction effect of SPC can be shown even after 4 h of global ischemia at 17°C and that this effect was blocked by 5-HD.[21] Thus, unlike the functional effect, the reduced infarction effect appears to be triggered by $mK_{ATP}$ channel opening.

We showed earlier that improved cardiac perfusion and mechanical and metabolic function, provided by SPC after 37°C ischemia, were antagonized by glibenclamide.[1] Exposure of hearts to sevoflurane until the onset of normothermic ischemia enhanced functional return;[18,42,43] protection can be blocked by glibenclamide.[42,43] These data suggest that improved cardiac function initiated by the anesthetic before 37°C ischemia is a result of $K_{ATP}$ channel opening. Two differences between our study and others are that we used only the more selective $mK_{ATP}$ channel blocker and that we used the guinea pig heart model. It remains specifically possible that $sK_{ATP}$ channel openers are also involved. Another important difference is that we only bracketed anesthetic exposure with 5-HD, so that 5-HD was washed out before the cold index global ischemia. Thus, our data do not support $mK_{ATP}$ channel opening as a factor involved in triggering functional improvements as they do for permanent cell damage, but we also cannot rule out $mK_{ATP}$ channel opening as a protective mechanism during cold ischemia and reperfusion.

In conclusion, these results demonstrate that brief anesthetic exposure before cold ischemia at 17°C affords additional cardioprotection provided by hypothermia against ischemia reperfusion injury and that this is associated with decreased cytosolic $[Ca^{2+}]$ loading. The infarct-reducing effect of APC may be the result of, in large part, opening of mitochondrial $K_{ATP}$ channels. This protective effect of even temporary anesthetic exposure may be clinically important because moderate hypothermia is widely used to protect hearts during open-heart surgery.

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