Isoflurane Pretreatment Inhibits Cytokine-induced Cell Death in Cultured Rat Smooth Muscle Cells and Human Endothelial Cells

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Background: Anesthetics are protective during ischemic–reperfusion injury and associated inflammation; therefore, the authors hypothesized that anesthetic pretreatment may provide protection in culture from cytokine-induced cell death.

Methods: Rat vascular smooth muscle (VSM) cell and human umbilical vascular endothelial cell (HUVEC) cultures were used to determine whether pretreatment with 30 min of isoflurane decreases cell death from tumor necrosis factor α (TNF-α), interleukin 1 (IL-1β), and interferon (IFN-γ) alone or in combination. Cell survival and viability were determined by trypan blue staining and cell proliferation assay, as well as by DNA fragmentation assays. The roles of protein kinase C (PKC) and adenosine triphosphate-sensitive potassium (KATP) channels in mediating isoflurane (and halothane) protection were evaluated with the antagonists staurosporine or glibenclamide in cytokine- and also hydrogen peroxide (H2O2)-induced cell death.

Results: Pretreatment with 1.5% isoflurane immediately prior to cytokine exposure increased cell survival and viability from cytokines by 10–60% for 24, 48, 72, and 96 h in VSMs and up to 72 h in HUVECs. DNA fragmentation (TUNEL) was also attenuated by isoflurane. Isoflurane was equally effective in VSMs at 0.75, 1.5, and 2.5%, whereas in HUVECs, 1.5 and 2.5% were more effective than 0.75%. In VSMs, isoflurane administered 1 h prior to or simultaneously with cytokines was also effective, whereas isoflurane 2 h prior to cytokines was less effective, and either 4 h prior to or 30 min after cytokines was not effective. In both cytokine- and H2O2-induced cell death, isoflurane and halothane pretreatment were equally protective, and staurosporine and glibenclamide attenuated the protective effect.

Conclusions: Thirty minutes of isoflurane attenuates cytokine-induced cell death and increases cell viability in VSMs for 96 h and in HUVECs for 72 h. Isoflurane must be administered less than 2 h prior to or simultaneously with the cytokines to be protective. These initial inhibitor studies suggest involvement of PKC and KATP channels in isoflurane and halothane protection against both cytokine- and H2O2-induced cell death of VSMs and HUVECs.

ENDOTHELIAL and vascular smooth muscle (VSM) cells play key roles in the physiologic function of the pulmonary and systemic vasculature. The endothelium synthesizes and releases vasoactive mediators that modulate vascular resistance by altering VSM tone. This important function may be altered by clinical conditions that cause ischemic–reperfusion injury and inflammation of the vascular. Cell death during ischemia–reperfusion and inflammation may result from necrosis or increased apoptosis (programmed cell death). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1β), and interferon (IFN-γ) are released during reperfusion injury and induce apoptosis. Isoflurane protects from cytokine-induced cell death, providing a protective effect against inflammatory cell death.

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preconditioning or anesthetic pretreatment on cell death and viability in cell culture have not been studied extensively. Cytokines have been shown to induce apoptosis in cell culture; therefore, we used this model to test the hypothesis that a brief period of isoflurane may protect VSM cells and endothelial cells. We investigated whether 30 min of pretreatment with 1.5% isoflurane inhibits cytokine-induced cell death and increases cell viability in rat VSM and human umbilical vascular endothelial cells (HUVEC). Since previous in vivo studies have indicated that the timing of IPC and anesthetic preconditioning is important, we evaluated the timing required for anesthetic administration to attenuate cytokine-induced cell death. To understand the mechanisms, we evaluated whether the effects of isoflurane pretreatment were similar to those with halothane, whether the pretreatment effects also occurred with H$_2$O$_2$-induced cell death, and whether PKC and/or $K_{ATP}$ are involved in the mechanisms associated with anesthetic pretreatment.

Materials and Methods

**Cell Cultures**

Rat aorta VSMs were donated by Dr. L. Palmer, Ph.D. (Associate Professor, Departments of Anesthesiology and Pediatrics, University of Virginia Health System, Charlottesville, Virginia). The HUVECs were a gift from Dr. Tom G. Obrig, Ph.D. (Department of Internal Medicine and Immunology, University of Virginia Health System). The SMCs were grown in Dulbecco’s minimal essential growth medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS). HUVECs were grown in medium bought from the Roswell Park Memorial Institute (Buffalo, New York), supplemented with 50 μg/ml endothelial mitogen (Biomedical Technologies, Fishers, IN), 29.2 mg/ml l-glutamine, 76 μg/ml heparin (Sigma, St. Louis, MO), and 20% FBS. The cytokines TNF-α, IFN-γ, and IL-1β were purchased from R&D Systems (Minneapolis, MN) and diluted into phosphate-buffered saline (PBS; Gibco) containing 0.1% bovine serum albumin (BSA; Sigma). The cells, passages 5–9, were plated at a density of 4×10⁴/ml.

**Pretreatment and Cytokine Treatment**

The initial experimental groups were pretreated with 1.5% isoflurane for 30 min in an airtight chamber before incubation with the cytokines at 37°C for periods of 24, 48, 72, or 96 h. Assessment was not possible for HUVECs at 96 h because of the need to change the medium for control cell survival. Isoflurane, 1.5%, was administered with an agent-specific vaporizer using a gas mixture of 95% O$_2$ and 5% CO$_2$ at a flow rate of 5 l/min. The isoflurane concentration in the chamber was measured with a gas analyzer (Datex Ohmeda, Finland). When the concentration remained constant at 1.5%, the chamber inlet and outlet were closed, and the chamber was placed in the incubator at 37°C for 30 min. The medium was then replaced with 1 ml fresh medium in which the cytokines were dissolved at the following concentrations: 0.1 ng/ml TNF-α, 5.0 ng/ml IFN-γ, and 5.0 ng/ml IL-1β. The cultures were incubated with no cytokines (negative control), one cytokine, or combinations of two or three cytokines. After the different incubation periods (24, 48, 72, or 96 h), the cells were washed twice with PBS (Gibco) and were then further treated according to the specific assay protocols.

**Measurement of Cell Survival and Viability**

Dead cells are incapable of excluding trypan blue; therefore, the uptake of this dye was used as a marker of cell death. Trypan blue (10 μl) was mixed with 50 μl cell suspension. For each sample, at least 100 cells per field were counted with a hemocytometer under a light microscope. Cell survival is the number of total cells minus the number of dead cells.

Cell viability was determined by proliferation assay (Chemicon International, Temecula, CA) and is based on cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenase as described by Krzniewski et al. Briefly, 100 μl of each sample was incubated with 10 μl of assay reagent in a 96-well plate for 2 h. The absorbance was measured with a spectrometer using a 450-nm filter. The viability of each sample was calculated as percentage of the absorbance of the sample cultured without cytokines.

**Assessment of DNA Fragmentation Assays by TUNEL and ELISA**

An apoptosis plus peroxidase detection kit (Intergen, Purchase, NY) was used to detect DNA fragmentation, by means of nick-end labeling (TUNEL) fragmented DNA with biotinylated deoxyribonucleotide triphosphate (dUTP) as previously described by Wang et al. Briefly, the cells were washed and treated with 20 μg/ml protein kinase K (Sigma). Endogenous peroxide was blocked by covering the sections with 3% H$_2$O$_2$ (Sigma). The biotinylated dUTP was applied to the slides, which then were incubated in a humidified chamber at 37°C for 1 h. The labeled DNA fragments were then stained with an antidigoxigenin monoclonal antibody, and the cells were counterstained by immersion of the slides in 0.5% methyl green (Sigma). For each sample, two slides were made, and the total of stained cells were counted.

The enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to characterize cell death by measurement of 5′-Bromo-2′-deoxy-Uridine (BrdU)-labeled DNA fragments in culture supernatants and cell lysate. Proliferating VSMs were incubated with BrdU, which is incorporated into the genomic DNA. The cells were exposed to all three cytokines or H$_2$O$_2$ (1 mM) for periods of 2, 4, 6, and 8 h.
Aliquots (100 μl) from the supernatant of each sample were transferred to an anti-BrdU antibody–coated 96-well plate. The remaining cells were lysed with a solution containing ethylenediaminetetraacetic acid, BSA, and polyoxyethylene sorbitan. The lysate was also transferred into a BrdU antibody–coated well plate. After incubation for 90 min at room temperature, the samples in both plates were heated by microwave irradiation to denature DNA. Anti-BrdU peroxidase conjugate was added followed by a 90-min incubation period at room temperature. The samples were incubated on a shaker in darkness with 3,3’-5,5’-tetramethylbenzidine until fluorescence developed. This reaction was stopped by 5.5% sulfuric acid, and the absorbance was measured with a spectrophotometer at 450 nm.

Statistical Analysis

Cell counting was performed in a blinded manner. Comparisons between groups (n = 6) were made with an analysis of variance and a Tukey post hoc test. Statistical analysis was performed with SigmaStat 2.0 (Jandel Scientific Software, San Rafael, CA). Data are presented as mean ± SD. P < 0.05 was considered significant.

Results

Effect of Isoflurane Pretreatment on Cytokine-induced Cell Death

The objective of the first experiment was to determine the effect of 30 min of pretreatment with 1.5% isoflurane on cell survival and viability. Isoflurane, 1.5%, was administered for 30 min in VSM and HUVEC cultures immediately prior to replacing the media with media in which TNF-α, IFN-γ, and IL-1β alone, in combinations of two, and all three together, were dissolved. Control cultures not exposed to isoflurane or cytokines showed less than 10% cell death and greater than 90% viability over 72 h for HUVECs and 96 h for VSMs. Isoflurane had no effect on cell viability or cell survival in cultures not exposed to cytokines. Cytokines decreased cell viability and cell survival with all combinations of cytokines and for all time periods. The effects of the cytokines were increased at longer time periods and in the presence of greater combinations of cytokines.

In VSMs, pretreatment with 1.5% isoflurane for 30 min immediately prior to cytokine increased cell survival and increased cell viability for all multiple combinations of cytokines at all time periods by 25–60%. In single cytokine experiments using VSMs, isoflurane pretreatment increased cell survival at all time points except for IL-1β at 24 h, TNF-α at 48 h, and IFN-γ at 72 h and increased cell viability for all time points except TNF-α alone and IL-1β alone at 24 h and TNF-α at 48 h. In HUVECs, isoflurane pretreatment increased cell survival in the presence of IFN-γ, IL-1β, and all three cytokines in combination at 24 h, for all cytokine combinations at 48 h, and all combinations except TNF-α alone at 72 h by 10–45%. Cell viability was increased for all combinations except IL-1β at 72 h. Isoflurane protection is illustrated at 96 h for VSMs (fig. 1) and 72 h for HUVECs (fig. 2).

The Dose-Response Effects of Isoflurane Pretreatment

Because of similar results with most parameters in the initial experiment, the remaining experiments were evaluated only in the presence of all three cytokines (TNF-α, IFN-γ, and IL-1β) and to the time periods at 24 and 72 h. To determine the dose-response effects of isoflurane pretreatment, VSMs and HUVECs were pretreated with 0.75, 1.5, or 2.5% isoflurane for 30 min prior to cytokines.

In VSMs, there was no significant difference between the effects of 0.75, 1.5, and 2.5% isoflurane pretreatment on cell viability and cell survival at either 24 or 72 h. In HUVECs, pretreatment with 0.75% isoflurane had signif-
The Effect of Isoflurane Pretreatment and Cell Protection

To determine whether isoﬂurane has similar effects as isoflurane, VSMs were exposed to 1.5% isoﬂurane or 1.2% halothane for 30 min prior to cytokine exposure. To determine whether isoﬂurane protection and halothane protection were similar in H2O2-induced cell death, pretreatment for 30 min was evaluated in VSMs containing H2O2 (1 mM).

In cytokines, 1.2% halothane signiﬁcantly increased VSM cell survival and increased cell viability to the same extent as did isoﬂurane at 24 and 72 h (data not shown). H2O2 signiﬁcantly decreased cell survival and viability. In H2O2, 1.5% isoﬂurane and 1.2% halothane had the same protective effect on cell survival and viability at 24 and 72 h (ﬁg. 5, 72-h survival only).

The Effect of Glibenclamide and Staurosporine on Isoflurane Pretreatment

We investigated whether glibenclamide, which can inhibit KATP channels, and staurosporine, which can inhibit PKCs, alter the effects of anesthetic pretreatment in VSMs and HUVECs. Pretreatment experiments with 1.5% isoﬂurane were performed in the presence of glibenclamide (10 μM) or staurosporine (4 nM) in cultures exposed to all three cytokines or H2O2 (1 mM).

In VSMs and HUVECs, glibenclamide and staurosporine had no effect on cell survival or viability in cells not exposed to cytokines or H2O2 in the presence or absence of isoﬂurane. Conversely, the pretreatment effects of isoﬂurane in VSM exposed to cytokines were attenuated by glibenclamide and staurosporine at 24 and 72 h. In HUVECs, glibenclamide and staurosporine completely abolished the pretreatment effect of isoﬂurane at 24 and 72 h (ﬁg. 6, 72 h only) Likewise, the effects of isoﬂurane and halothane on cell survival and viability were abolished with glibenclamide and staurosporine in H2O2-induced cell death at both 24 and 72 h (ﬁg. 5, 72-h survival only).

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DNA Fragmentation Studies

A TUNEL assay was used to evaluate the effects of 1.5% isoflurane pretreatment on DNA fragmentation in cytokine experiments. VSMs and HUVECs were evaluated after 72 h. Cytokines increased TUNEL staining by 78 ± 1% in HUVECs and 65 ± 1% in SMCs after 72 h. Isoflurane pretreatment almost completely abolished staining so that it only measured 8 ± 1% in SMCs and 7 ± 1% in HUVECs.

DNA fragmentation assay was measured every 2 h up to 8 h to determine whether there were differences between cytokines and H2O2. In VSMs, cytokines increased lysate DNA fragmentation by 2.5-fold after 8 h but did not alter supernatant DNA fragmentation compared to controls, which is suggestive of apoptosis. In contrast, H2O2 increased supernatant DNA fragmentation by 3.8-fold after 8 h but did not alter lysate DNA fragmentation, which is suggestive of necrosis.

Discussion

Previous in vivo studies indicated that anesthetic pretreatment protects the myocardium from ischemia-reperfusion injury, and in vitro studies indicated that continuous volatile anesthetic administration attenuates apoptosis and cell death.21–23 Thus, we hypothesized that isoflurane pretreatment would protect VSMs and HUVECs from cytokine-induced cell death and increase cell viability. In this study, we showed that 30 min of pretreatment with 1.5% isoflurane increased cell survival and cell viability due to cytokine-induced injury for at least 96 h in VSMs and 72 h in HUVECs. Pretreatment with isoflurane and halothane also increased VSM and HUVEC cell survival and viability for at least 72 h after exposure to H2O2.

The protective effects of isoflurane pretreatment from cytokine-induced cell death were evaluated at three different concentrations ranging from 0.75 to 2.5%. There was no significant difference in the protective effect among all three concentrations in VSMs; however, in HUVECs 1.5% and 2.5% provided greater protection than 0.75%. Lui et al.27 demonstrated that pretreatment with concentrations as low as 0.2% isoflurane were sufficient...
to protect cultured chick myocytes from ischemia-reperfusion injury. While we did not evaluate isoflurane concentrations below 0.75%, it is possible that similarly low concentrations may be protective in our model with VSMs. On the other hand, the decreased effect of 0.75% in HUVECs indicates that low concentrations may not be protective in all cell types. Our study indicates that concentrations above 1.5% may not offer additional protective effects. In fact, Johnson et al. reported that supraclinical concentration of isoflurane (4%) and halothane (2.7%) afforded less protection than clinical concentration during H2O2-induced cell death. Clinically relevant concentrations of volatile anesthetics also are protective in animal models of myocardial ischemia-reperfusion injury, suggesting a close correlation between in vivo and in vitro results. Possible clinical relevance is further supported by evidence that clinical concentrations of isoflurane may decrease myocardial damage in humans undergoing coronary artery bypass surgery.

Protection of VSMs by isoflurane (1.5%) and halothane (1.2%) pretreatment in cytokine- and H2O2-induced injury were the same. Similarly, Zaugg et al. showed that continuous administration of halothane and isoflurane inhibited norepinephrine-induced apoptosis to the same extent. In rat hearts, halothane and isoflurane have also been shown to have similar protective effects on myocardial injury. However, other studies have shown that
the effects of volatile anesthetics are not similar. Johnson et al.\textsuperscript{22} indicated that halothane was more protective than isoflurane in H\textsubscript{2}O\textsubscript{2}-induced cell death. The opposite result was demonstrated by Roscoe et al.,\textsuperscript{31} who indicated that isoflurane but not halothane induced protection of cultured human cardiomyocytes. Whether or not there are significant differences in protection from isoflurane and halothane are undoubtedly model and injury dependent; however, our study indicates that pretreatment with each increases cell survival and viability to the same extent in the presence of cytokines and H\textsubscript{2}O\textsubscript{2} in VSMs and HUVECs.

The timing of the isoflurane administration was critical for increasing cell survival and viability in VSMs. We administered isoflurane either immediately prior to cytokines; 1, 2, and 4 h prior to cytokines; simultaneously with cytokines; or 30 min after cytokines. As long as the 30 min of isoflurane was completed within 1 h prior to the administration of cytokines or administered at the same time as the cytokines, the maximal protective effects were observed. The protective effect decreases when the time between the completion of isoflurane administration and cytokine exposure is extended to 2 h and is abolished at 4 h. When isoflurane treatment is initiated 30 min after the cytokine exposure, there is also no protective effect. These results indicate several important findings. First, isoflurane must be present at the time of or prior to the initiating events of cytokine-induced cell death. In other words, isoflurane is effective in inhibiting the initiation of the cytokine cascade but is ineffective in halting the cascade once initiated. Second, the mechanisms by which isoflurane prevents the initiation of the cytokine-induced cell death are lost after 2–4 h. Previous studies have not evaluated this time period; however, in dog hearts, anesthetics may be delivered as early as 30 min prior to ischemia.\textsuperscript{26} The apparent 2- to 4-h window that we demonstrated is particularly interesting because once the cytokine cascade is inhibited, the increase in cell survival and viability lasts at least 96 h. Because the effects of isoflurane appear to be lost after 2–4 h, it is likely that this increase in long-term survival is explained by inhibition of the initiation of the cytokine cascade. The observation that simultaneous administration of isoflurane with cytokine exposure also was effective indicates that the protective mechanisms have a very rapid onset.

Previous \textit{in vivo} studies have suggested that stimulation of K\textsubscript{ATP} channels or PKC is the protective mechanism for IPC and anesthetic preconditioning against ischemic injury.\textsuperscript{32} Blockade of isoflurane and halothane protection by glibenclamide and staurosporine argues for involvement of similar mechanisms against cytokine- and H\textsubscript{2}O\textsubscript{2}-induced cell death in cultured VSMs and HUVECs. Similar protective effects of volatile anesthetics and associated mechanisms in our cell culture studies compared to \textit{in vivo} studies may not be surprising since ischemic–reperfusion injury is partly mediated by cytokine and H\textsubscript{2}O\textsubscript{2} release.\textsuperscript{5} Importantly, because of the 4-h window, the effects of isoflurane and halothane on PKC and/or K\textsubscript{ATP} channels appear to be transient. The precise mechanisms by which PKC and/or K\textsubscript{ATP} channels may alter cell death are unclear, although the mechanisms appear to be effective against different cytokines alone and in combination and against H\textsubscript{2}O\textsubscript{2}. Because isoflurane must be present at the initiation of cytokines, factors which are responsible for delayed protection, such as elevated heat shock proteins, are unlikely to be involved in our study.\textsuperscript{12,15} Whether these conclusions are also true for IPC or anesthetic preconditioning \textit{in vivo} remains to be studied.

It is well accepted that K\textsubscript{ATP} channels are involved in the mechanistic pathway of both ischemic and anesthetic preconditioning in \textit{in vivo} models of myocardial protection.\textsuperscript{24,26} Volatile anesthetics offer myocardial protection in coronary ligation models, an effect which is inhibited by blocking the K\textsubscript{ATP} channels.\textsuperscript{20} Similarly, K\textsubscript{ATP} channels have been shown to be involved in protection of coronary endothelium during ischemia–reperfusion \textit{in vivo}.\textsuperscript{35} Initial studies focused on the role of sarclemmal K\textsubscript{ATP} channel activity in IPC, but since the isolation of a mitochondrial K\textsubscript{ATP} channel, studies have shown that the mitochondrial channel is crucial to the protection elicited by IPC.\textsuperscript{18,19,54} Ockaili et al.\textsuperscript{17} reported that direct stimulation of the mitochondrial K\textsubscript{ATP} channel produced protection from ischemic injury in rats while blockade of this channel eliminated the protective effect. We evaluated only the effects of nonspecific K\textsubscript{ATP} channel blockade with glibenclamide, and, although it is likely that mitochondrial K\textsubscript{ATP} are more importantly involved, further studies will be required to determine whether the same applies to isoflurane and halothane protection during cytokine- and H\textsubscript{2}O\textsubscript{2}-induced cell death.

Activation of PKC appears to be a critical step in preconditioning because the phenomenon can be induced by PKC activators or blocked by PKC inhibitors. Wang et al.\textsuperscript{10} have shown that stimulation of PKC provides protection against Ca\textsuperscript{2+} overload injury in isolated rat hearts. Zhao et al.\textsuperscript{55} demonstrated that transvected, basally active PKC-\(\delta\) conferred protection from simulated ischemic injury in cell culture. Blockade of PKC abolishes the protective effects on various forms of preconditioning, including Ca\textsuperscript{2+} preconditioning and direct activation of mitochondrial K\textsubscript{ATP} channels in isolated rat hearts.\textsuperscript{10} There are numerous PKC isozymes, of which the \(\epsilon\) isozyme has recently been shown to be critical in IPC.\textsuperscript{57} Staurosporine is a nonspecific antagonist of a PKC, and further evaluation will be required to determine the role of specific PKC isoenzymes in the VSM and HUVECs cell cultures. It has been hypothesized that PKC is part of a linear cascade with the mitochondrial K\textsubscript{ATP} channel as the end.
effector. Sato et al.16 demonstrated that increased mitochondrial $K_{ATP}$ channel opening is protective during an ischemic injury and can be elicited through priming the channel with PKC activity or by direct stimulation of the channel. This linear cascade, however, is challenged by the findings both in vitro and in vivo that blockade of PKC activity with simultaneous direct stimulation of mitochondrial $K_{ATP}$ channels does not lead to any protective effect.10,36 This is supported by our study because if isoflurane increases $K_{ATP}$ channel activity, blockade of PKC by staurosporine should not have abolished the protective effect. Based on these findings, it appears that PKC and mitochondrial $K_{ATP}$ channels are involved in a network more complicated than a simple linear cascade. While previous studies have indicated that these mechanisms apply to ischemic-reperfusion injury, our study suggests that these mechanisms also may be involved in cellular protection from cytokines and $H_2O_2$.

The observation in our study that isoflurane and halothane inhibit cell death in both cytokine- and $H_2O_2$-induced injury may be important. Evaluation of DNA fragments in lysate versus supernatant suggests that cytokines induce apoptosis while $H_2O_2$ causes necrosis. Differentiation of apoptosis and necrosis is complex and is not conclusive by DNA fragmentation assays alone; however, our finding is consistent with other studies that have used additional methodologies.13,23 These results are also consistent with other studies that indicate that volatile anesthetics inhibit both apoptosis and necrosis.22,23 The role of PKC in inhibiting both cytokine-induced apoptosis and $H_2O_2$-induced necrosis is supported by a study by Lui et al.,37 who showed that IPC activation of PKC{alpha} attenuates both apoptosis and necrosis in cardiomyocytes. Furthermore, evidence of the role of isoflurane in inhibition of apoptosis and/or necrosis is suggested by our results indicating staining of DNA fragmentation by TUNEL assay is greatly attenuated in VSMCs and HUVECs.

In conclusion, this study demonstrates that 30 min of pretreatment with 1.5% isoflurane decreases cell death due to cytokine-induced injury for at least 96 h in VSMCs and 72 h in HUVECs. The timing of isoflurane administration is critical as isoflurane inhibits the initiation of the cytokine cascade but does not appear to halt it. These initial inhibitor studies suggest involvement of PKC and $K_{ATP}$ channels in isoflurane and halothane protection against both cytokine- and $H_2O_2$-induced cell death of VSMCs and HUVECs.

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