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Isoflurane Preconditioning Reduces the Rat NR8383 Macrophage Injury Induced by Lipopolysaccharide and Interferon γ

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Background: **Isoflurane exposure before an insult can reduce the insult-induced injury in various organs. This phenomenon is called *isoflurane preconditioning*. The authors hypothesize that isoflurane can precondition macrophages, cells that travel to all tissues and are important in the host defense and inflammation responses.**

Methods: **Rat NR8383 macrophages were pretreated with or without 1–3% isoflurane for 1 h at 30 min before they were incubated with or without 100 ng/ml lipopolysaccharide plus 50 U/ml interferon γ for 24 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Flow cytometry was performed after cells were stained with annexin V and propidium iodide. Inducible nitric oxide synthase protein expression in macrophages was quantified by Western blotting.**

Results: **Lipopolysaccharide plus interferon γ decreased cell viability by approximately 50%. This decrease was dose-dependently inhibited by aminoguanidine, an inducible nitric oxide synthase inhibitor. Lipopolysaccharide plus interferon γ caused inducible nitric oxide synthase expression. This expression was inhibited by pretreatment with 2% but not 1% or 3% isoflurane. Isoflurane at 2% inhibited lipopolysaccharide plus interferon γ–induced accumulation of nitrite, an oxidation product of nitric oxide. Pretreatment with 2% but not 1% or 3% isoflurane improved cell viability. Lipopolysaccharide plus interferon γ increased the number of propidium iodide–positive staining cells. This increase was attenuated by 2% isoflurane pretreatment. The protective effect of 2% isoflurane was abolished by chelerythrine, calphostin C, or bisindolylmaleimide IX, protein kinase C inhibitors.**

Conclusions: **Lipopolysaccharide plus interferon γ causes an inducible nitric oxide synthase–dependent macrophage injury. Isoflurane induces preconditioning effects that may be mediated by protein kinase C in macrophages.**

MACROPHAGES are involved in the host defense and inflammatory responses.[1] Phagocytosis of microorganisms by macrophages results in microbial killing. These cells also release factors, such as cytokines, which participate in immune and inflammatory responses.[1,2] Macrophages are long-lived cells and resistant to death. However, many bacterial infections are characterized by induction of macrophage apoptosis and necrosis.[3–5] Experimental sepsis significantly increases macrophage apoptosis and dysfunction or hyporesponsiveness to lipopolysaccharide,[6,7] indicating a marked decrease of immune functions with sepsis. Suppression of macrophage apoptosis prolongs survival of rats and mice with *Pneumocystis* pneumonia.[8]

Lipopolysaccharide, a component of the outer membrane of gram-negative bacteria, plays a major role in the development and the progress of sepsis and pulmonary inflammation, including acute respiratory distress syndrome.[9,10] Lipopolysaccharide has been a common agent used to induce experimental endotoxemia and acute lung injury.[11,12] Lipopolysaccharide can reduce the viability of macrophages *in vitro*, and interferon γ (IFN-γ) enhances this lipopolysaccharide effect.[13,14] It has been shown that exposure to high concentrations of lipopolysaccharide plus IFN-γ induces macrophage death or apoptosis by overexpression of inducible nitric oxide synthase (iNOS).[15,16]

Isoflurane anesthesia has been shown to decrease lipopolysaccharide-induced inflammatory responses and to improve survival of rats and mice with endotoxic shock.[11,17] It has been generally accepted that volatile anesthetics have antiinflammatory effects in various organs.[11,18,19] Because inflammation is involved in many pathologic processes, such as ischemia–reperfusion injury,[20,21] it is no surprise to see that the presence of volatile anesthetics during a lethal insult is protective. The presence of volatile anesthetics before the insult also has been shown to be protective.[22,23] This phenomenon is called *volatile anesthetic preconditioning*. Such preconditioning effects can be induced in various organs, such as heart and brain, and different cells, including myocytes, neurons, and endothelial cells.[22,24–26] In this study, we test the hypothesis that isoflurane can induce a preconditioning effect in macrophages. We used rat NR8383 macrophage cultures and stimulated these cells with lipopolysaccharide plus IFN-γ.

Materials and Methods

Materials

NR8383 cells (CRL-2192), a rat pulmonary alveolar macrophage cell line, were purchased from American

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Type Culture Collection (Manassas, VA). Heat-inactivated fetal bovine serum and recombinant rat IFN-γ produced from *Escherichia coli* were purchased from Invitrogen Corporation (Carlsbad, CA). Rabbit polyclonal antibody against iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–based colorimetric assay kit was obtained from Roche Applied Science (Roche, IN). Isoflurane was purchased from Abbott Laboratories (North Chicago, IL). Chelerythrine chloride, calphostin C, or bisindolylmaleimide IX were obtained from Biomol (Plymouth Meeting, PA). Alexa Fluor® 488 annexin V–propidium iodide (PI) staining kit and Griess Reagent Kit were purchased from Molecular Probes (Eugene, OR). Lipopolysaccharide (*E. coli* 0111:B4), 0.4% trypan blue solution, aminoguanidine bicarbonate salt, and other general chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture

NR8383 cells were cultured in Ham's F12K medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 15% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 pg/ml streptomycin in a humidified atmosphere of 95% air–5% CO_2 at 37°C. The medium was changed every 2 days. The cells were suspended in the culture medium at a density of 1×10^5 cells/ml and plated at a density of 2×10^4/well on 96-well tissue culture plates for viability experiments by MTT assay or 2×10^6 on 100-mm-diameter dishes for Western blotting and flow cytometry experiments.

Isoflurane Exposure and Application of Chemicals

Isoflurane pretreatment of the cells was performed in an airtight chamber as we described previously.[27,28] Briefly, 3 h after the cells were plated, they were placed in the airtight chamber. The chamber was then gassed with 95% air–5% CO_2 through an isoflurane vaporizer set at 0, 1, 2, or 3% for 15 min at 37°C. The isoflurane concentrations in the gases from the outlet of the chambers were monitored with a Datex infrared analyzer (Capnomac, Helsinki, Finland) and reached the target concentrations at approximately 3 min after the onset of gassing. The chamber was sealed and the incubation was for 45 min at 37°C. At the end of the incubation time, the isoflurane concentrations in the gases from the outlet of the chamber were confirmed to be at the target concentrations. The cells were then removed from the chamber and were kept under their normal and isoflurane-free culture conditions for 30 min before they were exposed to 100 ng/ml lipopolysaccharide plus 50 U/ml IFN-γ for 24 h at 37°C. Various concentrations of aminoguanidine, an iNOS inhibitor, were added to some cells just before the application of lipopolysaccharide plus IFN-γ. These cells were not pretreated with isoflurane. To determine the involvement of protein kinase C (PKC) in the isoflurane preconditioning, the PKC inhibitors chelerythrine (10 μM), calphostin C (20 nM), or bisindolylmaleimide IX (5 nM) were added just before the isoflurane application. After the isoflurane exposure, the incubation medium was replaced with fresh medium without PKC inhibitors before the cells were exposed to lipopolysaccharide plus IFN-γ.

In a separate experiment, the isoflurane concentrations in the incubation solutions were determined by gas chromatography as we described previously.[29] The aqueous isoflurane concentrations at 37°C were approximately 209, 415, and 620 μM, respectively, when 1, 2, and 3% isoflurane was delivered and the samples were taken at the end of the 1-h isoflurane incubation. There was no detectable isoflurane in the incubation solution at the end of the 30-min isoflurane-free period.

Cell Viability and Cell Death

Cell viability was determined by MTT assay according to the manufacturer's procedure and as we described previously.[30] Briefly, 10 μl of the MTT labeling reagent was added to each well that contained 100 μl culture medium and cells. Four hours later, 100 μl of the solubilization solution was added into each well. The plate was kept overnight at 37°C. Absorbance of the samples was measured at 570 nm with the reference wavelength of 650 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). In each experiment, the results of the MTT measurements from the controls without any treatments were set as 100%. The results from the sister cultures, subjected to various treatments, were then calculated as a percentage of the controls.

Macrophage survival was determined by trypan blue exclusion. In brief, 0.2 ml NR8383 cell suspension at a density of 1×10^5/ml was mixed thoroughly with 0.2 ml trypan blue, 0.4%, for 5 min at room temperature. Trypan blue–stained or unstained cells were counted under a microscope with the use of a hemocytometer.

Detection of Cell Necrosis/Apoptosis

Macrophages (approximately 2×10^6 cells) in 100-mm-diameter dishes were pretreated with or without 2% isoflurane for 1 h and then exposed to 100 ng/ml lipopolysaccharide plus 50 U/ml IFN-γ for 24 h at 37°C. Cells were then stained with annexin V–fluorescein isothiocyanate conjugate and PI using an Alexa Fluor® 488 annexin V–PI staining kit according to the manufacturer's protocol. Annexin V–fluorescein isothiocyanate conjugate labels apoptotic cells by binding to phosphatidyl serine exposed on the outer leaflet of the plasma membrane. PI is impermeant to live cells and the early phase of apoptotic cells and binds tightly to the nucleic acids in dead cells. After staining, the cells were analyzed with flow cytometry (FACSCalibur system; Becton, Dickinson and Company Biosciences, Franklin Lakes, NJ).

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Western Blot Analysis

Western blot analysis was performed as we detailed previously.[31] Cells were homogenized in 25 mM Tris hydrochloride, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) α-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. The homogenates were centrifuged at 1,000*g* for 10 min at 4°C. The supernatants were used for Western blotting and loaded at 20 μg protein per lane. The probed protein bands were visualized by the enhanced chemiluminescence reaction. The protein bands were densitometrically analyzed by an ImageQuant 5.0 densitometer (Amersham Biosciences, Piscataway, NJ).

Nitrite Concentration Measurement

Macrophages (approximately 2×10^6 cells) in 100-mm-diameter dishes were pretreated with or without 2% isoflurane for 1 h and then exposed to 100 ng/ml lipopolysaccharide plus 50 U/ml IFN-γ for 24 h at 37°C. The culture medium was collected. The concentrations of nitrite in the culture medium were measured with a Griess Reagent Kit according to the instructions. The absorbance of the samples was read at 570 nm against a standard curve using a microplate reader (Bio-Rad Laboratories).

Data Analysis

Results are presented as mean ± SD from at least three separate experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student-Newman-Keuls test for *post hoc* comparison or Student *t* test as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Lipopolysaccharide plus Interferon γ Induced an Inducible Nitric Oxide Synthase–dependent Decrease of NR8383 Macrophage Viability

Cell survival measured by trypan blue exclusion test decreased as the duration of incubation with 100 ng/ml lipopolysaccharide plus 50 U/ml IFN-γ increased, and approximately 55% cells were injured when the incubation time was 24 h (fig. 1). Consistent with this result, incubation of macrophages with 100 ng/ml lipopolysaccharide plus 50 U/ml IFN-γ for 24 h reduced cell viability assessed by MTT assay to approximately 50% of control cells (fig. 2). The iNOS inhibitor aminoguanidine dose-dependently inhibited the lipopolysaccharide plus IFN-γ–reduced cell viability (fig. 2). Lipopolysaccharide plus IFN-γ also induced the expression of iNOS proteins in these macrophages that did not express iNOS under control conditions (fig. 3). Consistent with the iNOS expression data, lipopolysaccharide plus IFN-γ increased the concentration of nitrite, a stable nitric oxide oxidation product, in the culture medium (fig. 4). These re-

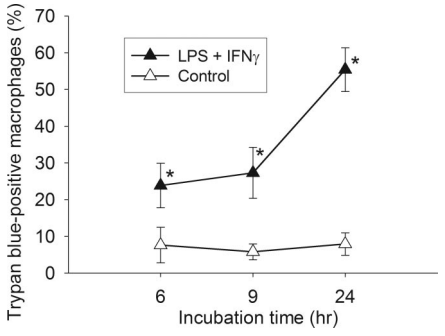

Fig. 1. Time course of lipopolysaccharide (LPS) plus interferon γ (IFN-γ)–reduced cell viability. Rat NR8383 macrophages were incubated with or without 100 ng/ml LPS plus 50 U/ml IFN-γ for various times. Cell survival was assessed by trypan blue exclusion test. Results are mean ± SD (n = 4). * $P < 0.05$ compared with the corresponding cells without the exposure to LPS plus IFN-γ.

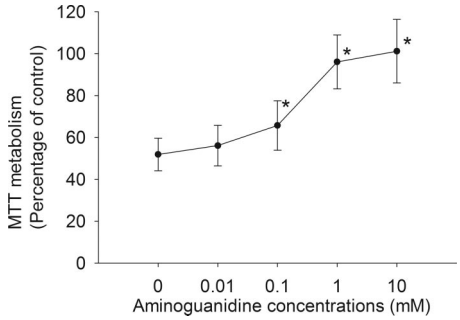

Fig. 2. Dose response of aminoguanidine reversal of lipopolysaccharide plus interferon γ–induced decrease of cell viability. Rat NR8383 macrophages were incubated with or without 100 ng/ml lipopolysaccharide plus 50 U/ml interferon γ in the presence or absence of various concentrations of aminoguanidine. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are mean ± SD (n = 28). * $P < 0.05$ compared with the corresponding cells exposed to lipopolysaccharide plus interferon γ only.

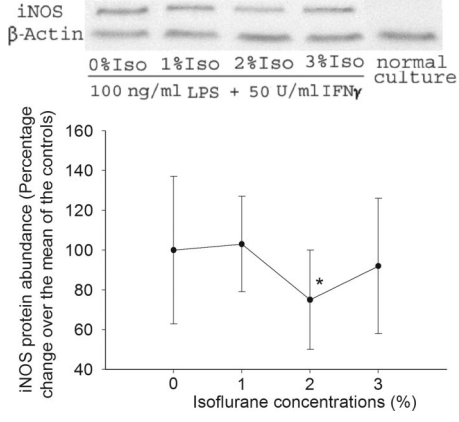

Fig. 3. Effects of isoflurane pretreatment on inducible nitric oxide synthase (iNOS) expression induced by lipopolysaccharide (LPS) plus interferon γ (IFN-γ). Rat NR8383 macrophages were pretreated with or without various concentrations of isoflurane (Iso). They were then exposed to 100 ng/ml LPS plus 50 U/ml IFN-γ for 24 h at 30 min after the isoflurane pretreatment. These cells and the cells that were not exposed to isoflurane and LPS plus IFN-γ (normal culture) were harvested for Western analysis. Results are mean ± SD (n = 9–25). * $P < 0.05$ compared with the corresponding cells exposed to LPS plus IFN-γ only.

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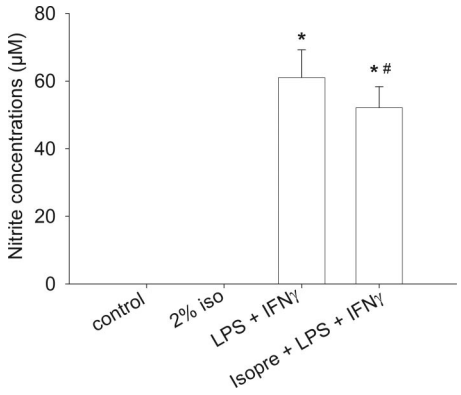

Fig. 4. Effects of isoflurane pretreatment on nitrite production induced by lipopolysaccharide (LPS) plus interferon γ (IFN-γ). Rat NR8383 macrophages were pretreated with or without 2% isoflurane (2% iso). They were then exposed to 100 ng/ml LPS plus 50 U/ml IFN-γ for 24 h at 30 min after the isoflurane pretreatment (Isopre). The culture medium was collected for nitrite measurement. Results are mean ± SD (n = 5). * $P < 0.05$ compared with the control cells. # $P < 0.05$ compared with the cells incubated with LPS plus IFN-γ.

sults suggest that iNOS mediates the lipopolysaccharide plus IFN-γ–reduced macrophage viability.

Isoflurane Pretreatment Induced a Protective Effect

Pretreatment of macrophages with 2% isoflurane, but not 1% or 3% isoflurane, at 30 min before the application of lipopolysaccharide plus IFN-γ significantly improved the viability of the cells (fig. 5). The cell viability assessed by MTT assay was 51.5 ± 7.2 and 61.5 ± 6.8%, respectively, of the control in the absence and presence of 2% isoflurane pretreatment ($P < 0.05$, n = 9). Of note, the viability of cells exposed to 1, 2, and 3% isoflurane alone was 99% ± 21, 97% ± 22, and 94% ± 18% of the control ($P > 0.05$, n = 36), suggesting that isoflurane does not affect cell viability under control conditions. Consistent with the cell viability results, only 2% isoflurane pretreatment significantly decreased the lipopolysaccharide plus IFN-γ–induced expression of iNOS proteins (fig. 3). The expression of β-actin after application of 1, 2, and 3% isoflurane with lipopolysaccharide plus IFN-γ was 98 ± 11, 100 ± 16, and 99 ± 16%, respectively, of that with lipopolysaccharide plus IFN-γ alone ($P > 0.05$, n = 9), indicating that the effects of 2% isoflurane pretreatment on iNOS expression after the application of lipopolysaccharide plus IFN-γ is specific. Isoflurane at 2% also decreased lipopolysaccharide plus IFN-γ–induced increase of nitrite production (fig. 4).

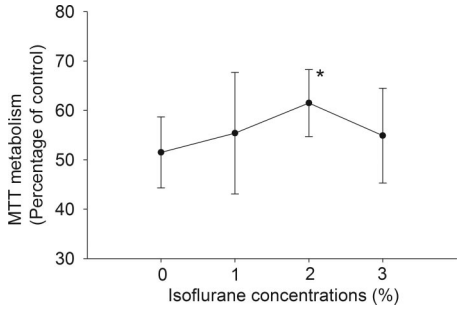

Fig. 5. Isoflurane preconditioning–induced protection. Rat NR8383 macrophages were pretreated with or without various concentrations of isoflurane. They were then exposed to 100 ng/ml lipopolysaccharide plus 50 U/ml interferon γ for 24 h at 30 min after the isoflurane pretreatment. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are mean ± SD (n = 9). * $P < 0.05$ compared with the corresponding cells exposed to lipopolysaccharide plus interferon γ only.

To determine whether cell death was *via* apoptosis or necrosis under our experimental conditions, we stained the cells with annexin V and PI and then sorted the cells by flow cytometry. There were 14 ± 3 and 24 ± 10% cells that were PI- and annexin V–positive staining cells, respectively, under control conditions (cells that were positive staining for both PI and annexin V contributed to both percentages). Incubation of macrophages with lipopolysaccharide plus IFN-γ for 24 h increased the PI-positive staining cells to 47 ± 2% (n = 8, $P < 0.05$ compared with the control) and annexin V–positive staining cells to 45 ± 26% (n = 8, $P < 0.05$ compared with the control). These results suggest that lipopolysaccharide plus IFN-γ increased macrophage necrosis and apoptosis. Pretreatment of macrophages with 2% isoflurane before the application of lipopolysaccharide plus IFN-γ significantly reduced the lipopolysaccharide plus IFN-γ–induced increase of PI-positive staining cells but not the annexin V–positive staining cells (fig. 6), indicating that isoflurane preconditioning reduces the macrophage necrosis but not the apoptosis after lipopolysaccharide plus IFN-γ application.

Isoflurane Preconditioning-induced Protection May Be Mediated by Protein Kinase C

The PKC inhibitors chelerythrine, calphostin C, and bisindolylmaleimide IX abolished the 2% isoflurane preconditioning–induced protection in macrophages stimulated by lipopolysaccharide plus IFN-γ, although these inhibitors did not affect the viability of macrophages after the application of lipopolysaccharide plus IFN-γ only (fig. 7). These results suggest a role of PKC in the isoflurane preconditioning–induced protection in macrophages.

Discussion

Isoflurane has been shown to induce a preconditioning effect in various organs and cell types against ischemia–reperfusion injury.[22,24–26] Because inflammation contributes to the ischemia–reperfusion injury and is a pathophysiologic process involved in many diseases, it is important to determine whether isoflurane induces a preconditioning effect in cells participating in inflammation. Our study showed that isoflurane induced a preconditioning effect in macrophages, cells that travel around the whole body and involve in the host defense and inflammation.

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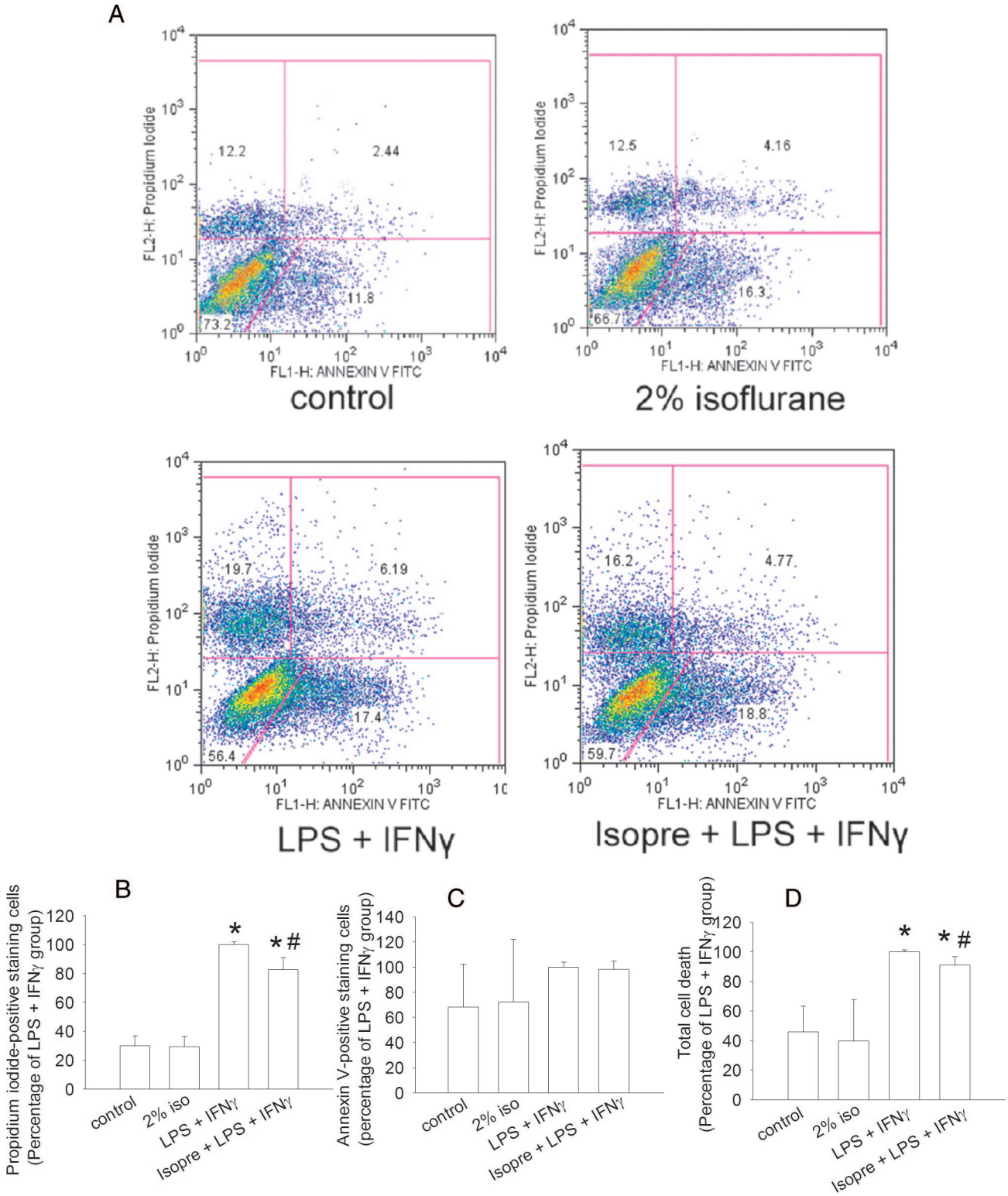

Fig. 6. Effects of isoflurane pretreatment on macrophage necrosis and apoptosis induced by lipopolysaccharide (LPS) plus interferon γ (IFN-γ). Rat NR8383 macrophages were pretreated with or without 2% isoflurane (2% iso). They were then exposed to 100 ng/ml LPS plus 50 U/ml IFN-γ for 24 h at 30 min after the isoflurane pretreatment (Isopre). These cells were stained with annexin V or propidium iodide, and then were analyzed by flow cytometry. (*A*) A representative of sorted cells by flow cytometry. (*B*) Propidium iodide–positive staining cells. (*C*) Annexin V–positive staining cells. (*D*) Cells that were either propidium iodide– or annexin V–positive staining. Results are mean ± SD (n = 8). * $P < 0.05$ compared with the control cells. # $P < 0.05$ compared with the corresponding cells exposed to LPS plus IFN-γ only. FITC = fluorescein isothiocyanate.

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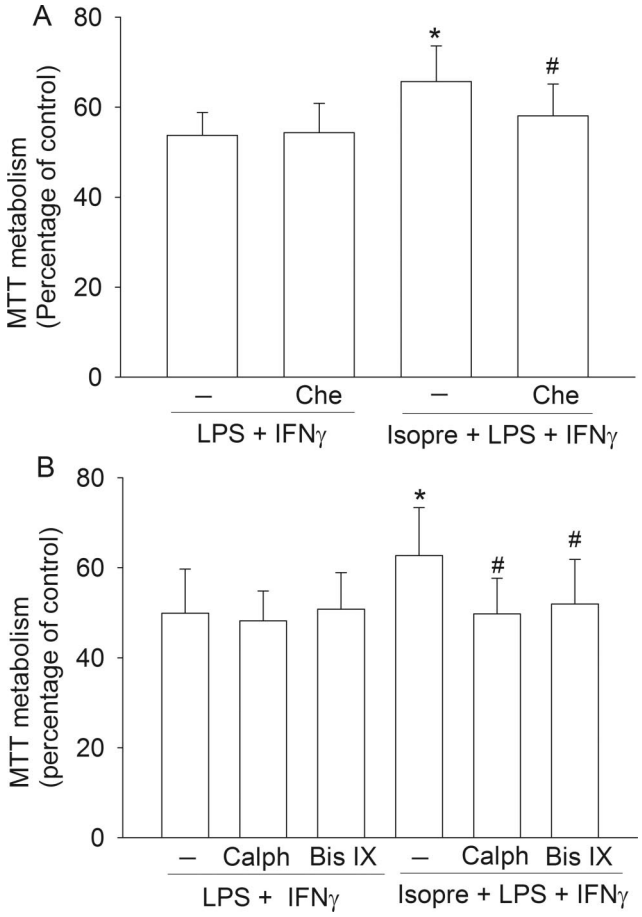

Fig. 7. Attenuation of isoflurane preconditioning-induced protection by protein kinase C inhibition. Rat NR8383 macrophages were pretreated with or without 2% isoflurane in the presence or absence of 10 μM chelerythrine (Che) (*A*), 20 nM calphostin C (Calph), or 5 nM bisindolylmaleimide IX (Bis IX) (*B*). They were then exposed to 100 ng/ml lipopolysaccharide (LPS) plus 50 U/ml interferon γ (IFN-γ) for 24 h at 30 min after the isoflurane pretreatment (Isopre). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are mean ± SD (n = 22–27). * $P < 0.05$ compared with the corresponding cells exposed to LPS plus IFN-γ only. # $P < 0.05$ compared with the cells pretreated with 2% isoflurane and then LPS plus IFN-γ.

It has been a well-known phenomenon that proinflammatory cytokines and bacterial products trigger iNOS expression and nitric oxide production in various cells, including macrophages.[31] Nitric oxide can then produce proinflammatory and cell-destructive effects. Our results showed that stimulation of NR8383 cells with lipopolysaccharide plus IFN-γ for 24 h induced a significant amount of iNOS protein expression and decreased the cell viability in rat macrophages. This decreased cell viability was dose-dependently reversed by aminoguanidine, an iNOS inhibitor. These results indicate that lipopolysaccharide plus IFN-γ–induced rat NR8383 macrophage injury is iNOS dependent.

It has been established that nitric oxide, lipopolysaccharide alone, and lipopolysaccharide plus IFN-γ induce macrophage apoptosis.[15,32–34] Consistent with this idea, our study showed that lipopolysaccharide plus IFN-γ increased the annexin V–positive staining cells. Annexin V binds to phosphatidyl serine exposed on the surface of the plasma membrane, a characteristic of cell apoptosis. Our study also showed that lipopolysaccharide plus IFN-γ increased the PI-positive staining cells. PI is a highly polar fluorescent dye and penetrates damaged plasma membrane to bind to DNA. It has been used as a marker to stain necrotic cells. Therefore, our results suggest that lipopolysaccharide plus IFN-γ increases macrophage necrosis. To support our finding, lipopolysaccharide has been shown to induce macrophage death *via* caspase-independent autophagy.[32]

Our results showed that 2% isoflurane induced a preconditioning effect in macrophages. Pretreatment with this concentration of isoflurane also decreased the lipopolysaccharide plus IFN-γ–induced iNOS expression and the nitrite production. These results suggest that isoflurane preconditioning reduces iNOS expression to protect the macrophages from lipopolysaccharide plus IFN-γ–induced injury. Interestingly, 2% isoflurane pretreatment attenuated the lipopolysaccharide plus IFN-γ–induced macrophage necrosis but did not affect the lipopolysaccharide plus IFN-γ–induced macrophage apoptosis. Isoflurane preconditioning has been shown to reduce cell apoptosis in other cells, such as myocardium and brain cells,[22,25] after ischemia. Future studies are needed to determine why isoflurane preconditioning did not reduce lipopolysaccharide plus IFN-γ–induced macrophage apoptosis.

Our results showed that chelerythrine, calphostin C, and bisindolylmaleimide IX abolished the 2% isoflurane pretreatment–induced protection in macrophages. Chelerythrine, bisindolylmaleimide IX, and calphostin C are structurally different PKC inhibitors. Therefore, our results strongly suggest a role of PKC in the isoflurane preconditioning effects on macrophages. Consistent with our results, PKC may play a role in the isoflurane preconditioning effect against cytokine-induced cell injury in smooth muscle cells and endothelial cells.[26] It has been shown that volatile anesthetics may directly activate PKC,[35] and many of isoflurane pharmacologic effects on various cells and tissues may be mediated by PKC.[28,36] In addition, although PKC activation can increase lipopolysaccharide- and cytokine-induced iNOS expression in macrophages,[37] activation of certain PKC isozymes, such as PKC-ε, also can lead to the inhibition of iNOS expression in macrophages stimulated by lipopolysaccharide, IFN-γ, or other cytokines.[38,39] Therefore, these previous findings are consistent with our identified mechanisms that isoflurane preconditioning reduces lipopolysaccharide plus IFN-γ–induced iNOS overexpression to provide protection in macrophages and that activation of PKC may play a role in this effect.

We observed that 2% isoflurane, but not 1% or 3% isoflurane, induced a preconditioning effect. This bell-

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shaped response has been reported previously in other biologic effects of isoflurane.[27,36] The mechanisms for loss of protection by a higher concentration of isoflurane are not clear. However, the phenomenon may not be due to the isoflurane toxicity because various concentrations of isoflurane alone did not affect the viability of macrophages in our study. Because 3% isoflurane pretreatment also did not inhibit lipopolysaccharide plus IFN-γ–induced iNOS overexpression and activation of different PKC isozymes may result in increase or decrease of iNOS expression in the macrophage stimulated by lipopolysaccharide or cytokines,[37,38] it is possible that 3% isoflurane may activate PKC isozymes that can increase iNOS expression and, therefore, cancel out the inhibition of iNOS expression by isoflurane pretreatment *via* other PKC isozymes. Activation of selective PKC isozymes by different concentrations of isoflurane was proposed to explain the biphasic effects of isoflurane on contraction in skinned pulmonary arterial strips.[36]

The antiinflammatory effects of isoflurane when it is present during the application of proinflammatory reagents have been shown in many studies.[11,18,19] Recently, isoflurane has been revealed to induce a preconditioning effect against lipopolysaccharide-induced inflammation and injury in lungs and vasculature in rats and mice.[12,18] Our findings that isoflurane pretreatment reduces iNOS expression in macrophages and improves the viability of these cells after the challenge of lipopolysaccharide plus IFN-γ may help to explain the results of these previous *in vivo* studies, especially the results from pulmonary study because our macrophages are derived from lungs. Our findings also may have implications for clinical medicine and experimental biology because isoflurane is frequently used in animal studies and in patients with ongoing pulmonary infection and inflammation.

In summary, we have shown that lipopolysaccharide plus IFN-γ causes an iNOS-dependent macrophage injury. Isoflurane preconditioning induces a protection against lipopolysaccharide plus IFN-γ–induced macrophage necrosis. This protection may be mediated by PKC. Considering that isoflurane is a commonly used anesthetic in clinical practice and that animal studies and surgery often induces pulmonary inflammation and infection, our study may have broad implications.

References

1. Nathan CF: Neutrophil activation on biological surfaces: Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J Clin Invest 1987; 80:1550–60
2. Aderem A: Role of Toll-like receptors in inflammatory response in macrophages. Crit Care Med 2001; 29:S16–8
3. Zychlinsky A, Sansonetti P: Perspectives series: Host/pathogen interactions. Apoptosis in bacterial pathogenesis. J Clin Invest 1997; 100:493–5
4. Williams TE, Ayala A, Chaudry IH: Inducible macrophage apoptosis following sepsis is mediated by cysteine protease activation and nitric oxide release. J Surg Res 1997; 70:113–8
5. Ayala A, Urbanich MA, Herdon CD, Chaudry IH: Is sepsis-induced apoptosis associated with macrophage dysfunction? J Trauma 1996; 40:568–73
6. Reddy RC, Chen GH, Newstead MW, Moore T, Zeng X, Tateda K, Standiford TJ: Alveolar macrophage deactivation in murine septic peritonitis: Role of interleukin 10. Infect Immun 2001; 69:1394–401
7. Deng JC, Cheng G, Newstead MW, Zeng X, Kobayashi K, Flavell RA, Standiford TJ: Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M. J Clin Invest 2006; 116:2532–42
8. Lasbury ME, Durant PJ, Ray CA, Tschang D, Schwendener R, Lee CH: Suppression of alveolar macrophage apoptosis prolongs survival of rats and mice with Pneumocystis pneumonia. J Immunol 2006; 176:6443–53
9. Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR: Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production *in vivo*. Am J Physiol Lung Cell Mol Physiol 2000; 278:L840–7
10. Tobias PS, Mathison JC, Ulevitch RJ: A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J Biol Chem 1988; 263:13479–81
11. Fuentes JM, Talamini MA, Fulton WB, Hanly EJ, Aurora AR, De Maio A: General anesthesia delays the inflammatory response and increases survival for mice with endotoxic shock. Clin Vaccine Immunol 2006; 13:281–8
12. Reutershan J, Chang D, Hayes JK, Ley K: Protective effects of isoflurane pretreatment in endotoxin-induced lung injury. ANESTHESIOLOGY 2006; 104: 511–7
13. Zhuang JC, Wogan GN: Growth and viability of macrophages continuously stimulated to produce nitric oxide. Proc Natl Acad Sci USA 1997; 94:11875–80
14. Lopez-Collazo E, Hortelano S, Bosca L: Interferon-alpha/beta inhibits the apoptosis induced by lipopolysaccharide and interferon-gamma in murine peritoneal macrophages. J Interferon Cytokine Res 1998; 18:461–7
15. Albina JE, Cui S, Mateo RB, Reichner JS: Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol 1993; 150:5080–5
16. Bosca L, Zeini M, Traves PG, Hortelano S: Nitric oxide and cell viability in inflammatory cells: A role for NO in macrophage function and fate. Toxicology 2005; 208:249–58
17. Fuentes JM, Hanly EJ, Aurora A, De Maio A, Talamini MA: Anesthesia-specific protection from endotoxic shock is not mediated through the vagus nerve. Surgery 2005; 138:766–71
18. Plachinta RV, Hayes JK, Cerilli LA, Rich GF: Isoflurane pretreatment inhibits lipopolysaccharide-induced inflammation in rats. ANESTHESIOLOGY 2003; 98: 89–95
19. Giraud O, Seince PF, Rolland C, Lecon-Malas V, Desmonts JM, Aubier M, Dehoux M: Halothane reduces the early lipopolysaccharide-induced lung inflammation in mechanically ventilated rats. Am J Respir Crit Care Med 2000; 162: 2278–86
20. Lipton P: Ischemic cell death in brain neurons. Physiol. Rev 1999; 79: 1431–568
21. Barone FC, Tuma RF, Legos JJ, Erhardt JA, Parsons AA: Brain Inflammation, Cytokines, and p38 MAP Kinase Signaling in Stroke. Edited by Lin RCS. Boca Raton, CRC, 2002, pp 204–44
22. Zheng S, Zuo Z: Isoflurane preconditioning induces neuroprotection against ischemia *via* activation of p38 mitogen-activated protein kinase. Mol Pharmacol 2004; 65:1172–80
23. Zheng S, Zuo Z: Isoflurane preconditioning decreases glutamate receptor overactivation-induced Purkinje neuronal injury in rat cerebellar slices. Brain Res 2005; 1054:143–51
24. Zheng S, Zuo Z: Isoflurane preconditioning reduces Purkinje cell death in an *in vitro* model of rat cerebellar ischemia. Neuroscience 2003; 118:99–106
25. Raphael J, Abedat S, Rivo J, Meir K, Beeri R, Pugatsch T, Zuo Z, Gozal Y: Volatile anesthetic preconditioning attenuates myocardial apoptosis in rabbits after regional ischemia and reperfusion *via* Akt signaling and modulation of Bcl-2 family proteins. J Pharmacol Exp Ther 2006; 318:186–94
26. de Klaver MJM, Manning L, Palmer L, Rich GF: Isoflurane pretreatment inhibits cytokine-induced cell death in cultured rat smooth muscle cells and human endothelial cells. ANESTHESIOLOGY 2002; 97:24–32
27. Huang Y, Zuo Z: Isoflurane enhances the expression and activity of glutamate transporter type 3 in C6 glioma cells. ANESTHESIOLOGY 2003; 99:1346–53
28. Huang Y, Zuo Z: Isoflurane induces a protein kinase C alpha-dependent increase in cell surface protein level and activity of glutamate transporter type 3. Mol Pharmacol 2005; 67:1522–33
29. Zuo Z, Johns RA: Halothane, enflurane, and isoflurane do not affect the basal or agonist-stimulated activity of partially isolated soluble and particulate guanylyl cyclases of rat brain. ANESTHESIOLOGY 1995; 83:395–404
30. Zuo Z, Wang Y, Huang Y: Isoflurane preconditioning protects human neuroblastoma SH-SY5Y cells against *in vitro* simulated ischemia-reperfusion through the activation of extracellular signal-regulated kinases pathway. Eur J Pharmacol 2006; 542:84–91
31. Zuo Z, Johns RA: Inhalational anesthetics up-regulate constitutive and lipopolysaccharide-induced inducible nitric oxide synthase expression and activity. Mol Pharmacol 1997; 52:606–12
32. Xu Y, Kim SO, Li Y, Han J: Autophagy contributes to caspase-independent macrophage cell death. J Biol Chem 2006; 281:19179–87
33. Hortelano S, Zeini M, Castrillo A, Alvarez AM, Bosca L: Induction of

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apoptosis by nitric oxide in macrophages is independent of apoptotic volume decrease. Cell Death Differ 2002; 9:643–50

34. Hortelano S, Traves PG, Zeini M, Alvarez AM, Bosca L: Sustained nitric oxide delivery delays nitric oxide-dependent apoptosis in macrophages: Contribution to the physiological function of activated macrophages. J Immunol 2003; 171:6059–64

35. Hemmings HJ, Adamo A, Hoffman M: Biochemical characterization of the stimulatory effects of halothane and propofol on purified brain protein kinase C. Anesth Analg 1995; 81:1216–22

36. Su JY, Vo AC: Role of PKC in isoflurane-induced biphasic contraction in skinned pulmonary arterial strips. ANESTHESIOLOGY 2002; 96:155–61

37. Salonen T, Sareila O, Jalonen U, Kankaanranta H, Tuominen R, Moilanen E: Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages. Br J Pharmacol 2006; 147:790–9

38. Paul A, Doherty K, Plevin R: Differential regulation by protein kinase C isoforms of nitric oxide synthase induction in RAW 264.7 macrophages and rat aortic smooth muscle cells. Br J Pharmacol 1997; 120:940–6

39. Muhl H, Pfeilschifter J: Possible role of protein kinase C-epsilon isoenzyme in inhibition of interleukin 1 beta induction of nitric oxide synthase in rat renal mesangial cells. Biochem J 1994; 303(pt 2):607–12

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