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Adenosine and a Nitric Oxide Donor Enhances Cardioprotection by Preconditioning with Isoflurane through Mitochondrial Adenosine Triphosphate–sensitive K^+ Channel–dependent and –independent Mechanisms

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Background: **Preconditioning with isoflurane has been shown to confer cardioprotection *via* activation of mitochondrial adenosine triphosphate–sensitive K^+ (mito K_{ATP}) channels. However, the relative contribution of mito K_{ATP} channel and non–mito K_{ATP} channel mechanisms to isoflurane-mediated cardioprotection has not been investigated.**

Methods: **Isolated and buffer-perfused rat hearts were used. Flavoprotein fluorescence was monitored as an index for mito K_{ATP} channel activity. Isovolumic left ventricular function and infarct size were measured as indices for cardioprotection.**

Results: **Flavoprotein fluorescence, which was monitored as an index for mito K_{ATP} channel activity, was increased by isoflurane and a known mito K_{ATP} channel opener, diazoxide, in a 5-hydroxydecanoate–sensitive manner. Although flavoprotein oxidation induced by diazoxide was dissipated soon after its removal from the buffer, flavoprotein oxidation induced by isoflurane was sustained after cessation of the treatment. The sustained increase in flavoprotein oxidation was associated with a significant reduction in infarct size after 30 min of ischemia followed by 120 min of reperfusion. Although adenosine and S-nitroso-*N*-acetyl-penicillamine each alone did not increase flavoprotein fluorescence, nor did they confer significant cardioprotection, coadministration of adenosine and S-nitroso-*N*-acetyl-penicillamine with isoflurane conferred a highly significant reduction of infarct size and improvement of left ventricular function without increasing flavoprotein oxidation over isoflurane alone. The early treatment with 5-hydroxydecanoate before and during preconditioning completely reversed flavoprotein oxidation and inhibited the infarct-sparing effect of isoflurane and combined preconditioning with isoflurane, adenosine, and S-nitroso-*N*-acetyl-penicillamine. The late treatment with 5-hydroxydecanoate after preconditioning abolished flavoprotein oxidation and the infarct-sparing effect of isoflurane but only partially inhibited cardioprotection conferred by the combined preconditioning, despite complete abrogation of flavoprotein oxidation.**

Conclusions: **Mito K_{ATP} channel activation is the essential trigger of both preconditioning with isoflurane and combined preconditioning with isoflurane, adenosine, and S-nitroso-*N*-acetyl-penicillamine. Mito K_{ATP} channel activation is also a crucial mediator of cardioprotection afforded by preconditioning with isoflurane. However, enhanced cardioprotection conferred by combined preconditioning is mediated through both mito K_{ATP} channel–dependent and –independent mechanisms.**

BRIEF periods of cardiac ischemia and reperfusion exert a protective effect against subsequent, more prolonged period of ischemia, a phenomenon termed *ischemic preconditioning* (IPC).[1] IPC-like effects have also been observed by administration of various pharmacologic agents. Volatile anesthetics, including isoflurane, have long been known as such cardioprotective agents against ischemia–reperfusion injury, and this cardioprotective effect has at least in part been attributed to opening of mitochondrial adenosine triphosphate–sensitive K^+ (mito K_{ATP}) channels.[2–8] Despite accumulating evidence showing cardioprotection afforded by isoflurane, the exact role of mito K_{ATP} channels in isoflurane-induced cardioprotection remains to be investigated.

Pharmacologic preconditioning (PPC) has been performed by using IPC mimetic drugs, such as adenosine, a G protein–coupled receptor agonist (GPCRA)[9,10]; diazoxide, a mito K_{ATP} channel opener[11]; and nitric oxide donors.[12,13] However, acute cardioprotection by single PPC with adenosine, diazoxide, or nitric oxide donors remains a controversial issue. There are a number of studies showing that PPC with each drug alone confers significant cardioprotection,[11–15] but also, a considerable number of studies have failed to substantiate acute cardioprotection by PPC with single-drug administration.[16–18] It is not surprising that a single PPC strategy failed to completely mimic IPC because IPC is mediated by complex signaling cascades arising from multiple triggers. Ninomiya *et al.*[19] have demonstrated that PPC with adenosine had no acute benefit in functional protection after global ischemia, whereas administration of adenosine during IPC was capable of significantly enhancing IPC-induced protection. This observation suggests that adenosine enhancement of IPC-induced cardioprotection is elicited by robust activation of G protein–coupled receptors in concert with other, yet unidentified triggers produced by preconditioning ischemia and reperfusion. Our subsequent studies[20,21] have shown that adenosine in combination with a mito K_{ATP} channel opener and a nitric oxide donor is necessary to completely mimic IPC, and pretreatment with a Gi/o protein inhibitor, pertussis toxin, a mito K_{ATP} channel inhibitor, 5-hydroxydecanoate (5-HD), or a nitric oxide

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scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, completely abrogated cardioprotection afforded by combined preconditioning. This observation has led us to hypothesize that GPCRA and nitric oxide are the likely cotriggers of successful preconditioning with a mito K_{ATP} channel opener. Requirement of a GPCRA and a nitric oxide donor may also be the case for isoflurane to confer optimal cardioprotection. Patel *et al.*[22] have demonstrated that a known GPCRA, δ opioid, enhances an infarct-sparing effect of isoflurane. Moreover, the fact that functional protection mediated by the nitric oxide donor nicorandil[23] was enhanced by isoflurane, which by itself exerted no functional benefit,[24] points to the conclusion that isoflurane requires coadministration with a nitric oxide donor to mediate functional protection. Therefore, we attempted to address the issue of whether coadministration with a GPCRA and a nitric oxide donor potentiates cardioprotection by preconditioning with isoflurane and whether such enhanced cardioprotection occurs solely through activation of mito K_{ATP} channels or also through mito K_{ATP} channel–independent mechanisms.

Materials and Methods

Perfusion Technique and Flavoprotein Fluorescence Measurement

Male Sprague-Dawley rats weighing 250–300 g were used in the current study. All experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals*[25] and were approved by Animal Care Committee of Kansai Medical University (Moriguchi, Japan). The rats were anesthetized intraperitoneally with pentobarbital sodium (100 mg/kg). After thoracotomy, the heart was rapidly excised, placed in a temperature-regulated heart chamber installed in a CAF-110 intracellular ion analyzer (Japan Spectroscopic Co., Tokyo, Japan), and perfused at a constant mean pressure of 60–70 mmHg with Krebs-Henseleit bicarbonate buffer solution with the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 11 mM glucose, with a pH of 7.4 at 37°C when equilibrated with a mixture of 95% O_2 and 5% CO_2 gas.

During the stabilization period, a latex balloon was inserted into the left ventricle through the left atrium to measure isovolumic left ventricular (LV) function. The balloon was filled with saline solution to produce an LV end-diastolic pressure of 5–10 mmHg at baseline, and the balloon volume was kept constant throughout the experiment. The hearts with LV developed pressure less than 80 mmHg or heart rate less than 240 beats/min at baseline were excluded from the study. LV developed pressure and heart rate were expressed as percentage of the baseline values.

Flavoprotein fluorescence of the heart was monitored as an index for mito K_{ATP} channel activity as described previously.[20,26] Briefly, fluorescence excitation was provided by a xenon lamp with a band-pass filter centered at 480 nm. The excitation light was diverted onto a circular region of the LV epicardium with a diameter of 1 cm by means of a dichroic mirror. Fluorescence then passed through an emission filter centered at 530 nm before reaching a photomultiplier. The photomultiplier output was digitized, and the fluorescence was displayed on a strip-chart recorder. At the end of each experiment, dinitrophenol (1 mM) was added to elicit full oxidation of mitochondrial flavoprotein. Data were expressed as percentage of the dinitrophenol-induced fluorescence.

Experimental Protocol

First, we studied the dose–response effect of a known mito K_{ATP} channel opener, diazoxide, and isoflurane on mito K_{ATP} channel activity. Applied concentrations of isoflurane in the perfusate buffer (0, 0.5, 1.0, 2.0, 3.0, and 4.0%) were measured by gas chromatography with a flame ionization detector (GC14-B; Shimazu, Kyoto, Japan). The isoflurane concentrations in the sample portion were 70 ± 8% of those in the flask at all concentrations of isoflurane tested. To confirm that the observed increase in flavoprotein fluorescence was a result of mito K_{ATP} channel activation, 5-HD (0.5 mM), a relatively selective inhibitor of mito K_{ATP} channels, was used.

Left ventricular function and infarct size were evaluated in the protocol as shown in figure 1, in which the hearts were subjected to 30 min of global ischemia followed by 120 min of reperfusion. In protocol 1, we compared the effect of preconditioning with 50 μM diazoxide and 1 minimum alveolar concentration (MAC) of isoflurane on mito K_{ATP} channel activity and cardioprotection. This concentration of diazoxide was chosen because it is known to confer maximum cardioprotection with fewer nonspecific effects.[27] One MAC of isoflurane in the rat[28] was obtained by means of a 2% mixture of this volatile anesthetic agent in our experimental model. Diazoxide and isoflurane were administered for 25 min and were discontinued 20 min before the index ischemia. 5-HD was given 10 min before and during and after the treatment with diazoxide and isoflurane. Reperfusion was performed for 120 min to assess myocardial infarct size.

In protocol 2, we compared the cardioprotective effect of preconditioning with isoflurane, adenosine, and a nitric oxide donor, S-nitroso-*N*-acetyl-penicillamine (SNAP), each alone or in combination. The dose of adenosine used in the current study (10 μM) seems to be appropriate as preconditioning stimulation because this dose of adenosine has been shown to increase cardiac interstitial adenosine to a level comparable with that produced by IPC.[29] SNAP at a dose of 10 μM was chosen in this experimental model because we have found that

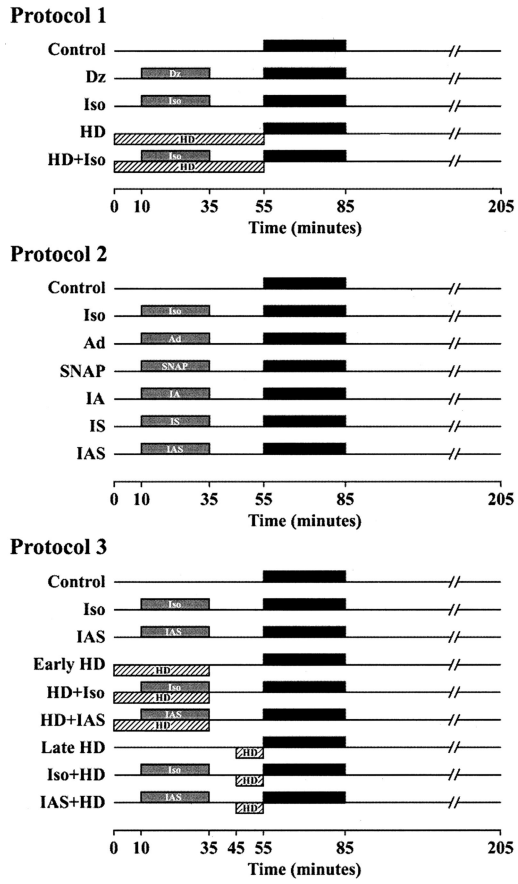

Fig. 1. Experimental protocol. Protocol 1: Control = 30 min of ischemia (*filled boxes*) followed by 120 min of reperfusion; Dz = preconditioning with 50 μM diazoxide; HD = pretreatment with 0.5 mM 5-hydroxydecanoate; HD + Iso = pretreatment with 0.5 mM HD 10 min before and throughout preconditioning with Iso; Iso = preconditioning with 2% isoflurane. Protocol 2: Ad = preconditioning with 10 μM adenosine; IA = preconditioning with Iso and Ad; IAS = preconditioning with Iso, Ad, and SNAP; IS = preconditioning with Iso and SNAP; SNAP = preconditioning with 10 μM S-nitroso-*N*-acetyl-penicillamine. Protocol 3: Early HD = treatment with HD between 55 and 25 min before ischemia; HD + IAS = treatment with HD 10 min before and during combined preconditioning with Iso, Ad, and SNAP; HD + Iso = treatment with HD 10 min before and during preconditioning with Iso; IAS + HD = combined preconditioning with Iso, Ad, and SNAP followed by treatment with HD for 10 min just before ischemia; Iso + HD = preconditioning with Iso followed by treatment with HD for 10 min just before ischemia; Late HD = treatment with HD for 10 min just before ischemia.

SNAP at concentrations up to 50 μM had no adverse effect on LV function.[21]

In the protocol 3, we investigated the role of mito K_{ATP} channels in cardioprotection conferred by combined preconditioning with isoflurane, adenosine, and SNAP. In this set of experiments, 5-HD was administered 10 min before and during preconditioning with isoflurane or the combined PPC as the early treatment modality or for 10 min just before 30 min of ischemia as the late treatment modality.

Isoflurane was purchased from Abbott Laboratories (Abbott Park, IL). Diazoxide, adenosine, and dinitrophenol were obtained from Sigma-Aldrich Japan (Tokyo, Japan). SNAP was purchased from Wako Pure Chemical (Osaka, Japan), and 5-HD was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Diazoxide and SNAP were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.05%.

Infarct Size Measurement

After termination of experiments, the heart was trimmed to remove the atrium, the right ventricular free wall, and the connective tissues and was sliced transversely in a plane perpendicular to the apical–basal axis into approximately 1-mm-thick slices. The slices were immersed in phosphate-buffered saline containing 2% triphenyltetrazolium chloride for 15 min at 37°C and fixed with 10% formaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature. The brick red area was traced with the use of National Institutes of Health 1.61 image-processing software (Bethesda, MD), and each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contract enhancement for improved clarity and distinctness. The areas at risk (equivalent to total LV mass), as well as the infarct zones of each slice, were calculated in terms of pixels. The infarct volume was calculated, and the sum of all slices was used to compute a ratio of percent infarct to total LV mass.

Statistics

All numerical data are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance, followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Isoflurane Provokes Flavoprotein Oxidation in a Concentration-dependent Manner

Flavoprotein fluorescence was stabilized within 15 min after Langendorff perfusion with Krebs-Henseleit bicarbonate buffer and remained stable over 120 min (not shown). Addition of diazoxide increased flavoprotein fluorescence, which reached a plateau within 5 min (fig. 2A). The increase in flavoprotein fluorescence induced by diazoxide was abolished by treatment with 0.5 mM 5-HD, which by itself did not affect baseline flavoprotein fluorescence. The dose–response study showed that 50 μM diazoxide provoked a nearly maximal increase in flavoprotein fluorescence (fig. 2B). Isoflurane increased flavoprotein fluorescence, but the increase in flavoprotein fluorescence was slower than that observed with diazoxide, taking 15–20 min to reach a plateau (fig. 2C). This flavoprotein oxidation induced by isoflurane was also sensitive to 5-HD, suggesting that mito K_{ATP}

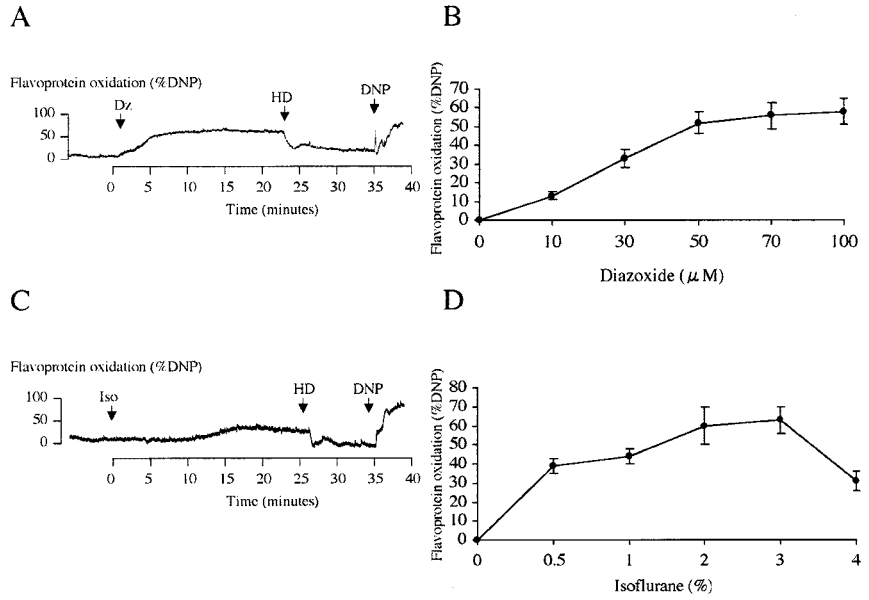

Fig. 2. Effect of 5-hydroxydecanoate (HD) on diazoxide (Dz)- and isoflurane (Iso)-induced changes in flavoprotein oxidation. (*A*) Representative trace of the change in flavoprotein fluorescence by treatment with 50 μM diazoxide followed by cotreatment with 0.5 mM HD. Dinitrophenol (DNP; 1 mM) was added to maximally activate flavoprotein oxidation. The data are expressed as a percentage of dinitrophenol-induced flavoprotein oxidation. (*B*) Dose–response effect of diazoxide on flavoprotein oxidation. (*C*) Representative trace of the change in flavoprotein fluorescence by treatment with 2% isoflurane followed by cotreatment with HD. (*D*) Dose–response effect of isoflurane on flavoprotein oxidation. The data are expressed as mean ± SEM of seven experiments.

channel activation is responsible for the increase in flavoprotein fluorescence induced by isoflurane. The amplitude of flavoprotein fluorescence was increased with increasing isoflurane concentrations in a range between 0.5 and 3.0% (fig. 2D), and further increase in isoflurane concentration reduced flavoprotein fluorescence as expressed by percent of dinitrophenol-induced flavoprotein oxidation (2% isoflurane: 60 ± 10; 3% isoflurane: 63 ± 7; 4% isoflurane: 31 ± 5).

Isoflurane but Not Diazoxide Induces Sustained Activation of Mito K_{ATP} Channels

Although both diazoxide and isoflurane increased flavoprotein fluorescence, a major difference in the pattern of flavoprotein fluorescence between these two agents was observed after cessation of the treatment. The increase in flavoprotein fluorescence induced by diazoxide was dissipated soon after its removal from the buffer, whereas that induced by isoflurane was sustained after cessation of the treatment (fig. 3).

Preconditioning with Isoflurane but Not with Diazoxide Confers Cardioprotection

We compared the effect of preconditioning with diazoxide to that of isoflurane on cardioprotection (protocol 1). Preconditioning with 50 μM diazoxide did not significantly reduced infarct size compared with control ($P = 0.201$; fig. 4). In contrast, preconditioning with isoflurane was effective in significantly reducing infarct size.

Baseline LV function was not significantly different in all groups. We compared LV function 45 min after reperfusion because maximum recovery of LV systolic function was observed between 30 min and 60 min after reperfusion in all the hearts. LV function was not significantly improved by preconditioning with diazoxide (table 1). Isoflurane was also without a significant effect on the recovery of LV function.

Treatment with 5-HD by itself had no significant effect on infarct size and postischemic recovery of LV function. However, the infarct-sparing effect of preconditioning with isoflurane was abolished by 5-HD, suggesting that activation of mito K_{ATP} channels is involved in the infarct-sparing effect mediated by preconditioning with isoflurane.

Combined Preconditioning with Isoflurane, Adenosine, and SNAP Potentiates Cardioprotection without Enhancement of Flavoprotein Oxidation

We investigated whether cardioprotection afforded by preconditioning with isoflurane can be enhanced by coadministration with adenosine and SNAP (protocol 2). In contrast to preconditioning with isoflurane, preconditioning with adenosine and SNAP alone did not significantly reduce infarct size compared with control ($P = 0.274$ and $P = 0.225$, respectively; fig. 5). Coadministration of isoflurane with adenosine but not SNAP significantly improved LV function (table 2) and enhanced the infarct-sparing effect of preconditioning with isoflurane. Moreover, only preconditioning with isoflurane in combination with adenosine and SNAP significantly enhanced postischemic recovery of LV function and reduced infarct size over preconditioning with isoflurane alone.

Neither adenosine nor SNAP by itself significantly affected baseline flavoprotein fluorescence (fig. 6). Isoflurane significantly increased flavoprotein oxidation as described before. However, isoflurane in combination with adenosine and SNAP did not further increase in flavoprotein oxidation over isoflurane alone during and after treatment.

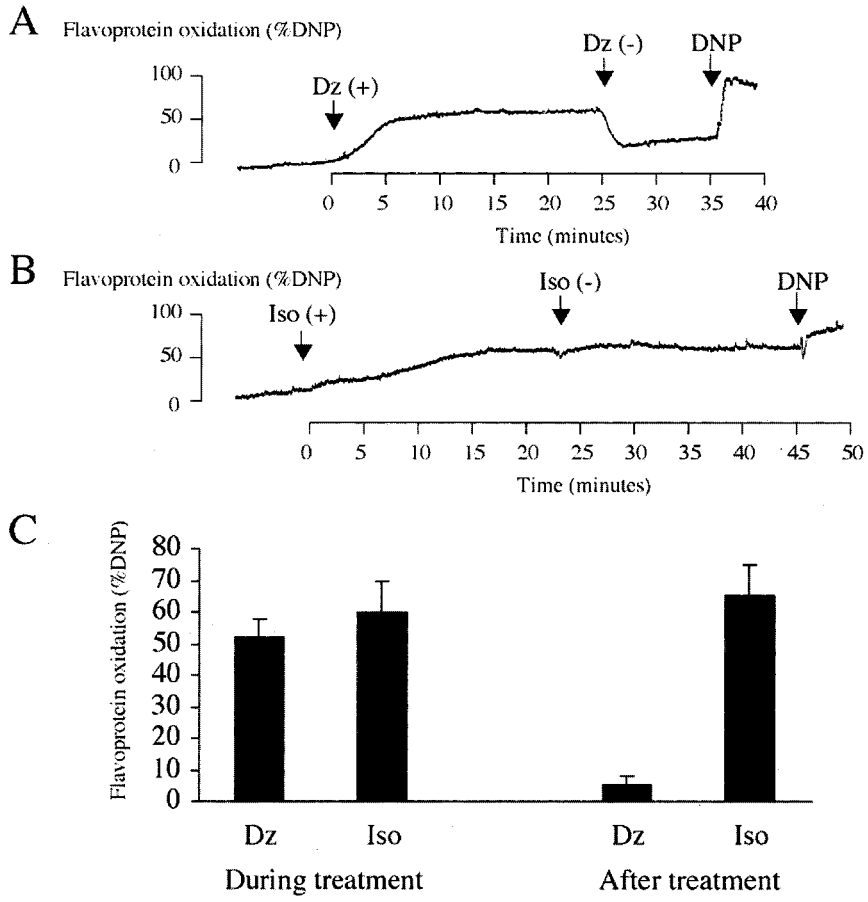

Fig. 3. Change in flavoprotein oxidation during treatment with and after washout of diazoxide and isoflurane. (*A*) Representative trace of the change in flavoprotein fluorescence by treatment with 50 μM diazoxide [Dz (+)] followed by removal of diazoxide [Dz (−)]. Dinitrophenol (DNP; 1 mM) was added to maximally activate flavoprotein oxidation. The data were expressed as a percentage of 1% dinitrophenol–induced flavoprotein oxidation. (*B*) Representative trace of the change in flavoprotein fluorescence by treatment with 2% isoflurane [Iso (+)] followed by removal of isoflurane [Iso (−)]. (*C*) Quantitative analysis of flavoprotein oxidation during and after treatment with diazoxide and isoflurane. Each *bar* represents the mean ± SEM of seven experiments.

Early Treatment with 5-HD Completely Inhibits but Late Treatment with 5-HD Only Partially Inhibits Cardioprotection Afforded by Combined Preconditioning with Isoflurane, Adenosine, and SNAP

Mito K_{ATP} channels may act as a trigger of cardioprotection conferred by combined preconditioning with isoflurane, adenosine, and SNAP. The trigger role of mito K_{ATP} channels was tested by administration with 5-HD 10 min before and during the combined preconditioning (protocol 3). As was the case for preconditioning with isoflurane alone, the early treatment modality abolished the infarct-sparing effect (fig. 7) and inhibited improvement of LV function conferred by combined preconditioning (table 3). Because powerful cardioprotection afforded by combined preconditioning was not correlated with the magnitude of flavoprotein oxidation, it was thought that mito K_{ATP} channels might not be a sole mediator of the combined preconditioning. The mediator role of mito K_{ATP} channels was tested by late treatment with 5-HD. The late treatment with 5-HD by itself had no effect on the recovery of LV function and infarct size. Although the late treatment with 5-HD completely inhibited the infarct-sparing effect conferred by precon-

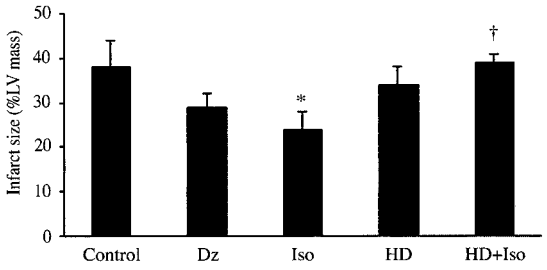

Fig. 4. Effect of preconditioning with diazoxide (Dz) and isoflurane (Iso) on myocardial infarct size. Experimental protocol is shown in protocol 1 in figure 1. Each *bar* represents the mean ± SEM of seven experiments. * $P < 0.05$ compared with control. † $P < 0.05$ compared with isoflurane. HD = 5-hydroxydecanoate; LV = left ventricular.

Table 1. Effect of Preconditioning with Diazoxide and Isoflurane on the Recovery of Left Ventricular Function

	LVDP, % Baseline	HR, % Baseline	LVEDP, mmHg
Control	55 ± 5	82 ± 3	31 ± 3
Diazoxide	63 ± 4	77 ± 5	28 ± 3
Isoflurane	60 ± 5	85 ± 5	34 ± 4
5-HD	48 ± 5	75 ± 12	40 ± 4
5-HD + isoflurane	60 ± 6	95 ± 2	30 ± 2

Left ventricular function was measured 45 min after reperfusion following 30 min of ischemia. The experimental protocol is shown in protocol 1 in figure 1.

Data are expressed as mean ± SEM of seven experiments.

5-HD = 5-hydroxydecanoate; HR = heart rate; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure.

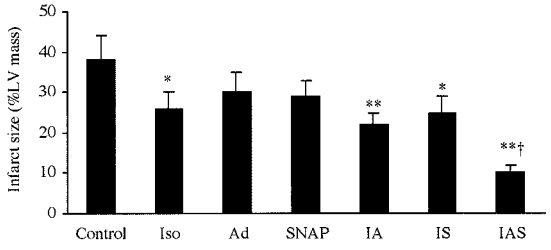

Fig. 5. Effect of preconditioning with isoflurane (Iso) in combination with or without adenosine (Ad) and S-nitroso-*N*-acetyl-penicillamine (SNAP) on myocardial infarct size. Experimental protocol is shown in protocol 2 in figure 1. * $P < 0.05$, ** $P < 0.01$ compared with control. Each *bar* represents the mean ± SEM of seven experiments. † $P < 0.05$ compared with isoflurane. IA = isoflurane + adenosine; IAS = isoflurane + adenosine + SNAP; IS = isoflurane + SNAP; LV = left ventricular.

ditioning with isoflurane, the same treatment exerted significant but only partial inhibition against enhanced postischemic recovery of LV function and infarct size reduction afforded by combined preconditioning with isoflurane, adenosine, and SNAP. Either the early treatment or the late treatment with 5-HD abolished flavoprotein oxidation induced by combined preconditioning with isoflurane, adenosine, and SNAP (fig. 8).

Discussion

The current study compared the efficacy of preconditioning with isoflurane to that of a mito K_{ATP} channel opener, diazoxide; GPCRA; adenosine; and a nitric oxide donor, SNAP, all of which are known to be mimetic of IPC. The results showed that preconditioning with isoflurane but not with diazoxide, adenosine, or SNAP conferred significant cardioprotection. The inability of preconditioning with diazoxide, adenosine, or SNAP alone to confer significant cardioprotection contradicts many former studies that showed significant cardioprotection by preconditioning with each drug alone.[11–15] The mechanism underlying the discrepant observation remains unclear. Although the statistical power with respect to the infarct-sparing effect of these drugs may be increased when the sample size is increased, such a statistical power would not substantially affect the conclusion that a cardioprotective effect of each drug alone is modest compared with that afforded by IPC. Alternatively, drug concentrations are possible explanations. We have tested a preconditioning effect of only single doses of drugs and did not attempt to undertake a dose–response study. We used 50 μM diazoxide, a concentration known to confer maximum cardioprotection with less nonspecific effects.[27] This concentration of diazoxide was found to cause nearly maximum activation of flavoprotein oxidation, indicating that mito K_{ATP} channels were fully activated by this concentration of diazoxide. The doses of adenosine and SNAP seem to be lower than those used in other studies. However, this dose of adenosine was found to increase its interstitial concentration up to several times higher than that observed in IPC.[29] In addition, we have demonstrated in a previous study[20] that five-times higher doses of adenosine and SNAP than in the current study could not provoke significant flavoprotein oxidation and cardioprotection when each was used alone. Therefore, the inability of these drugs to put the heart into the preconditioned state may not solely be attributed to lower drug doses. The rationale for using lower doses of drugs is to elicit only a specific effect while avoiding unwanted side ef-

Table 2. Effect of Preconditioning with Isoflurane in Combination with or without Adenosine and SNAP on the Recovery of Left Ventricular Function

	LVDP, % Baseline	HR, % Baseline	LVEDP, mmHg
Control	55 ± 5	82 ± 3	31 ± 3
Isoflurane	60 ± 5	85 ± 5	34 ± 4
Adenosine	58 ± 6	80 ± 4	29 ± 3
SNAP	67 ± 7	89 ± 5	30 ± 4
IA	74 ± 5*	87 ± 5	25 ± 5
IS	68 ± 6	86 ± 3	31 ± 2
IAS	81 ± 7†‡	95 ± 4	13 ± 2†§

Left ventricular function was measured 45 min after reperfusion following 30 min of ischemia. The experimental protocol is shown in protocol 1 in figure 2.

Data are expressed as mean ± SEM of seven experiments.

* $P < 0.05$, † $P < 0.01$ compared with control. ‡ $P < 0.05$, § $P < 0.01$ compared with isoflurane.

HR = heart rate; IA = isoflurane + adenosine; IAS = isoflurane + adenosine + SNAP; IS = isoflurane + SNAP; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; SNAP = S-nitroso-*N*-acetyl-penicillamine.

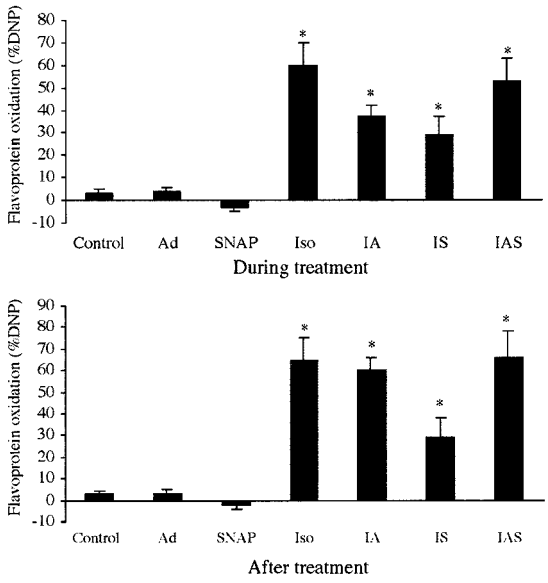

Fig. 6. Change in flavoprotein oxidation during and after treatment with 10 μM adenosine (Ad), 10 μM S-nitroso-*N*-acetyl-penicillamine (SNAP), 2% isoflurane (Iso), isoflurane + adenosine (IA), isoflurane + SNAP (IS), and isoflurane + adenosine + SNAP (IAS). The data are expressed as a percentage of 1% dinitrophenol (DNP)–induced flavoprotein oxidation. Each *bar* represents the mean ± SEM of seven experiments. * $P < 0.01$ compared with control.

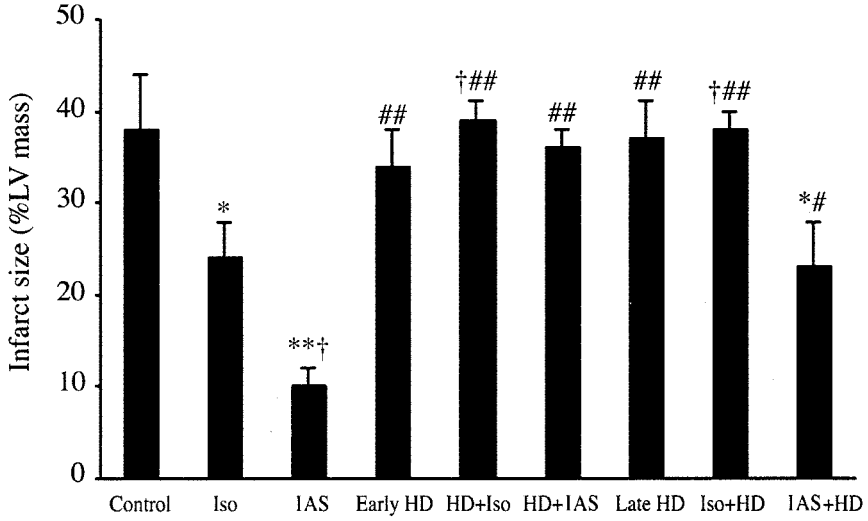

Fig. 7. Effect of early and late treatment with 5-hydroxydecanoate (HD) on the infarct-sparing effect of preconditioning with isoflurane (Iso) and combined preconditioning with isoflurane, adenosine, and S-nitroso-*N*-acetyl-penicillamine (IAS). Experimental protocol is shown in protocol 3 in figure 1. Each *bar* represents the mean ± SEM of seven experiments. * $P < 0.05$, ** $P < 0.01$ compared with control. † $P < 0.05$ compared with isoflurane. # $P < 0.05$, ## $P < 0.01$ compared with IAS. LV = left ventricular.

fects, which may be meritorious in the clinical setting of myocardial protection.

Although PPC with adenosine or SNAP was unable to confer cardioprotection, coadministration of these drugs with isoflurane dramatically potentiated cardioprotective effects conferred by isoflurane. We have previously demonstrated that preconditioning with adenosine, diazoxide, or SNAP alone was not capable of mediating cardioprotection, even at a higher dose than that which was used in this study, but the combination of these drugs conferred powerful cardioprotection that was as effective as IPC.[20,21] Moreover, cardioprotective signaling as evaluated by protein kinase C (PKC)-ϵ activity was increased only by IPC and the combined preconditioning. These observations suggested that enhanced cardioprotection by the combined preconditioning was not simply due to an additive effect but was a synergistic effect through integration of distinct signals arising from each drug to robust cardioprotective signaling. The current study also points to the conclusion that subthreshold inputs of cardioprotective signaling provoked by adenosine and SNAP can augment isoflurane-induced cardioprotection.

Table 3. Effect of Early and Late Treatment with 5-HD on Improved Left Ventricular Function Afforded by Preconditioning with Isoflurane and Combined Preconditioning with Isoflurane, Adenosine, and SNAP

	LVDP, % Baseline	HR, % Baseline	LVEDP, mmHg
Control	55 ± 5	82 ± 3	31 ± 3
Iso	60 ± 5	85 ± 5	34 ± 4
IAS	81 ± 7*	95 ± 4	13 ± 2*
Early 5-HD	57 ± 6	87 ± 7	30 ± 3
5-HD + Isoflurane	60 ± 6	95 ± 2	30 ± 2
5-HD + IAS	63 ± 6	85 ± 8	25 ± 2
Late 5-HD	58 ± 6	84 ± 5	34 ± 4
Isoflurane + 5-HD	58 ± 4	78 ± 5	35 ± 2
IAS + 5-HD	69 ± 7	99 ± 8	20 ± 3*

Left ventricular function was measured 45 min after reperfusion following 30 min of ischemia. The experimental protocol is shown in protocol 3 in figure 1.

Data are expressed as mean ± SEM of seven experiments.

* $P < 0.01$ compared with control.

5-HD = 5-hydroxydecanoate; HR = heart rate; IAS = isoflurane + adenosine + SNAP; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; SNAP = S-nitroso-*N*-acetyl-penicillamine.

We have studied the difference in the potency of cardioprotection between isoflurane and other known triggers of IPC with respect to the activity of mito K_{ATP} channels. Because there is currently no direct means to measure mito K_{ATP} channel activity in the intact heart and cardiomyocytes, we[21,26] and others[30] have adopted flavoprotein oxidation as an indirect index for mito K_{ATP} channel activity. Using this technique, we showed in the current study that isoflurane and diazoxide increase flavoprotein oxidation in a concentration-dependent and a 5-HD–sensitive manner. A significant increase in flavoprotein fluorescence occurred with isoflurane concentrations ranging between 0.5 and 4%, but this effect was maximal at the concentrations between 2 and 3%, indicating that mito K_{ATP} channels were fully activated by the isoflurane concentration used in the preconditioning study. The increase in flavoprotein oxidation induced by diazoxide and isoflurane was abolished in the presence of 5-HD, which did not affect flavoprotein fluorescence at baseline and flavoprotein oxidation induced by dinitrophenol,[20] suggesting that mito K_{ATP} channel activation may be responsible for flavoprotein oxidation induced by diazoxide and isoflurane. A similar dose–response effect of volatile anesthetics on mito K_{ATP} channels has been observed in isolated cardiomyocytes.[31]

The increase in flavoprotein oxidation induced by diazoxide reached a plateau within 5 min, whereas that induced by isoflurane was a slow process, taking 15–20 min to reach a plateau. Moreover, the increase in flavoprotein oxidation induced by isoflurane was sustained after cessation of the treatment, while that induced by diazoxide was dissipated soon after its removal from the buffer. The sustained increase in flavoprotein oxidation

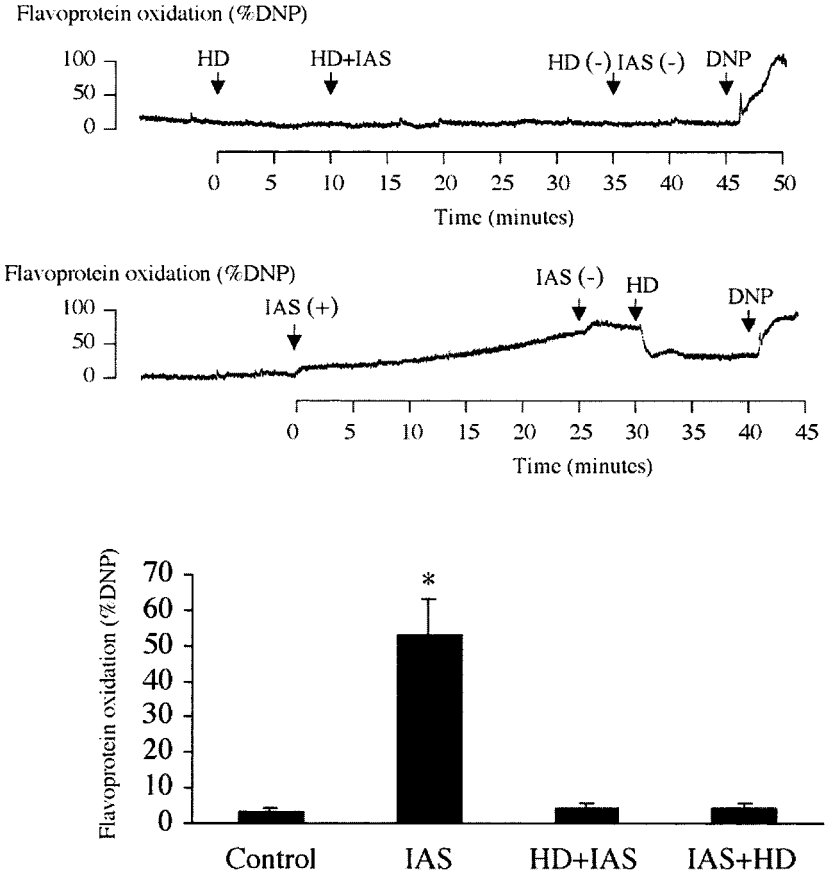

Fig. 8. Effect of early and late treatment with 5-hydroxydecanoate (HD) on flavoprotein oxidation induced by combined preconditioning with isoflurane, adenosine, and S-nitroso-*N*-acetyl-penicillamine (IAS). In the pretreatment modality, 0.5 mM HD was administered 10 min before and during treatment with IAS. In the late treatment modality, HD was administered after treatment with IAS. The data are expressed as a percentage of 1% dinitrophenol (DNP)–induced flavoprotein oxidation. Each *bar* represents mean ± SEM of seven experiments. * $P < 0.01$ compared with control.

induced by preconditioning with isoflurane but not with diazoxide was associated with significant reduction of infarct size. Therefore, there was correlation between flavoprotein oxidation at the time of index ischemia and cardioprotection. These findings provide an explanation for the discrepant results regarding the efficacy of cardioprotection between preconditioning with diazoxide and isoflurane and imply that the mechanism of activation of mito K_{ATP} channels may be different between these two agents. Because isoflurane is a lipophilic substance that partitions into membranes, it presumably takes longer than diazoxide to be fully expelled from tissues. A more intriguing hypothesis is that additional triggers of preconditioning besides mito K_{ATP} channels may be involved in isoflurane-induced sustained activation of mito K_{ATP} channels. Accumulating evidence suggests that isoflurane not only activates mito K_{ATP} channels, but also activates other signaling cascades. Isoflurane has been shown to induce protection of human myocardium from anoxic injury *via* adenosine A_1 receptors and K_{ATP} channels.[32] Involvement of G protein–coupled receptors in isoflurane-induced cardiac preconditioning was further suggested by the fact that the blockade of Gi/o proteins by pertussis toxin abolished the infarct size–limiting effect of isoflurane in the canine heart.[33] Furthermore, another volatile anesthetic agent, halothane, has been shown to directly interact with G protein–coupled receptors.[34] Isoflurane is also capable of producing nitric oxide in the mouse brain.[35] Finally, the cardioprotective effect of volatile anesthetics was found to be dependent on PKC.[36] Although mito K_{ATP} channels could be opened by mito K_{ATP} channel openers without PKC activation, a priming effect of PKC activation on enhanced mito K_{ATP} channel opening has been demonstrated,[30] and this mechanism may also be crucial in the sustained activation of mito K_{ATP} channels that was attributed to the "acute memory" of cardioprotection afforded by isoflurane.[6] Therefore, it is likely that isoflurane provokes sustained activation of mito K_{ATP} channels even after cessation of the treatment, presumably through activation of multiple signaling pathways that converge on PKC activation, as has been proposed by Zaugg *et al.*[36]

The current study investigated the role of mito K_{ATP} channels in cardioprotection afforded by isoflurane and combined preconditioning with isoflurane, adenosine, and SNAP. The early treatment with 5-HD completely abolished the infarct size–limiting effect of isoflurane and the combined preconditioning, indicating that mito K_{ATP} channels are the essential trigger of preconditioning with isoflurane and the combined preconditioning. However, although cardioprotection conferred by preconditioning with isoflurane was completely abrogated by the late treatment with 5-HD, that afforded by the

combined preconditioning was only partially inhibited by the late treatment with 5-HD, which abolished flavoprotein oxidation mediated by isoflurane and the combined preconditioning. These observations suggest that cardioprotection afforded by isoflurane is mediated through mito K_{ATP} channel activation, while the combined preconditioning–mediated cardioprotection occurs through both mito K_{ATP} channel–dependent and –independent mechanisms. The evidence that the combined preconditioning conferred superior cardioprotection over isoflurane alone is consistent with a recent study[17] showing that the K_{ATP} channel opener bimakalim alone had no infarct size–limiting effect, whereas the simultaneous administration of adenosine and bimakalim resulted in a marked decrease in infarct size, suggesting a synergism between adenosine and K_{ATP} channels in the combined preconditioning–mediated cardioprotection. Taken together, our study reinforces the hypothesis that a synergistic interaction of multiple triggers, including mito K_{ATP} channels, GPCRA, and a nitric oxide donor, promotes signaling pathways that culminate in robust cardioprotection through mito K_{ATP} channel–dependent and –independent mechanisms. Whether these cardioprotective mechanisms are promoted by PKC or in concert with other signaling pathways remains to be determined.

Conclusions

The current study showed that preconditioning with isoflurane in combination with low doses of adenosine and SNAP, which by themselves had no significant benefit in cardioprotection, significantly enhanced cardioprotection over preconditioning with isoflurane alone. The powerful cardioprotection mediated by the combined PPC technique is not simply additive to isoflurane of which cardioprotection may be ascribed solely to mitochondrial K_{ATP} channel activation, but is synergistic through both mitochondrial K_{ATP} channel–dependent and –independent mechanisms. Therefore, the current study suggests that exogenous addition of a GPCRA and a nitric oxide donor represents a reasonable approach to enhance cardioprotection afforded by isoflurane. However, clinical evidence has been lacking as to whether combined preconditioning is in fact necessary to confer superior cardioprotection over volatile anesthetic agents alone despite the fact that volatile anesthetic agents are often used in conjunction with GPCRAs such as adenosine and norepinephrine and nitric oxide donors such as nitroglycerine and sodium nitroprusside in cardiac surgery. Future clinical trials are warranted to address this issue.

References

1. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36

2. Davis RF, Sidi A: Effect of isoflurane on the extent of myocardial necrosis and on systemic hemodynamics, regional myocardial blood flow, and regional myocardial metabolism in dogs after coronary artery occlusion. Anesth Analg 1989; 69:575–86

3. Oguchi T, Kashimoto S, Yamaguchi T, Nakamura T, Kumazawa T: Comparative effects of halothane, enflurane, isoflurane and sevoflurane on function and metabolism in the ischaemic rat heart. Br J Anaesth 1995; 74:569–75

4. Kersten JR, Schmeling TJ, Hettrick DA, Pagel PS, Gross GJ, Warltier DC: Mechanism of myocardial protection by isoflurane: Role of adenosine triphosphate–regulated potassium (K_{ATP}) channels. ANESTHESIOLOGY 1996; 85:794–807

5. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. ANESTHESIOLOGY 1997; 86:699–709

6. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K_{ATP} channels: Reduction of myocardial infarct size with an acute memory phase. ANESTHESIOLOGY 1997; 87:361–70

7. Cason BA, Gamperl AK, Slocum RE, Hickey RF: Anesthetic-induced preconditioning: Previous administration of isoflurane decreases myocardial infarct size in rabbits. ANESTHESIOLOGY 1997; 87:1182–90

8. Piriou V, Chiari P, Knezynski S, Bastien O, Loufoua J, Lehot JJ, Foex P, Annat G, Ovize M: Prevention of isoflurane-induced preconditioning by 5-hydroxydecanoate and gadolinium: Possible involvement of mitochondrial adenosine triphosphate–sensitive potassium and stretch-activated channels. ANESTHESIOLOGY 2000; 93:756–64

9. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM: Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. Circulation 1991; 84:350–6

10. Toombs CF, McGee S, Johnston WE, Vinten-Johansen J: Myocardial protective effects of adenosine: Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. Circulation 1992; 86:986–94

11. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM: Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. Circ Res 2000; 87:460–6

12. Nakano A, Liu GS, Heusch G, Downey JM, Cohen MV: Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. J Mol Cell Cardiol 2000; 32:1159–67

13. Lochner A, Marais E, Genade S, Moolman JA: Nitric oxide: A trigger for classic preconditioning? Am J Physiol Heart Circ Physiol 2000; 279:H2752–65

14. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM: Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K_{ATP} channels. Circ Res 2001; 89:273–8

15. Thornton JD, Liu GS, Olsson RA, Downey JM: Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. Circulation 1992; 85:659–65

16. Cave AC, Collis CS, Downey JM, Hearse DJ: Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. Cardiovasc Res 1993; 27:663–8

17. Yao Z, Mizumura T, Mei DA, Gross GJ: KATP channels and memory of ischemic preconditioning in dogs: Synergism between adenosine and KATP channels. Am J Physiol 1997; 272:H334–42

18. Belhomme D, Peynet J, Florens E, Tibourtine O, Kitakaze M, Menasche P: Is adenosine preconditioning truly cardioprotective in coronary artery bypass surgery? Ann Thorac Surg 2000; 70:590–4

19. Ninomiya H, Otani H, Lu K, Uchiyama T, Kido M, Imamura H: Enhanced IPC by activation of pertussis toxin-sensitive and -insensitive G protein-coupled purinoceptors. Am J Physiol Heart Circ Physiol 2002; 282:H1933–43

20. Uchiyama Y, Otani H, Okada T, Uchiyama T, Ninomiya H, Kido M, Imamura H, Nakao S, Shingu K: Integrated pharmacological preconditioning in combination with adenosine, a mitochondrial K_{ATP} channel opener and a nitric oxide donor. J Thorac Cardiovasc Surg 2003; 126:148–59

21. Otani H, Okada T, Fujiwara H, Uchiyama T, Sumida T, Kido M, Imamura H: Combined pharmacological preconditioning with a G-protein-coupled receptor agonist, a mitochondrial K_{ATP} channel opener and a nitric oxide donor mimics ischaemic preconditioning. Clin Exp Pharmacol Physiol 2003; 30:684–93

22. Patel HH, Ludwic LM, Fryer RM, Hsu AK, Warltier DC, Gross GJ: Delta opioid agonists and volatile anesthetics facilitate cardioprotection via potentiation of K_{ATP} channel opening. FASEB J 2002; 16:1468–70

23. Ivanova K, Schaefer M, Drummer C, Gerzer R: Effects of nitric oxide-containing compounds on increases in cytosolic ionized Ca^{2+} and on aggregation of human platelets. Eur J Pharmacol 1993; 244:37–47

24. Piriou V, Ross S, Pigott D, Evans R, Foex P: Beneficial effect of concomitant administration of isoflurane and nicorandil. Br J Anaesth 1997; 79:68–77

25. National Institutes of Health: Guidelines for the Care and Use of Laboratory Animals. Bethesda, National Institutes of Health, revised 1996. NIH publication No. 85-23

26. Uchiyama Y, Otani H, Wakeno M, Okada T, Uchiyama T, Sumida T, Kido M, Imamura H, Nakao S, Shingu K: Role of mitochondrial K_{ATP} channels and protein kinase C in ischaemic preconditioning. Clin Exp Pharmacol Physiol 2003; 30:426–36

27. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB,

D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ: Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: Possible mechanism of cardioprotection. Circ Res 1997; 81:1072–82

28. Quasha AL, Eger EI II, Tinker JH: Determination and applications of MAC. ANESTHESIOLOGY 1980; 53:315–34

29. Ninomiya H, Otani H, Lu K, Uchiyama T, Kido M, Imamura H: Complementary role of extracellular ATP and adenosine in ischemic preconditioning in the rat heart. Am J Physiol Heart Circ Physiol 2002; 282:H1810–20

30. Sato T, O'Rourke B, Marban E: Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. Circ Res 1998; 83:110–4

31. Kohro S, Hogan QH, Nakae Y, Yamakage M, Bosnjak ZJ: Anesthetic effects on mitochondrial ATP-sensitive K channel. ANESTHESIOLOGY 2001; 95:1435–40

32. Roscoe AK, Christensen JD, Lynch C III: Isoflurane, but not halothane, induces protection of human myocardium *via* adenosine A_1 receptors and adenosine triphosphate–sensitive potassium channels. ANESTHESIOLOGY 2000; 92:1692–701

33. Toller WG, Kersten JR, Gross ER, Pagel PS, Warltier DC: Isoflurane preconditions myocardium against infarction *via* activation of inhibitory guanine nucleotide binding proteins. ANESTHESIOLOGY 2000; 92:1400–7

34. Ishizawa Y, Pidikiti R, Liebman PA, Eckenhoff RG: G-protein coupled receptors as direct targets of inhaled anesthetics. Mol Pharmacol 2002; 61:945–52

35. Kehl F, Shen H, Moreno C, Farber NE, Roman RJ, Kampine JP, Hudetz AG: Isoflurane-induced cerebral hyperemia is partially mediated by nitric oxide and epoxyeicosatrienoic acids in mice *in vivo*. ANESTHESIOLOGY 2002; 97:1528–33

36. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC: Volatile anesthetics mimic cardiac preconditioning by priming the activation mitochondrial K_{ATP} channels *via* multiple signaling pathways. ANESTHESIOLOGY 2002; 97:4–14