Background: Volatile anesthetics have protective effects against cytokine-induced injury in endothelial and vascular smooth muscle cells. The authors hypothesized that isoflurane pretreatment may trigger immediate and delayed protection that is modulated by adenosine triphosphate–sensitive potassium channels.

Methods: Human and bovine endothelial cells and rat vascular smooth muscle cells were pretreated with isoflurane (1.5% for 30 min) and then exposed to cytokines (tumor necrosis factor-α, interferon-γ, and interleukin-β) for 72 h. Cytokine exposure was initiated immediately after isoflurane pretreatment or after a delay of 1–48 h. Cell survival and viability were evaluated by trypan blue exclusion and lactate dehydrogenase release. The role of mitochondrial and cell membrane adenosine triphosphate–sensitive potassium channels, or both, were evaluated with the antagonists 5-hydroxydecanoate, HMR-1098, or glybenclamide.

Results: Immediate isoflurane pretreatment was approximately 70% effective in increasing cell survival and prevented lactate dehydrogenase release in all cell lines. However, cellular protection was completely lost if the time between isoflurane and cytokine exposure was extended to 2–12 h, depending on the cell type. Delayed protection was equal to immediate protection when the interval was extended to 12–24 h, with protection being sustained at ≥48 h in human endothelial and rat vascular smooth muscle cells. The immediate and delayed protection was inhibited by glybenclamide and 5-hydroxydecanoate but not by HMR-1098, whereas diazoxide, a mitochondrial adenosine triphosphate–sensitive potassium channels agonist, mimicked the time course of isoflurane-induced immediate and delayed protection in all cell lines.

Conclusion: Isoflurane pretreatment has immediate and delayed protective effects against cytokine-induced injury in endothelial and vascular smooth muscle cells. This protection being sustained at 48 h in human endothelial and rat vascular smooth muscle (RVSM) cells. This protection against cytokine-induced injury was most pronounced when isoflurane pretreatment immediately preceded or was administered 1 h before cytokine exposure, whereas the protective effect was significantly decreased after a delay of 2–4 h.1

Several investigators have demonstrated that volatile anesthetic preconditioning may mimic ischemic myocardial preconditioning.2–6 Anesthetic preconditioning and ischemic preconditioning (IPC) seem to be similar in protecting myocardial cells by regulation of adenosine triphosphate–sensitive potassium (K_{ATP}) channels.7–9 In addition, the protection by volatile anesthetics and the classic IPC is lost if the time interval between the preconditioning stimulus and the more prolonged period of ischemia is increased from several minutes to 2–3 h.2–4,6 However, a number of studies have described a second window of protection or delayed IPC in myocardium.10–12 The delayed effects of IPC are described as periods extending beyond 12 h between the brief exposure to ischemia and the final ischemic insult, that results in myocardial and coronary artery protection.10,13 It was recently shown that isoflurane (1%) induces delayed (24 h) cardioprotection in rabbit hearts, and that this protection is abolished by sarcolemmal and mitochondrial K_{ATP} inhibitors.14 Conversely, Kehl et al.15 reported that isoflurane (1.28%) did not induce delayed (24 h) cardioprotection from ischemia–reperfusion injury in dogs hearts.

Research on immediate and delayed ischemic and volatile anesthetic preconditioning has been limited to protection against ischemia–reperfusion injury in myocardium. There are no studies investigating delayed anesthetic preconditioning or the role of K_{ATP} channels in other cell types, such as endothelial and RVSM cells, nor are there investigations of delayed protection against inflammation or cytokine-induced injury. Furthermore, previous studies have focused on delayed preconditioning after 12 or 24 h, whereas insight into the time course of immediate and delayed preconditioning remains unexplored. We hypothesized that isoflurane pretreatment would have immediate and delayed protective effects in human microvascular endothelial cells (HMEC), bovine pulmonary endothelial cells (BPEC) and RVSM cells from cytokine-induced cell injury. Therefore, we evaluated cell survival and viability when cytokine exposure was initiated at different time points after isoflurane pretreatment (1.5% for 30 min), thus creating a time course of immediate and delayed preconditioning for multiple cell.

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IMMEDIATE AND DELAYED ISOFLURANE PRETREATMENT

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Materials and Methods

Cell Cultures, Cytokines, and Isoflurane Pretreatment

The endothelial and RVSM cells were gifts from Lisa A. Palmer, Ph.D., Associate Professor, and Tom G. Obrig, Ph.D., Professor at the University of Virginia. RVSM cells were grown in Dulbecco’s Minimal Essential Medium (HAM’s/F12; Gibco, Rockville, MD) supplemented with 20% fetal bovine serum (Gibco). BPECs were cultured in Modified Eagle Medium (Gibco), supplemented with 4.8 μg/ml thymidine and 10% fetal bovine serum. HMECs were grown in molecular cellular and developmental biology medium (Gibco), supplemented with 29.2 mg/ml L-glutamine, 1 μg/ml hydrocortisone (Sigma Chemical, St. Louis, MO), 10 μg/ml epidermal growth factor (BD Biosciences, Bedford, MA), and 15% fetal bovine serum (Gibco). Confluent cell cultures of passages 7–12 were seeded with a density of 4 × 10^5/ml in 24-well plates and allowed to attach overnight.

The cytokines were purchased from R&D Systems (Minneapolis, MN) and dissolved into phosphate-buffered saline (Gibco) and further diluted into the medium at the following concentrations: 0.1 ng/ml tumor necrosis factor-α (5.0 ng/ml), interferon-γ (5.0 ng/ml), and interleukin-1β (5.0 ng/ml). The cells were pretreated with isoflurane (1.5%) for 30 min in an airtight chamber by ventilating the chamber with 100% oxygen and isoflurane. The isoflurane concentration in the chamber was measured with a gas analyzer (Datex Ohmedea, Madison, WI). After isoflurane pretreatment, the cells were washed with phosphate-buffered saline and incubated with fresh medium.

Immediate and Delayed Pretreatment Protocols

To evaluate immediate pretreatment, the 72-h exposure to cytokines was initiated directly after isoflurane in each cell type (fig. 1A). Delayed effects of isoflurane pretreatment were obtained in the same experiment by delaying the cytokine exposure by 1, 2, 4, 8, 12, 16, 20, 24, or 48 h (fig. 1B), enabling direct comparison between the immediate and delayed effects of isoflurane pretreatment. In the control groups, cells were exposed to 100% oxygen with or without isoflurane for 30 min, washed with phosphate-buffered saline, and incubated in fresh medium for 72 h or, for the delay experiments, 72 plus 1–48 h. Cells in the cytokine-only group were exposed to 100% oxygen only and either directly incubated in medium with cytokines for 72 h or incubated after a delay of 1–48 h.

Measurement of Cell Survival and Viability

Cell survival and viability were evaluated after cytokine exposure. Because dead cells are incapable of excluding trypan blue (Sigma), the uptake of this dye was used as a marker of cell death. Trypan blue (10 μl) was mixed with 50 μl of cell suspension. For each sample, at least 100 cells per field were counted with a hemocytometer under a light microscope. Cell survival was calculated as the number of total cells minus the number of dead cells and expressed as percentage of the total number of cells per field.

Cell viability was evaluated by measuring the release of lactate dehydrogenase (LDH) into the medium, which is a result of disruption of the cell plasma membrane. The LDH assay, performed according to the protocol provided by the vendor (Sigma), is based on the reduction of nicotinamide-adenine dinucleotide by the action of LDH, and the resulting hydrolyzed nicotinamide-adenine dinucleotide is used in the stoichiometric conversion of a tetrachloride dye. The absorbance was measured spectrophotometrically at 490 nm and the background absorbance of the plates at 690 nm. The resulting difference correlates with the extent of LDH release.

Role of K<sub>ATP</sub> Channels

To evaluate the role of the mitochondrial and cell membrane K<sub>ATP</sub> channels in immediate and delayed (12 or 24 h only) isoflurane protection, we examined the effects of the nonselective K<sub>ATP</sub> channel antagonists gly-

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benclamidine, (10 μM, Sigma), the mitochondrial K<sub>ATP</sub> channel antagonist 5-hydroxydeconoate (5-HD, 100 μM; ICN Biomedicals, Aurora, OH) or the cell membrane K<sub>ATP</sub> channel antagonist 1-[5-[2-(5-chloro-o-anisamido) ethyl]-2-methoxyphenyl]sulfonyl-3-methylthiourea (HMR-1098, 10 μM-1 mm; a gift from Aventis Pharma, Frankfurt am Main, Germany). The K<sub>ATP</sub> channel antagonists were administered directly before the isoflurane pretreatment (fig. 1C and D). Diazoxide (100 μM, ICN Biomedicals) was administered for 30 min (then washed out with phosphate-buffered saline) before cytokines to evaluate if the mitochondrial K<sub>ATP</sub> channel agonist would mimic the protective effects of isoflurane pretreatment.

**Statistical Analysis**

Cell counting was performed in a blinded manner. Comparisons between immediate and delayed protection, within each cell line and between cell lines, the effects of K<sub>ATP</sub>-antagonists within each cell line, and comparison between isoflurane and diazoxide were made with two-way (multiple) analysis of variance and a student Newman-Keuls post hoc test. Statistical analysis was performed with SigmaStat 2.0 (Jandel Scientific Software, San Rafael, CA). Data (n = 6 each group) are presented as mean ± SD. A P < 0.05 was considered significant.

**Results**

**Immediate and Delayed Protective Effects of Isoflurane Pretreatment against Cytokine-induced Injury**

The objective of the first experiment was to compare immediate and delayed protection against cytokine-induced cell death. In all cell lines, control cultures in the presence or absence of isoflurane showed less then 10% cell death, whereas cells in cultures only exposed to cytokines for 72 h had more than 80% cell death as determined by trypan blue uptake. In control experiments in the presence or absence of isoflurane, or in experiments with cytokines only, cell death was not altered in any cell line if there was a delay of 1–48 h before the 72-h incubation period. In contrast, in cells exposed to isoflurane followed by cytokines, there were immediate and delayed protective effects of isoflurane pretreatment on cell survival and viability from cytokine-induced injury exposure in BPECs, HMECs, and RVSM cells (fig. 2). Isoflurane pretreatment administered immediately before or 1 h before cytokine exposure equally attenuated cytokine-induced cell death by approximately 70% in all three cell lines. The immediate protective effect of isoflurane pretreatment was equal in all cell types but was lost after a 4-h delay in HMECs and BPECs, and after 2 h in RVSM cells.

In BPECs, cell survival was restored when the interval between isoflurane pretreatment and cytokine exposure was extended to 8 h and was equal to the immediate effects after a 12-h delay. The delayed protective effect in BPEC was less than the immediate effect by 24–48 h but greater than without isoflurane pretreatment. In HMECs and RVSMs, cell survival was increased with isoflurane pretreatment compared with cells not pretreated, but not equal to immediate effects, when cytokine exposure was delayed for 8 h. When the time between isoflurane pretreatment and cytokine exposure was extended to 24–48 h, cell survival was equal to the immediate protective effect. In cultures with a delay of 4–16 h, BPEC showed greater cell survival than HMECs and RVSM cells, whereas in cultures with a delay of 20–48 h, HMECs and RVSM cells showed greater survival than BPECs. There was a delay period of 4–8 h in HMECs, 2–8 h in RVSM cells, and 4 h in BPECs, during which time isoflurane pretreatment did not significantly increase cell survival.

The LDH release in controls was 0.63 ± 0.05, 0.64 ±
levels when isoflurane and cytokines, LDH release was equal to control in the presence or absence of isoflurane in human microvascular endothelial cells. K\textsubscript{ATP}-antagonists had no effect in any group. Data are mean ± SD (n = 6). 5-HD = 5-hydroxydecanoate; HMR-1098 = 1-[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl-3-methylthiourea.

0.03, and 0.15 ± 0.02 in the absence of isoflurane and 0.66 ± 0.03, 0.065 ± 0.02, and 0.14 ± 0.02 in the presence of isoflurane in BPECs, HMECs and RVSM cells, respectively. LDH release in cultures exposed to cytokines only was 1.23 ± 0.15, 1.20 ± 0.82 and 2.20 ± 0.80, respectively. In control experiments in the presence or absence of isoflurane, or in experiments with only cytokines in human microvascular endothelial cells, isoflurane exposure was delayed to 24 h. When cytokine exposure was delayed to 24 h after isoflurane pretreatment, LDH release was increased compared with cytokines only if there was a delay of 1–48 h before the 72-h incubation period. In contrast, in cells exposed to isoflurane and cytokines, LDH release was equal to control levels when isoflurane was administered immediately before or 1 h before cytokines, but significantly increased when isoflurane was administered 2–4 h before cytokines. In BPECs, LDH release decreased to control levels when the time between isoflurane pretreatment and cytokines was extended to 12 h. When cytokine exposure was delayed to 24–48 h after isoflurane pretreatment, LDH release was increased compared with control levels but remained attenuated compared with cytokines only. In contrast, in HMECs and RVSM cells, LDH release decreased when isoflurane pretreatment was administered more than 4 h before cytokine exposure and reached levels equal to control at 20 h in HMECs and 24 h in RVSM cells (fig. 2).

The Role of K\textsubscript{ATP} Channels in Immediate and Delayed Effects of Isoflurane Pretreatment

The role of K\textsubscript{ATP} channels in modulating the effects of isoflurane pretreatment were evaluated during immediate and delayed pretreatment of 12 and 24 h. In all cell lines and at each period, (0, 12, and 24 h), K\textsubscript{ATP} channel antagonists had no effect on cell survival or viability in control cultures in presence or absence of isoflurane and did not alter cytokine-induced cell injury in any cell line under immediate or delayed protocols (fig. 3 shows an example in HMECs under the immediate protocol). In contrast, glybenclamide (10 μM) and the mitochondrial K\textsubscript{ATP} channel antagonist, 5-HD (100 μM), abolished or attenuated the isoflurane-induced immediate and delayed protection (figs. 4–6). The cell membrane K\textsubscript{ATP} channel antagonists HMR-1098 (10 μM, 100 μM, and 1 mM) did not produce immediate or delayed protection in BPECs, HMECs, or RVSM cells (figures show 100 μM only).

In all cell lines the control cultures in presence of absence of diazoxide showed 10% or less cell death, whereas cells in cultures only exposed to cytokines for 72 h had more then 85% cell death. These results were not altered in any cell line if there was a delay of 1–48 h before the 72-h incubation period. In contrast, in BPECs, HMECs, and RVSM cells pretreated with diazoxide and exposed to cytokines, diazoxide induced immediate and delayed protection on cell survival (fig. 7). When diazoxide was administered immediately before or 1 h before cytokine exposure, cell survival was increased by approximately 70% in all three cell lines compared with cells not pretreated. Cell protection was significantly

![Fig. 3. The effects of adenosine triphosphate-sensitive potassium (K\textsubscript{ATP}) antagonists on cell survival in control cells in the presence or absence of isoflurane, or in experiments with only cytokines in human microvascular endothelial cells. K\textsubscript{ATP}-antagonists had no effect in any group. Data are mean ± SD (n = 6). 5-HD = 5-hydroxydecanoate; HMR-1098 = 1-[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl-3-methylthiourea.](image)

![Fig. 4. Cell survival (A) and lactate dehydrogenase (LDH) release (B) in bovine pulmonary endothelial cells after immediate or delayed (12 h or 24 h) isoflurane pretreatment in absence or presence of adenosine triphosphate-sensitive potassium (K\textsubscript{ATP}) inhibitors (glybenclamide, 5-hydroxydecanoate [5-HD], or 1-[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl-3-methylthiourea [HMR-1098]). Asterisk denotes that the K\textsubscript{ATP} inhibitors abolished the protective effects of isoflurane pretreatment. Number sign denotes that K\textsubscript{ATP} inhibitors significantly attenuated the protective effects of isoflurane pretreatment. Data are mean ± SD (n = 6). Significance was considered as P < 0.05.](image)
decreased when diazoxide was administered 2 h before cytokines, and abolished after a 4–8-h delay. In HMECs and RVSM cells, diazoxide pretreatment 12–20 h before cytokine exposure significantly increased cell survival compared with cells not pretreated. When administered 24–48 h before cytokine exposure, diazoxide was equally protective compared with the immediate effects. In contrast, cell survival in BPECs was equal to the immediate effects when cytokine exposure was delayed 12 h. However, the delayed protective effects then decreased, but remained greater than nonpretreated cells when cytokines exposure was delayed by 24–48 h.

The LDH release in controls was 0.20 ± 0.02, 0.23 ± 0.07, and 0.18 ± 0.01 in the absence of diazoxide and 0.18 ± 0.02, 0.24 ± 0.04, and 0.17 ± 0.01 in the presence of diazoxide in BPECs, HMECs, and RVSM cells, respectively. LDH release in cultures exposed to cytokines only was 1.07 ± 0.15, 1.46 ± 0.14, and 1.34 ± 0.06, respectively. These results were not altered in any cell type if there was a delay of up to 48 h before the 72-h incubation period. In contrast, in cells exposed to diazoxide followed by cytokines, LDH release was equal to control levels when diazoxide was administered immediately before or 1 h before cytokine exposure, but significantly increased when diazoxide was administered 2 h before cytokine exposure in all three cell lines. LDH release was equal to cytokine levels after a 4-h delay in BPEC, a 4–16-h delay in HMECs, and a 4–8-h delay in RVSM cells. In BPECs, LDH release decreased to control levels when the time between diazoxide pretreatment and cytokines was extended to 12 h. When cytokine exposure was delayed by 24–48 h, LDH release was increased compared with control levels but remained attenuated compared with cytokines only. In contrast, LDH release was significantly attenuated when the time between diazoxide and cytokines was extended to 20 h in HMECs and to 12 h in RVSM cells, and equal to immediate pretreatment at 24–48 h in both cell lines.

**Discussion**

Based on our previous study, which showed that isoflurane and halothane have protective effects against cytokine-induced cell injury in cell culture, we hypothesized that isoflurane pretreatment may trigger immedi-
there were no protective effects of isoflurane pretreatment, the protective effects reappeared after a longer delay in all three cell lines. In BPECs, delayed protection by isoflurane was equal to the immediate effect when the time between isoflurane pretreatment and cytokines exposure was extended to 12 h, but the delayed protective effect continuously decreased from 16–48 h (however, the effect remained greater than without pretreatment). In contrast, in HMECs and RVSM cells, the delayed protective effects were equal to the immediate effects after 24 h and sustained until 48 h. The difference in the time course of delayed protection might be due to differences in cell type and species. No other studies have extensively evaluated the time course of immediate and delayed protection; however, the delayed isoflurane-induced protection reported in our study at 12 and 24 h is consistent with other in vivo studies of delayed myocardial IPC,10,16 the studies showing delayed volatile anesthetic myocardial preconditioning,14 and delayed IPC in vitro in myocytes.12

Glybenclamide and 5-HD abolished or attenuated both the immediate and delayed protective effects of isoflurane pretreatment whereas HMR-1098 had no effect. The observation that cell membrane KATP channels may not be involved in preconditioning is consistent with some in vivo and in vitro studies showing that anesthetic preconditioning and IPC of the myocardium are unaffected by inhibition of sarcolemmal (cell membrane) KATP channels.17,18 However, volatile anesthetics have been reported to modulate sarcolemmal KATP channels in ventricular myocyte whole cell patch clamp models.19 Both sarcolemmal and mitochondrial KATP channels have been shown to be involved in delayed anesthetic preconditioning with isoflurane in rabbit myocardium against ischemia–reperfusion injury.14 In addition, a study using knockout mice illustrated the importance of sarcolemmal KATP channels in cardioprotection against ischemia–reperfusion injury.20 Although cell membrane KATP channels have been isolated in endothelial21 and vascular smooth muscle cells,22 our study suggests that in endothelial and vascular smooth muscle cells the membrane KATP channels do not play a significant role in isoflurane-induced protection. It is possible that sarcolemmal or cell membrane KATP channels only modulate protection in excitable cells such as myocytes.

Glybenclamide and 5-HD had no effect in controls in the absence or presence of isoflurane or in experiments with cytokines only, yet attenuated the protection of isoflurane from cytokine-induced injury. In addition, diazoxide induced a similar time course of immediate and delayed protection as isoflurane. These results suggest that mitochondrial KATP channels modulate both the immediate and delayed protective effects of isoflurane pretreatment. Volatile anesthetics have been reported to activate mitochondrial KATP channels in vitro (myocytes) as suggested by flavoprotein oxidation.17 Several
studies have provided evidence that glybenclamide and 5-HD prevent the protection induced by volatile anesthetic preconditioning in myocardium,23–24 and that opening of the mitochondrial K\textsubscript{ATP} channels by the selective agonist diazoxide mimics anesthetic preconditioning and IPC.25 The precise mechanism by which opening of the mitochondrial K\textsubscript{ATP} channels modulates immediate protective effects remains unclear. Opening of mitochondrial K\textsubscript{ATP} channels has been suggested to result in membrane depolarization, matrix swelling, and slowing of adenosine triphosphate phosphate-buffered saline synthesis, accelerated respiration, and reduced calcium overload.25 It has also been hypothesized that opening of mitochondrial K\textsubscript{ATP} channels may maintain the tight apposition of the inner and outer membranes of the mitochondria, thereby preserving the structure and function of the mitochondria.\textsuperscript{26} In addition, activation of mitochondrial K\textsubscript{ATP} channels may have antiapoptotic effects by inhibiting cytochrome c release and the loss of mitochondrial membrane potential,27 both of which are early events in the cell death cascade.28 In our study, however, K\textsubscript{ATP} antagonists had no effect on cell death secondary to exposure to cytokines alone. Although our previous study provided evidence that cytokine-induced cell death might be caused by apoptosis,\textsuperscript{1} further research is necessary to determine whether the antiapoptotic effects of mitochondrial K\textsubscript{ATP} channel activation could be associated with the protection from cytokine-induced cell injury.

The mitochondrial K\textsubscript{ATP} channel activity that modulates immediate protection seems to diminish or become insufficient to prevent cell injury after 2–4 h, as shown by the loss of the protective effects following this time period in all three cell lines. The time course during which there is no protection between immediate and delayed preconditioning suggests there is a period between the decline of the immediate protective effects of mitochondrial K\textsubscript{ATP} activation and the appearance of delayed protective mechanism; i.e., synthesis of de novo proteins.\textsuperscript{20} It seems that these proteins are activated 8–12 h after isoflurane pretreatment because the delayed protection appears at this time.

The delayed protective effects of isoflurane pretreatment were attenuated or abolished by glybenclamide and 5-HD suggesting that mitochondrial K\textsubscript{ATP} channels modulate delayed protection against cytokine-induced cell injury. Activation of K\textsubscript{ATP} channels is thought to enhance the synthesis of inducible nitric oxide synthase, heat shock proteins, and antioxidant enzymes.\textsuperscript{13} Nitric oxide derived from inducible nitric oxide synthase may be involved in the antioxidant actions and activation of cyclooxygenase-2 with the consequent production of cytoprotective prostanoids such as prostaglandin.\textsuperscript{30} Heat shock proteins are hypothesized to protect cells against stress by stabilizing proteins in an adenosine triphosphate-dependent reaction.\textsuperscript{51–52} Delayed preconditioning also has been correlated with increased expression of antioxidant enzymes, such as superoxide dismutase, that may decrease free-radical generation during reperfusion and increase the availability of nitric oxide.\textsuperscript{15} It is also likely that many of these proteins increase activity of K\textsubscript{ATP} channels. Therefore, we do not know if 5-HD blocked the delayed protective effects of isoflurane pretreatment because K\textsubscript{ATP} initiates delayed protection, or if K\textsubscript{ATP} channels are the end effector. Further investigation is necessary to determine the details of the protective mechanisms that are responsible for the delayed protection of endothelial and RVSM cells from cytokine-induced injury, if they are similar to delayed protection in myocardium, and why the time course of immediate and delayed protection is not identical between different cell lines.

The uptake of trypan blue has been described to measure loss of cell permeability rather than cell death, and it is possible that apoptotic cells may be identified as viable cells.\textsuperscript{53} However, the increase in cell survival secondary to isoflurane pretreatment in the present study (\(= 70\%\)) is comparable with the isoflurane-induced decrease of nick end–labeled fragmented DNA (from 78 ± 1% to 7 ± 1%) in our previous study.\textsuperscript{1} Furthermore, the results obtained with trypan blue uptake are consistent with the cell viability results measured by cell proliferation in our previous study\textsuperscript{1} and LDH release in this study. Nevertheless, we cannot rule out that more detailed evaluation of cell viability, including DNA fragmentation and cell surface protein expression, may yield different results regarding isoflurane-induced protection.

This \textit{in vitro} study, which shows that isoflurane pretreatment has immediate and delayed protective effects in endothelial and RVSM cells against cytokine-induced injury, is consistent with our \textit{in vivo} study showing that isoflurane pretreatment attenuates the detrimental changes in endothelial function and pathology associated with lipopolysaccharide-induced inflammation in rats.\textsuperscript{34} These \textit{in vivo} and \textit{in vitro} studies suggest that isoflurane pretreatment of the endothelium and vascular smooth muscle has similar protective effects as anesthetic preconditioning and IPC of the myocardium. Although immediate and delayed volatile anesthetic preconditioning have not previously been demonstrated in endothelial and RVSM cells, it may not be surprising that volatile anesthetics precondition these cells against both ischemia–reperfusion and inflammation, because ischemia–reperfusion injury causes an inflammatory response with release of proinflammatory cytokines during reperfusion.\textsuperscript{35}

Protection of the endothelium and vascular smooth muscle may have significant clinical implications and important physiologic consequences. The endothelium produces modulators responsible for vasodilatation, provides an antithrombogenic surface, and also plays an important role in the regulation of adhesion and migra-
tion of leukocytes. Protection of the endothelium may be important not only during ischemia-reperfusion injury, but also during the systemic inflammatory response associated with cardiopulmonary bypass and other vascular diseases involving inflammation.

In conclusion, this study shows that isoflurane pretreatment has immediate and delayed protective effects against cytokine-induced injury in BPECs, HMECs, and RVSM cells. The time course of immediate and delayed protection in the three cell lines is similar but not identical, and each contains a period of 2–12 h (depending on the cell line) after isoflurane pretreatment during which there is no protection. Mitochondrial but not cell membrane K<sub>ATP</sub> Channels seem to modulate immediate and delayed isoflurane-induced protection of endothelial and vascular smooth muscle cells.

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References
