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Research Paper

# Isoflurane preconditioning increases endothelial cell tolerance to in-vitro simulated ischaemia

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## Abstract

**Objectives** Isoflurane preconditioning has been shown to protect endothelial cells against lipopolysaccharide and cytokine induced injury. This study was designed to determine whether isoflurane preconditioning increased endothelial cell tolerance to ischaemia.

**Methods** Bovine pulmonary arterial endothelial cells were exposed or not exposed to various concentrations of isoflurane for 1 h. After a 30-min isoflurane-free period, cells were subjected to oxygen-glucose deprivation (OGD) for 3 h and reoxygenation for 1 h. Lactate dehydrogenase release from cells was used to measure cell injury. In some experiments, various protein kinase C (PKC) inhibitors and ATP-sensitive potassium channel ($K_{ATP}$ channel) inhibitors were present from 30 min before isoflurane treatment to the end of isoflurane treatment.

**Key findings** Isoflurane preconditioning dose-dependently decreased the OGD induced lactate dehydrogenase release. This protection was inhibited by 2 $\mu$M chelerythrine, a general PKC inhibitor, or 10 $\mu$M Gö6976, an inhibitor for the conventional PKCs. This protection was also inhibited by 0.3 $\mu$M glybenclamide, a general $K_{ATP}$ channel inhibitor, and 500 $\mu$M 5-hydroxydecanoate, a mitochondrial $K_{ATP}$ channel blocker. In addition, pretreatment with 100 $\mu$M diazoxide, a $K_{ATP}$ channel activator, for 1 h also reduced OGD induced endothelial cell injury. This diazoxide induced protection was inhibited by chelerythrine.

**Conclusions** The results suggest that isoflurane preconditioning induces endothelial protection against in-vitro simulated ischemia. This protection may be mediated at least in part by conventional PKCs and mitochondrial $K_{ATP}$ channels. The results also indicate that PKCs may be downstream of $K_{ATP}$ channels in causing endothelial protection.

**Keywords** endothelial cells; isoflurane; mitochondrial $K_{ATP}$ channel; preconditioning; protein kinase C

## Introduction

Endothelial cells line blood vessels in all organs and systems. They form a unique barrier to prevent free access of chemicals and cells in the blood to the vascular smooth muscle cells and parenchymal cells in organs. Also, endothelial cells release various molecules, such as nitric oxide and endothelin, to affect the functions of other cells.[1,2] Thus, maintaining structural and functional integrity of endothelial cells is critical under physiological and pathophysiological conditions. However, many insults can threaten this integrity. Ischaemia, for example, can injure endothelial cells.

Preconditioning is a phenomenon in which a prior exposure to short episodes of ischaemia or other stimuli can induce ischaemic tolerance. Since the concept 'preconditioning' was introduced in 1986,[3] numerous studies have shown that preconditioning is a promising approach to reduce ischaemic injury in various organs.[4,5] Although many stimuli can induce a preconditioning effect, the use of a relatively safe agent to induce preconditioning effects represents a potentially useful approach to provide organ protection in clinical practice. It has been shown that commonly used volatile anaesthetics, such as isoflurane, can induce a preconditioning effect against ischaemia in many organs including brain and heart.[6,7] Isoflurane preconditioning also reduces inflammation induced endothelial injury.[8] Application of sevoflurane, another volatile anaesthetic used in clinical practice, during the period from 15 min before ischaemia to 5 min after the onset of reperfusion,

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improved the function of endothelial cells in human forearm arteries after a 15-min forearm ischaemia.[9] We hypothesised that preconditioning with volatile anaesthetics would reduce ischaemic injury of endothelial cells. To test this hypothesis, we used bovine pulmonary arterial endothelial cells and subjected them to oxygen-glucose deprivation (OGD to simulate ischaemia *in vitro*. Since protein kinase C (PKC) and ATP-sensitive potassium channels ($K_{ATP}$ channels) are implicated to be involved in isoflurane preconditioning induced protection in many organs and cell types,[7,10,11] we tested the role of PKC and $K_{ATP}$ channels in the isoflurane preconditioning induced endothelial protection. Despite numerous studies on preconditioning induced protection of various organs and tissues since 1986, very few studies have been focused on preconditioning induced protection of endothelial cells. This study was designed not only to determine whether isoflurane could induce a preconditioning effect against in-vitro simulated ischaemia/reperfusion in endothelial cells, but also to reveal the mechanisms for this protection.

## Materials and Methods

### Materials

Isoflurane was purchased from Abbott Laboratories (North Chicago, IL, USA). Chelerythrine chloride was obtained from Biomol (Plymouth Meeting, PA, USA). Other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA), unless otherwise specified.

### Cell culture

Bovine pulmonary arterial endothelial cells were isolated and characterised as described previously.[12,13] The cells were cultured in a T75 flask containing 12 ml of culture medium composed of Dulbecco's modified Eagle's medium (containing 1000 mg/l D-glucose, L-glutamine and pyridoxine HCl), 110 mg/l sodium pyruvate, 10% heat-inactivated fetal bovine serum, 90 $\mu$g/ml thymidine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. The cells were kept in a humidified atmosphere containing 95% air/5% $CO_2$ at 37°C. Culture medium was changed three times per week. Cells were subcultured when they were 70–80% confluent. The cells between passage 8 and 20 were used in the experiments.

### Isoflurane and oxygen-glucose deprivation exposure

The cells were placed into 6-well plates at a density of $5 \times 10$ cells/ml[3] (2 ml/well) and cultured overnight (approx. 17 h). Glucose-free buffer contained 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 3.6 mM $CaCl_2$ and 5.0 mM HEPES. Glucose was added to make glucose-containing buffer that contained 4.5 g/l glucose. Isoflurane was delivered by air through an agent-specific vaporiser. The glucose-containing buffer was pregassed with isoflurane for 10 min. This isoflurane-containing buffer was added to the cells. The cells were immediately placed into an air-tight chamber and this chamber was gassed with isoflurane containing air for 10 min. The anaesthetic concentrations in the outlet gases were monitored by a Datex infrared analyser (Capnomac, Helsinki, Finland) and the target isoflurane concentrations were reached in <2 min. After closure of the inlet and outlet of the chamber, the chamber was then placed in an incubator for 1 h at 37°C. Cells were then removed from the chamber and placed in the incubator for 30 min at 37°C before they were subjected to OGD.

OGD buffer was prepared by bubbling the glucose-free buffer with 100% $N_2$ for 30 min. Cells in the control group were washed with, and incubated in, glucose-containing buffer in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. The OGD condition in cells was created by washing cells with OGD buffer three times and then placing cells in this OGD buffer. These plates were then placed in an air-tight chamber gassed with 100% $N_2$ for 10 min. The oxygen content in the outlet of the chamber was monitored with a Datex infrared analyser and was below 2% at ~3 min after the onset of gassing. The inlet and outlet of the chamber were closed and the chamber was kept at 37°C for 3 h. After the oxygen content in the chamber at the end of incubation was confirmed to be <2%, the chamber was opened and glucose was added to the incubation solutions to give a final concentration of glucose of 4.5 g/l. In a separate preliminary experiment, the $O_2$ partial pressure in the incubation solutions during the OGD exposure was measured to be <10 mmHg. The cells were kept in glucose-containing solution in air for 1 h at 37°C. The incubation buffer and cells were then used for assay of lactate dehydrogenase (LDH) activity.

### Application of chemicals

$K_{ATP}$ channel blockers (0.3 $\mu$M glybenclamide or 500 $\mu$M 5-hydroxydecanoate) or PKC inhibitors (2 $\mu$M chelerythrine chloride or 10 $\mu$M Gö6976) were added to the cells at 30 min before exposure to 3% isoflurane for 1 h. After the incubation (total incubation time with these inhibitors was 1.5 h), the solutions were replaced with fresh glucose-containing buffers without these reagents. The solutions in the control, OGD only or isoflurane preconditioning plus OGD groups in the same set of experiments were also changed in the same way. In another set of experiments, a mitochondrial $K_{ATP}$ channel activator (100 $\mu$M diazoxide) was applied for 1 h in the presence or absence of 2 $\mu$M chelerythrine chloride. After a 30-min drug-free period, these cells were also exposed to OGD for 3 h followed by a 1-h simulated reperfusion.

### Lactate dehydrogenase activity assay

LDH activity was determined using an LDH cytotoxicity detection kit (Clontech Laboratory, La Jolla, CA, USA). Briefly, the incubation solution was centrifuged at 96*g* for 10 min and the cell-free supernatant was transferred to 96-well plates. A 100-$\mu$l supernatant was incubated with the same amount of reaction mixture. LDH activity was determined by a colorimetric assay. The absorbance of samples was measured at 492 nm with the reference wavelength of 655 nm in a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Background absorbance from the cell-free buffer solution was subtracted from all absorbance measurements. After removal of the buffer from 6-well plates, 0.5% Triton X-100 lysing solution was applied to the remaining cells. The percentage of LDH released into the incubation buffer in total LDH was calculated: $100 \times$ spontaneously

released LDH in the buffer/(spontaneously released LDH in the buffer + intracellular LDH released by Triton X-100).

### Statistical analysis

Data are expressed as mean ± SD ($n \geq 26$ for each experimental condition). One-way analysis of variance followed by the Tukey test for *post hoc* analysis was performed for normally distributed data. One-way analysis of variance on ranks followed by Dunn's test for *post hoc* analysis was performed for non-normally distributed data. A value of $P < 0.05$ was considered statistically significant.

## Results

The 3-h OGD and 1-h simulated reperfusion significantly increased LDH release from bovine pulmonary arterial endothelial cells (Figure 1), suggesting that this condition caused injury of these cells. This injury was significantly attenuated by pretreatment with 2 or 3% isoflurane (Figure 1). The attenuation of cell injury caused by 3% isoflurane pretreatment was inhibited by glybenclamide, a general $K_{ATP}$ channel inhibitor, and by 5-hydroxydecanoate, a mitochondrial $K_{ATP}$ channel blocker, whereas glybenclamide and 5-hydroxydecanoate did not affect the LDH release under the OGD and simulated reperfusion condition alone (Figure 2). Similarly, the 3% isoflurane pretreatment induced attenuation of cell injury was inhibited by chelerythrine, a general PKC inhibitor, and 10 $\mu$M Gö6976, an inhibitor for the conventional PKCs. These two PKC inhibitors did not affect the LDH release caused by the OGD and simulated reperfusion alone (Figure 3). Pretreatment with diazoxide, a $K_{ATP}$ channel activator, also reduced the OGD and simulated reperfusion induced LDH release. This diazoxide induced protection was inhibited by chelerythrine (Figure 3). Of note, the LDH release under the OGD and simulated reperfusion only condition in Figure 3 was higher than that presented in Figure 1 or Figure 2. This difference may be due to different batches of cells being used. In support of this possibility, LDH release under control conditions in Figure 3 was higher than that presented in Figures 1 and 2.

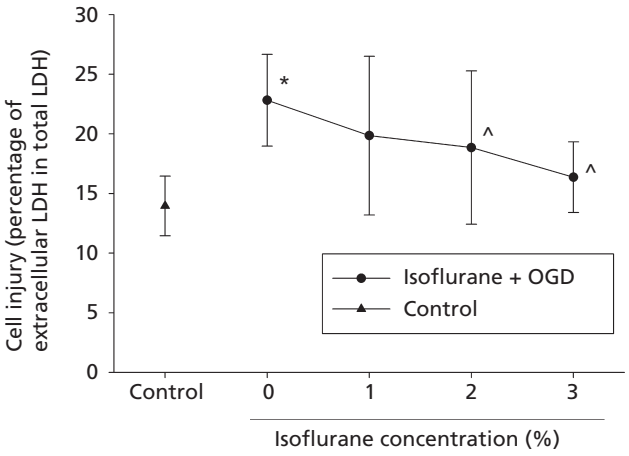


**Figure 1** Isoflurane preconditioning effect. Bovine pulmonary arterial endothelial cells were pretreated with various concentrations of isoflurane for 1 h and then exposed or not exposed to oxygen-glucose deprivation (OGD) for 3 h. Results are means ± SD ($n = 29$–$34$). $^{*}P < 0.05$ compared with control. $^{\wedge}P < 0.05$ compared with OGD only. Statistical analysis was performed by one-way analysis of variance. LDH, lactate dehydrogenase.

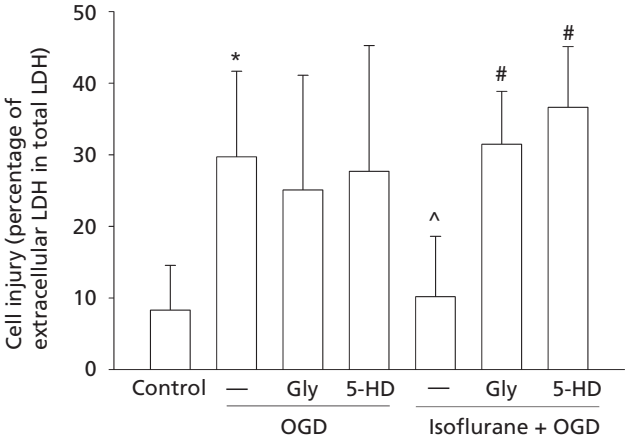


**Figure 2** Involvement of mitochondrial $K_{ATP}$ channels in the isoflurane preconditioning effect. Bovine pulmonary arterial endothelial cells were pretreated with or without 3% isoflurane for 1 h in the presence or absence of 0.3 $\mu$M glybenclamide (Gly) or 500 $\mu$M 5-hydroxydecanoate (5-HD) and then were exposed or not exposed to oxygen-glucose deprivation (OGD) for 3 h. Results are mean ± SD ($n = 26$–$30$). $^{*}P < 0.05$ compared with control. $^{\wedge}P < 0.05$ compared with OGD only. $^{\#}P < 0.05$ compared with isoflurane preconditioning + OGD. Statistical analysis was performed by one-way analysis of variance on ranks. LDH, lactate dehydrogenase.

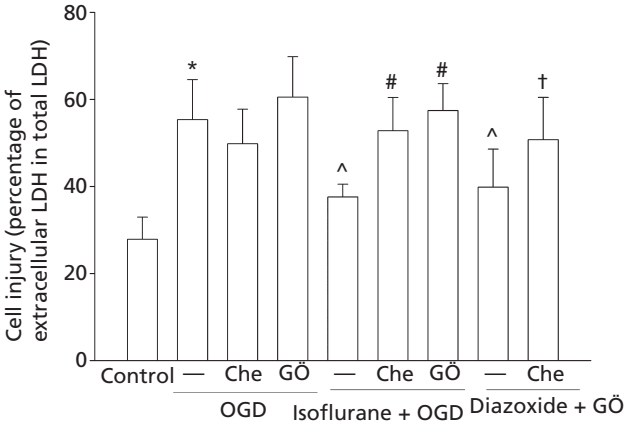


**Figure 3** Involvement of protein kinase C in the isoflurane preconditioning effect. Bovine pulmonary arterial endothelial cells were pretreated with or without 3% isoflurane for 1 h in the presence or absence of 2 $\mu$M chelerythrine (Che) or 10 $\mu$M Gö6976 (Gö), or were pretreated with 100 $\mu$M diazoxide for 1 h in the presence or absence of 2 $\mu$M chelerythrine. The cells were then subjected to oxygen-glucose deprivation (OGD) for 3 h. Results are mean ± SD ($n = 29$–$30$). $^{*}P < 0.05$ compared with control. $^{\wedge}P < 0.05$ compared with OGD only. $^{\#}P < 0.05$ compared with isoflurane preconditioning + OGD. $^{\dagger}P < 0.05$ compared with diazoxide + OGD. Statistical analysis was performed by one-way analysis of variance. LDH, lactate dehydrogenase.

## Discussion

Isoflurane preconditioning has been shown to reduce cytokine induced endothelial cell injury.[8] We showed in this study that isoflurane preconditioning concentration dependently decreased the OGD induced endothelial cell injury. The results suggest that isoflurane preconditioning can induce ischaemic tolerance in endothelial cells. Consistent with this suggestion, a previous study showed that sevoflurane improved endothelial function after ischaemia.[9] However, sevoflurane was present throughout the period from 15 min before to 5 min after a 15-min ischaemia. It is difficult to determine the contribution of sevoflurane preconditioning induced protection to the overall improvement of endothelial function after the ischaemia.

In our study, the isoflurane preconditioning induced endothelial cell protection was abolished by glybenclamide, a general $K_{ATP}$ channel blocker,[14] and 5-hydroxydecanoate, a mitochondrial $K_{ATP}$ channel blocker.[14,15] These results suggest that mitochondrial $K_{ATP}$ channels are involved in this isoflurane preconditioning induced protection. Consistent with this idea, diazoxide, a mitochondrial $K_{ATP}$ channel activator,[16] also induced a preconditioning effect. In addition, previous studies have suggested the involvement of the mitochondrial $K_{ATP}$ channels in the volatile anaesthetic preconditioning induced protection in the heart and brain.[11,17]

At least 11 isozymes of PKC have been identified. They are classified into three groups: conventional PKCs ($\alpha$, $\beta$I, $\beta$II and $\gamma$), novel PKCs ($\delta$, $\varepsilon$, $\eta$ and $\theta$), and atypical PKCs ($\zeta$ and $\iota/\lambda$).[18] We showed here that the isoflurane preconditioning induced endothelial cell protection was abolished by chelerythrine, a general PKC inhibitor, and Gö6976, an inhibitor for the conventional PKCs.[19,20] These results suggest the involvement of conventional PKCs in the isoflurane preconditioning effect. Consistent with this suggestion, we and others have shown that volatile anaesthetics including isoflurane can activate conventional PKCs.[21–23] Also, PKC has been shown to be involved in isoflurane preconditioning induced cardioprotection and neuroprotection.[10,24]

$K_{ATP}$ channels, including the mitochondrial $K_{ATP}$ channels, have been considered as a putative end-effector in organ protection induced by ischaemic or anaesthetic preconditioning.[10] Activation of mitochondrial $K_{ATP}$ channels reduces ischaemia induced $Ca^{++}$ influx into the mitochondria and subsequent mitochondrial swelling.[25] Activation of these channels may also restore mitochondrial membrane potential and ATP production.[10,25] In addition, activation of mitochondrial $K_{ATP}$ channels can produce signalling molecules, such as free radicals that can then activate PKC.[26–28] Thus, the mitochondrial $K_{ATP}$ channels are also signalling molecules. Here, we showed that the protection induced by diazoxide was abolished by chelerythrine, a PKC inhibitor.[29] These results suggest that the mitochondrial $K_{ATP}$ channels may be a molecule upstream of PKC to induce endothelial protection. These results, along with the findings that suggest the involvement of the conventional PKCs and mitochondrial $K_{ATP}$ channels in the isoflurane preconditioning induced protection, indicate that the conventional PKCs may be a molecule downstream of the mitochondrial $K_{ATP}$ channels to mediate the isoflurane preconditioning induced endothelial protection.

Our results suggest that specific activators for conventional PKCs or mitochondrial $K_{ATP}$ channels may be used to maintain the function and structural integrity of endothelial cells under physiological and pathophysiological conditions. This protection of endothelial cells may reduce organ injury under various pathological conditions, such as ischaemia.

## Conclusions

Preconditioning of endothelial cells with isoflurane dose-dependently reduced endothelial cell injury after in-vitro simulated ischaemia and reperfusion. This effect may be mediated at least partially by the mitochondrial $K_{ATP}$ channels and conventional PKCs. It appears that PKCs may be signalling molecules downstream of the mitochondrial $K_{ATP}$ channels in causing endothelial protection.

## Declarations

### Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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