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ORIGINAL PAPER

Sevoflurane and nitrous oxide exert cardioprotective effects against hypoxia-reoxygenation injury in the isolated rat heart

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Abstract It is unclear whether nitrous oxide (N_2O) has a protective effect on cardiac function in vitro. In addition, little is known about the cardioprotective effect of anesthesia administered during hypoxia or ischemia. We therefore studied the cardioprotective effects of N_2O and sevoflurane administered before or during hypoxia in isolated rat hearts. Rat hearts were excised and perfused using the Langendorff technique. For hypoxia-reoxygenation, hearts were made hypoxic (95% N_2, 5% CO_2) for 45 min and then reoxygenated (95% O_2, 5% CO_2) for 40 min (control: CT group). Preconditioning was achieved through three cycles of application of 4% sevoflurane (sevo-pre group) or 50% N_2O (N_2O-pre group) for 5 min with 5-min washouts in between. Hypoxic conditions were achieved by administering the 4% sevoflurane (sevo-hypo group) or 50% N_2O (N_2O-hypo group) during the 45-min hypoxic period. L-type calcium channel currents ($I_{Ca,L}$) were recorded on rabbit myocytes. (1) Both 4% sevoflurane and 50% N_2O significantly reduced left ventricular developed pressure (LVDP). Sevoflurane also increased left ventricular end-diastolic pressure, though N_2O did not. (2) The recoveries of LVDP and pressure-rate product (PRP) after hypoxia-reoxygenation were better in the sevo-pre group than in the CT or N_2O-pre group. (3) Application of either sevoflurane or N_2O during hypoxia improved recovery of LVDP and PRP, and GOT release was significantly lower than in the CT group. (4) Sevoflurane and N_2O reduced $I_{Ca,L}$ to similar extents. Although sevoflurane administered before or during hypoxia exerts a cardioprotective effect, while N_2O shows a cardioprotective effect only when administered during hypoxia.

Keywords Cardiac function · Cardioprotection · Hypoxia

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Introduction

It is now well established that preconditioning with volatile anesthetics protects the heart against ischemia and reperfusion injury [1–7]. Comparatively little is known about the effects of anesthetic preconditioning (APC) with nitrous oxide (N_2O), though it has been shown that brief, repetitive administrations of 60% N_2O before prolonged coronary occlusion and reperfusion does not protect rat myocardium in vivo [8]. Little is also known about cardioprotective effect of anesthetic, including sevoflurane and N_2O, administered during hypoxia or ischemia. Our aim, therefore, was to determine whether preconditioning with sevoflurane or N_2O, or their administration during hypoxia, might exert a cardioprotective effect against hypoxia-reperfusion injury in the isolated rat heart.

Materials and methods

This investigation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institute of Health publication, DHEW publication No. (NIH) 85-23, 1996) and was approved by the Juntendo University School of Medicine (Tokyo, Japan) animal experimentation committee.

Langendorff perfusion

Male Sprague-Dawley (SD) rats weighing 280–340 g were used. After anesthetizing the rats with diethyl ether, they were decapitated and their hearts were quickly excised for Langendorff perfusion. Each heart was perfused with modified Krebs–Henseleit solution (containing in mM: NaCl 116.0, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.0, glucose 5.5; pH 7.4) in a retrograde direction at a constant flow rate of 13 ml min^{-1} without recirculation. The perfusate was warmed to 38°C and oxygenated with a 95% O_2–5% CO_2 gas mixture to maintain the partial pressure of O_2 above 400 mmHg. During the period of hypoxia, glucose in the perfusate was replaced to adjust osmotic pressure with equimolar sucrose, and the solution was saturated with a 95% N_2–5% CO_2 gas mixture to maintain the partial pressure of O_2 at approximately 20 mmHg (hypoxic solution). In addition, a latex balloon was inserted through the mitral annulus into the left ventricular cavity, and distilled water (0.1–0.2 ml) was injected into the balloon until it was inflated to just above the level required to produce a visible (1–2 mmHg) elevation of the left ventricular end-diastolic pressure (LVEDP). Left ventricular developed pressure (LVDP), LVEDP, heart rate (HR) and coronary perfusion pressure were monitored throughout the experiments. The extent of irreversible myocardial damage was assessed by measuring the amount of glutamic oxaloacetic transaminase (GOT) released into the coronary effluent. GOT activities in 10-μl aliquots of coronary effluent were estimated using a dry chemical method with a commercially available kit (Fuji Film Ltd. Co., Tokyo, Japan). The released GOT activity was normalized to IU g^{-1} (dry tissue) min^{-1}.

Experimental protocols

Effects of 4% sevoflurane or 50% N_2O on the function of rat hearts (Fig. 1a)

After stabilization for 20 min, hearts were perfused for 45 min with Krebs solution equilibrated with 95% O_2 plus 5% CO_2 in the time-matched control (TC; $n = 6$) group. In the N_2O ($n = 5$), sevoflurane (sevo; $n = 6$) and N_2 ($n = 6$) groups, hearts were, respectively, perfused for 30 min with solutions equilibrated with N_2O (50% N_2O, 45% O_2, 5% CO_2), sevoflurane (4% sevo, 91% O_2, 5% CO_2), or N_2 (50% N_2, 45% O_2, 5% CO_2), and then with normal Krebs solution for 15 min. Sevoflurane was vaporized using a vaporizer (Sevotec 3, Ohmeda, West Yorkshire, UK) and then mixed into 95% O_2, 5% CO_2, subsequently bubbled through the bathing solution. The volume percentage of sevoflurane in the gas phase above the Krebs–Henseleit solution was continuously monitored using an anesthetic gas analyzer (Capnomac Ultima, Datax, Helsinki, Finland).

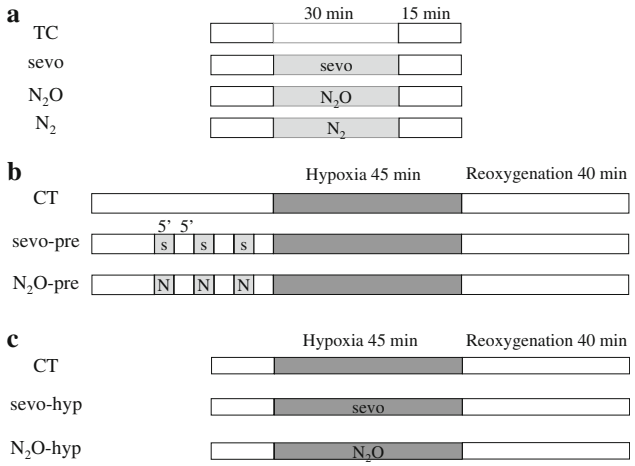

Fig. 1 Experimental protocols. **a** Experimental protocol for testing the effect of sevoflurane, N_2O and N_2 on cardiac function. **b** Experimental protocol for preconditioning with 4% sevoflurane or 50% N_2O. **c** Experimental protocol for treatment with 4% sevoflurane or 50% N_2O during hypoxia. *TC* time-matched control, *CT* control, *S* sevoflurane, *N* N_2O

Effects of preconditioning with 4% sevoflurane or 50% N_2O (Fig. 1b)

After stabilization for 20 min, hearts in the control group (CT; $n = 7$) were subjected to hypoxia for 45 min and reoxygenated for 40 min. In the sevoflurane-preconditioning (sevo-pre; $n = 7$) and N_2O-preconditioning (N_2O-pre; $n = 6$) groups, three cycles of application of sevoflurane (4% sevo, 91% O_2, 5% CO_2) or N_2O (50% N_2O, 45% N_2, 5% CO_2) for 5 min with 5-min washouts in between the anesthetics application were followed by hypoxia-reoxygenation.

Effects of 4% sevoflurane or 50% N_2O administered during hypoxia (Fig. 1c)

After stabilization for 20 min, hearts in the sevoflurane-hypoxia (sevo-hypo; $n = 6$) and N_2O-hypoxia (N_2O-hypo; $n = 7$) groups were perfused for 45 min with Krebs solution (sucrose), respectively, equilibrated with sevoflurane (4% sevo, 95% N_2, 5% CO_2) or N_2O (50% N_2O, 45% N_2, 5% CO_2), respectively, and then reoxygenated for 40 min.

We calculated the pressure-rate product (PRP = LVDP × HR) as an index of myocardial oxygen consumption. PRP values were expressed as percentages of the baseline value (the value at the end of stabilization expressed as *N* in Fig. 4).

L-type Ca^{2+} ($I_{Ca,L}$) current recording

For electrophysiological experiments, ventricular myocytes were enzymatically isolated from the hearts of Japanese white rabbits (1.6 kg). After anesthetizing the rabbits by injection of pentobarbital sodium (50 mg kg^{-1}) into the auricular vein, the hearts were excised, prepared for Langendorff perfusion, and then sequentially perfused at 37°C with normal Tyrode solution (in mM: NaCl 135, KCl 5.4, $CaCl_2$ 1.8, HEPES 10, glucose 10, pH 7.4) for 5 min, Ca^{2+}-free Tyrode for 5 min, collagenase (0.6 mg ml^{-1}); Type I, Sigma, Chemical Company; St Louis, MO, USA) solution for 30 min and Kraft–Brühe (KB; in mM: taurine 10, oxalic acid 10, glutamic acid 70, KCl 25, KH_2PO_4 10, EGTA–Tris 0.5, HEPES 5, glucose 10, pH 7.4) for 5 min. The digested ventricles were cut into small pieces with scissors and gently shaken in a warmed water bath for 1 min. The tissue fragments were then passed through a filter (mesh: 100 μm) into a beaker containing KB solution. Isolated cells were stored in normal Tyrode solution at room temperature (22–25°C) prior to experimentation.

Whole cell patch-clamp recordings were made in normal Tyrode solution at room temperature. Cells were gently dispersed onto a cover glass fixed at the bottom of an experimental chamber. Once a giga-seal was made, the whole cell configuration was established by applying negative pressure to rupture the patch membrane. The myocytes were then allowed to equilibrate with the pipette solution for about 10 min. To record $I_{Ca,L}$, normal Tyrode solution was replaced with Cs^+ solution (in mM: NaCl 135, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4). After obtaining baseline recordings, solution bubbled with 50% N_2O plus 50% O_2 ($n = 6$) or 4 vol% sevoflurane ($n = 8$) plus air was perfused for >20 min.

Membrane $I_{Ca,L}$ were recorded in the whole cell patch-clamp configuration using an Axopatch 1-D or Axopatch 200B patch-clamp amplifier (MDS Analytical Technologies, Toronto, ON, Canada). Analysis of the recorded data was carried out using pClamp 9.2 (Axon Instruments Inc., USA). Patch pipettes were pulled from plain hematocrit capillary tubes made of soda lime glass (Chase Instruments, NY, USA), coated with Sylgard and heat-polished under a microscope; the resultant electrode tip resistances ranged from 2 to 5 MΩ when filled with the pipette solution (in mM: CsCl 120, $CaCl_2$ 1, TEA–Cl 20, EGTA–CsOH 11, HEPES 10, Mg–ATP 5, pH 7.3). $I_{Ca,L}$ were evoked by applying 300-ms depolarizing pulses in 10-mV increments from −40 to +50 mV or from −20 to +20 mV after 55-ms prepulses to −40 mV that were imposed after a 20-ms ramp pulse from a holding potential of −80 mV.

Statistical analysis

Data are presented as means ± SEM. Differences between means were analyzed using Student's *t* test or ANOVA and the Bonferroni method, as deemed appropriate. Values of $P < 0.05$ were considered significant.

Results

Effects of 4% sevoflurane or 50% N_2O on cardiac function

As a preliminary experiment, we examined the effects of 4% sevoflurane or 50% N_2O on cardiac function to determine equivalency in cardiac depression. Perfusion of hearts with 4% sevoflurane or 50% N_2O for 30 min significantly ($P < 0.001$ vs. time-matched control; TC) reduced LVDP to 64.9 ± 3.1% and 57.2 ± 3.6% of baseline, respectively (Fig. 2a), and significantly ($P < 0.001$ vs. TC) reduced PRP to 47.4 ± 5.5% and 53.3 ± 5.0% of baseline, respectively (Fig. 2c). On the other hand, 4% sevoflurane increased LVEDP significantly from 2.0 ± 0.5 to 5.2 ± 0.8 mm Hg, though 50% N_2O did not (Fig. 2b). Perfusion with 50% N_2 for 30 min reduced LVDP to 76.0 ± 4.6% (not significant vs. TC) and reduced PRP to 65.9 ± 3.6% of baseline ($P < 0.001$ vs. TC), which were significantly higher than those of hearts perfused with 50% N_2O. 50% N_2 did not increase LVEDP.

Effects of preconditioning with 4% sevoflurane or 50% N_2O

Perfusion with hypoxic solution rapidly reduced PRP during the first 5 min. This was followed a continued gradual decline until, after 45 min of hypoxia, and PRP reached 4.8 ± 1.7% of baseline in the CT group, 8.2 ± 0.3% in the sevo-pre group, and 5.6 ± 3.7% in the N_2O-pre group. There was no significant difference among these three groups (Fig. 3b). After 40 min of reoxygenation, LVDP recovered to 60.1 ± 3.8% in the CT group, 77.0 ± 4.3% in the sevo-pre group, and 66.7 ± 6.4% in the N_2O-pre group. PRP recovered to 54.4 ± 2.6% of baseline in the CT group, 68.2 ± 4.5% in the sevo-pre group, and 58.3 ± 6.3% in the N_2O-pre group. The recoveries of LVDP and PRP in the sevo-pre group, but not N_2O-pre group, were significantly ($P < 0.05$) greater than those in the CT group (Fig. 3a, b). There were no significant differences in HR among the three groups (data not shown).

GOT release remained near baseline during the 45 min hypoxic period, but it increased rapidly during the first 5 min of reoxygenation and then declined gradually in all

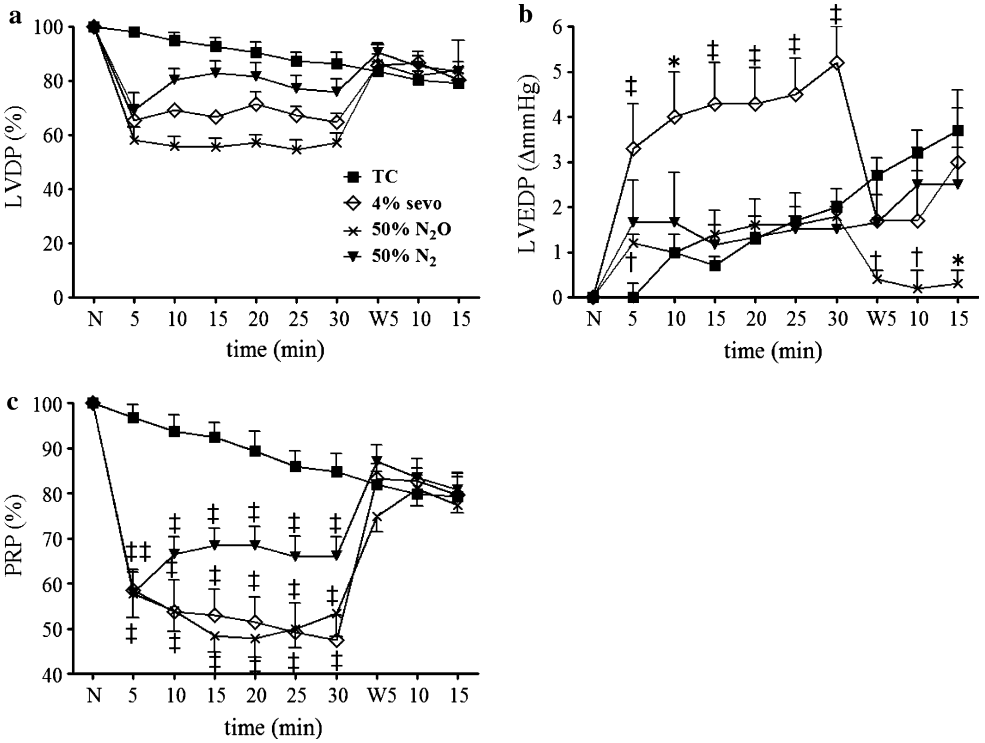

Fig. 2 Time course of the changes in pressure-rate product (*PRP*), left ventricular developed pressure (*LVDP*) and left ventricular end-diastolic pressure (*LVEDP*) during and after exposure to 4% sevoflurane or 50% N_2O for 30 min. **a** Sevoflurane and N_2O reduced PRP to similar degrees. **b** Sevoflurane and N_2O reduced LVDP. **c** Sevoflurane increased LVEDP, though N_2O did not. *TC* time-matched control, *N* normal, *W* washout. $*P < 0.05$ versus TC; $\dagger P < 0.01$ versus TC; $\ddagger P < 0.001$ versus TC

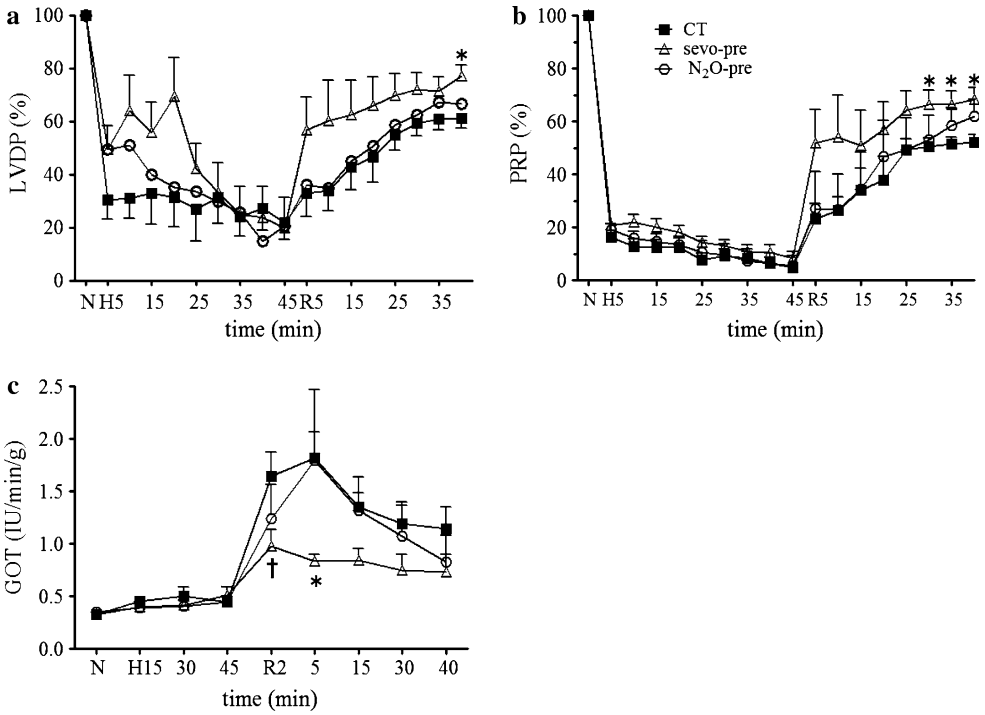

Fig. 3 Time course of the changes in PRP and glutamic oxaloacetic transaminase (*GOT*) release during hypoxia (H)-reoxygenation (R) after preconditioning with 4% sevoflurane or 50% N_2O. **a**, **b** In the sevo-pre group, LVDP and PRP recovered significantly better than in the CT group. In the N_2O-pre group, however, recoveries of LVDP and PRP were not different from CT. **c** The amount of GOT released was significantly lower in sevo-pre group than in the N_2O-pre group. *N* normal, *H* hypoxia, *R* reoxygenation. $*P < 0.05$ versus CT; $\dagger P < 0.01$ versus CT

groups. After the first 2 min of reoxygenation, the amount of GOT released was significantly lower in the sevo-pre group (0.8 ± 0.1, $P < 0.01$) than in the CT group (1.8 ± 0.3). By contrast, preconditioning with N_2O (1.8 ± 0.7 IU g^{-1} min^{-1}) did not reduce the amount of GOT released (Fig. 3c).

Effects of 4% sevoflurane or 50% N_2O administered during hypoxia

When 4% sevoflurane or 50% N_2O was administered during the hypoxic period, the recoveries of LVDP and PRP upon reoxygenation in the N_2O-hypo and sevo-hypo

groups were facilitated in comparison to those in the CT group. After 20 min of reoxygenation, LVDP had recovered to 32.3 ± 9.3% in the CT group, 56.7 ± 7.9% in the sevo-hypo group ($P < 0.05$ vs. CT), and 50.6 ± 8.6% in the N_2O-hypo group ($P < 0.05$ vs. CT) (Fig. 4a). PRP had recovered to 41.8 ± 7.1% of baseline ($P < 0.05$ vs. CT) in N_2O-hypo group and 58.0 ± 5.0% ($P < 0.001$ vs. CT) in sevo-hypo group, whereas it remained at 22.4 ± 5.3% in the CT group (Fig. 4b). In addition, after the first 2 min of reoxygenation, the amounts of GOT released in the N_2O-hypo (1.5 ± 0.3, $P < 0.05$) and sevo-hypo (1.2 ± 0.2, $P < 0.001$) groups were significantly lower than in the CT group (2.6 ± 0.2 IU g^{-1} min^{-1}) (Fig. 4c). There were no significant differences in HR among the three groups (data not shown).

Effects of 50% N_2O or 4% sevoflurane on $I_{Ca,L}$

Sevoflurane and N_2O administered during hypoxia protected heart from injury induced by hypoxia-reoxygenation. These effects may occur through the inhibitory actions of the anesthetics on $I_{Ca,L}$, and reductions in cytosolic and mitochondrial Ca^{2+} overload. Therefore, we examined the effects of N_2O or sevoflurane on $I_{Ca,L}$. Isolated rabbit cardiomyocytes were superfused with 50% N_2O or 4% sevoflurane beginning about 8 min after initiation of the whole cell patch clamp, once the basal $I_{Ca,L}$ had stabilized. Fifty percent N_2O reduced $I_{Ca,L}$ from 5.2 ± 0.3 to 3.8 ± 0.2 pA pF^{-1} ($P < 0.001$), while 4% sevoflurane reduced $I_{Ca,L}$ from 7.2 ± 0.6 to 5.8 ± 0.4 pA pF^{-1} ($P < 0.05$) (Fig. 5B). Normalizing effects to the CT group revealed that 50% N_2O and 4% sevoflurane significantly decreased $I_{Ca,L}$ by 18.7 ± 2.3 and 26.3 ± 1.5%, respectively ($P < 0.001$), as compared to control (Fig. 5C). There was no significant difference in the reductions in $I_{Ca,L}$ elicited by N_2O and sevoflurane.

Discussion

In this study, preconditioning with sevoflurane, but not N_2O, improved recoveries of LVDP and PRP, and reduced GOT release during reperfusion following hypoxia, suggesting sevoflurane, but not N_2O, exerts a protective effect against reoxygenation-induced injury (Fig. 3). In addition, application of either N_2O or sevoflurane during hypoxia suppressed GOT release and facilitated recoveries of LVDP and PRP, but the levels of recoveries at 35–40 min reoxygenation were not different from those of the control (Fig. 4). The increase in GOT release at the early phase of reperfusion or reoxygenation indicates the development of reperfusion injury on oxygen paradox. Thus, these anesthetics during hypoxia can protect heart from reperfusion-on reoxygenation-induced injury.

Both 50% N_2O and 4% sevoflurane reversibly reduced PRP to a similar degree under normoxic conditions, and there was a corresponding reduction in $I_{Ca,L}$. Although the contractile depression induced by 50% N_2O could be due, in part, to a reduction in O_2, the contractile depression was significantly larger than that in the 50% N_2 group

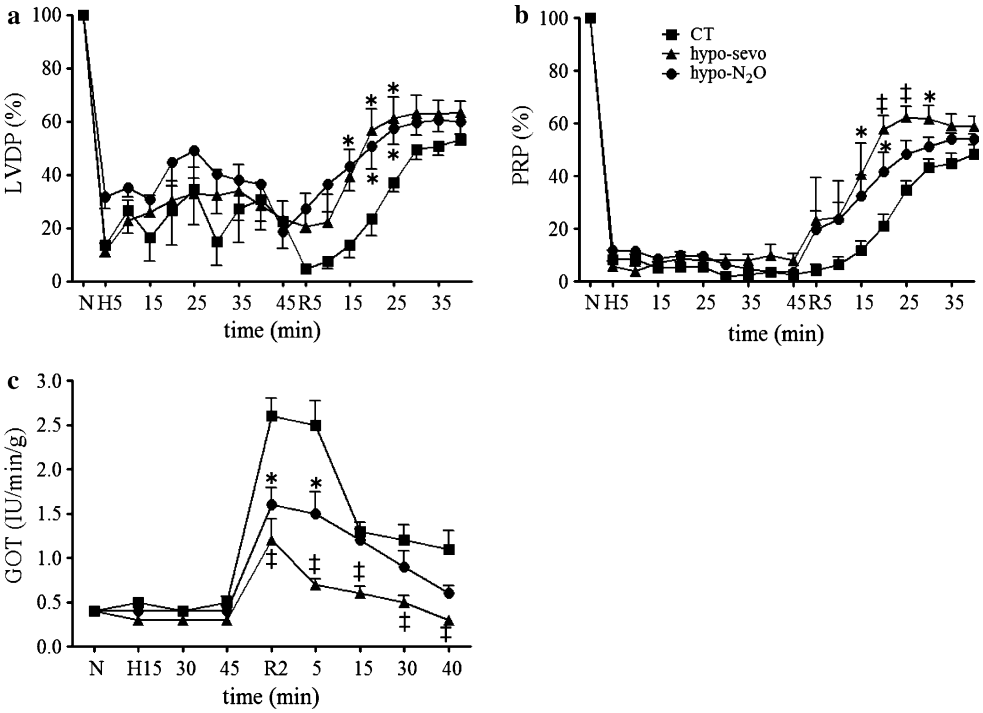

Fig. 4 Time course of the changes in PRP and GOT release during hypoxia (H)-reoxygenation (R) after treatment with 4% sevoflurane or 50% N_2O during the hypoxic period. **a**, **b** The recoveries of LVDP and PRP were faster in the N_2O-hypo and sevo-hypo groups than in the CT group. **c** The amounts of GOT released were significantly lower in N_2O-hypo and sevo-hypo groups than in the CT group. *N* normal, *H* hypoxia, *R* reoxygenation. *$P < 0.05$ versus CT; ‡$P < 0.001$ versus CT

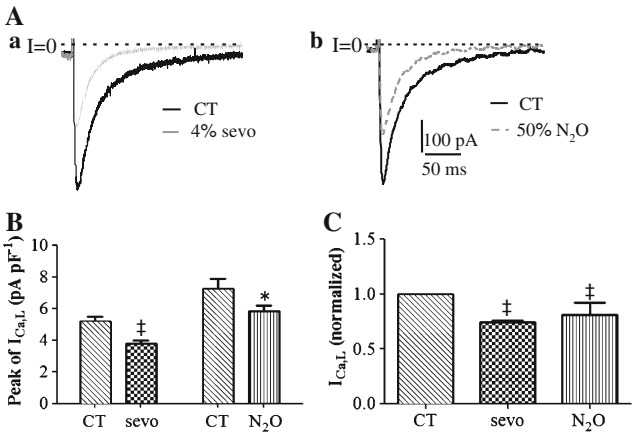

Fig. 5 Effects of 4% sevoflurane or 50% N_2O on L-type calcium channel currents ($I_{Ca,L}$). **A** Recordings showing the typical effects of sevoflurane (*a*) or N_2O (*b*) on peak amplitude of $I_{Ca,L}$. **B** Group data showing the effects of N_2O or sevoflurane of peak of $I_{Ca,L}$. **C** Ratio of sevoflurane- and N_2O-induced reductions in $I_{Ca,L}$. $^*P < 0.05$ versus CT; ‡$P < 0.001$ versus CT

(Fig. 2a, c). Sevoflurane also increased LVEDP significantly, suggesting it diminishes ventricular dilatation. By contrast, N_2O did not impair cardiac dilatation. The failure of cardiac preconditioning with N_2O to exert a cardioprotective effect may be due to that volatile anesthetics induce a change in metabolic state of the heart. For example, several volatile anesthetics have been shown to increase levels of NADH, an index of mitochondrial electron transport [9–11]. Thus, the increase in NADH suggests attenuated mitochondrial electron transport [9]. Anesthetic-induced inhibition of mitochondrial electron transport could lead to increased O_2^- levels and downstream reactions, which could trigger APC [9]. Moreover, isoflurane and sevoflurane both inhibit mitochondrial electron transport [12], whereas N_2O does not inhibit mitochondrial electron transport or ATP formation [13, 14]. Many investigators have shown that protein kinase C (PKC), ATP-sensitive mitochondrial and sarcolemmal potassium (mito$K^+_{ATP}$and sarc K^+_{ATP}) channels, and ROS are related to the signal transduction of APC [15–19]. Furthermore, myocardial protection by sevoflurane could also be related to its anti-inflammatory effect [20].

In the present study, administration of either N_2O or sevoflurane during hypoxia protected heart from injury induced by hypoxia-reoxygenation. This is consistent with an earlier report that desflurane administered during ischemia attenuates myocardial stunning in dogs [21] and reduces myocardial infarct size in adult rats [22], but there are no similar reports on the effects of sevoflurane or N_2O administered during hypoxia or ischemia. Our study suggests that N_2O protects the heart when administered during the hypoxia in vitro, although the use of this anesthetic gas in patients with ischemic heart disease remains controversial [23].

Volatile anesthetics reportedly depress Ca^{2+} currents (I_{Ca}) in atrial and ventricular myocytes [24, 25]. Our finding that 50% N_2O or 4% sevoflurane reduces $I_{Ca,L}$ in isolated rabbit ventricular myocytes (Fig. 5) is consistent with those earlier reports. This suggests that N_2O and sevoflurane may mitigate Ca^{2+} overload in hypoxic hearts by inhibiting Ca^{2+}-influx through L-type Ca^{2+} channels. Consistent with that idea, carbon monoxide and the L-type Ca^{2+} channel blockers nifedipine and diltiazem each alleviate Ca^{2+} overloading and cell death in ischemic H9c2 cells [26]. Sevoflurane has been shown to suppress sarcoplasmic reticulum Ca^{2+} release and depress myofilament Ca^{2+} sensitivity [27]. Administration of sevoflurane after ischemia also reduced cytosolic Ca^{2+} and myocardial damages [28]. Therefore, sevoflurane-induced protection may also occur through modulations of L-type Ca^{2+} channels and sarcoplasmic reticulum to reduce Ca^{2+} overload [29, 30].

Several limitations of this study should be noted. Firstly, we did not identify the area of necrosis or apoptosis. Secondly, the sevoflurane concentration used in this study was 4 vol%, other concentrations may have greater or lesser effects. Thirdly, further investigation is needed to identify essential components in these complex signal transduction cascades that mediate APC in our study.

In summary, our findings suggest that preconditioning with 4% sevoflurane, but not 50% N_2O, exerts a protective effect on the myocardium against hypoxia-reoxygenation injury, while administration of either 4% sevoflurane or 50% N_2O during hypoxia is cardioprotective. Both sevoflurane and N_2O reduced $I_{Ca,L}$. These results suggest that administration of 4% sevoflurane or 50% N_2O during hypoxia prevents Ca^{2+} overload, resulting in a protective effect. On the other hand, the absence of metabolic inhibition means that preconditioning with N_2O will not have a protective effect.

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