

Delayed Treatment with Isoflurane Attenuates Lipopolysaccharide and Interferon γ -induced Activation and Injury of Mouse Microglial Cells

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Background: Isoflurane pretreatment can induce protection against lipopolysaccharide and interferon γ (IFN γ)-induced injury and activation of mouse microglial cells. This study's goal was to determine whether delayed isoflurane treatment is protective.

Methods: Mouse microglial cells were exposed to various concentrations of isoflurane for 1 h immediately after the initiation of lipopolysaccharide (10 or 1000 ng/ml) and IFN γ (10 U/ml) stimulation or to 2% isoflurane for 1 h at various times after initiation of the stimulation. Nitrite production, lactate dehydrogenase release, and cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were assessed after stimulation with lipopolysaccharide and IFN γ for 24 h. Inducible nitric oxide synthase (iNOS) protein expression was quantified by Western blotting. The iNOS expression in mouse brain was also studied.

Results: Isoflurane applied 0 and 2 h after the initiation of lipopolysaccharide and IFN γ stimulation improved cell viability. Isoflurane at 2%, but not at 1 or 3%, reduced the lipopolysaccharide and IFN γ -induced nitrite production and decreased cell viability. Aminoguanidine, an iNOS inhibitor, also attenuated this decreased cell viability. Chelerythrine and bisindolylmaleimide IX, protein kinase C inhibitors, abolished isoflurane effects on cell viability and iNOS expression after lipopolysaccharide and IFN γ application. Isoflurane also decreased lipopolysaccharide-induced iNOS expression in mouse brain. Late isoflurane application to microglial cells reduced lipopolysaccharide and IFN γ -induced lactate dehydrogenase release that was not inhibited by aminoguanidine.

Conclusions: These results suggest that delayed isoflurane treatment can reduce lipopolysaccharide and IFN γ -induced activation and injury of microglial cells. These effects may be mediated by protein kinase C.

INFLAMMATION is an underlying pathophysiological process for almost all of the human diseases acquired later in life. For example, it is recognized that inflammatory process is involved in a broad range of common human brain diseases, such as brain trauma, stroke, brain infection, and neurodegenerative diseases.^{1,2} Microglial cells are macro-

phage-like cells resided in brain and play an important role in brain inflammatory reactions and host defense.² These cells can be activated by various agents, such as bacterial products, virus, and β -amyloid, to produce cytokines and to express inducible nitric oxide synthase (iNOS), which then induce inflammatory and host defense reactions that can cause death of brain cells or affect their functions.²⁻⁴ Despite these prominent roles of microglia, we know very little about how to reduce microglial activation and preserve these cells to provide neuroprotection under various pathologic conditions in the brain.

Pretreatment with anesthetics, such as isoflurane, can reduce endotoxin-induced lung injury⁵ and injury of endothelial cells,⁶ macrophages,⁷ and microglia.⁴ This pretreatment-induced protection may be useful if we can predict the occurrence of endotoxemia and severe inflammation. Also, it has been shown that anesthesia with isoflurane decreases lipopolysaccharide-induced inflammatory responses and improves survival of rats and mice with endotoxic shock.^{8,9} We recently showed that isoflurane applied during reperfusion reduced infarct volumes and improved neurologic functions after brain ischemia in rats.¹⁰ This isoflurane posttreatment-induced neuroprotection was also apparent under *in vitro* conditions.¹⁰ In this study, we hypothesize that delayed treatment with isoflurane can induce a protective effect in microglial cells. We used mouse microglial cultures and stimulated them by lipopolysaccharide plus interferon- γ (IFN γ), a commonly used combination to simulate clinical situations of endotoxemia and the subsequent inflammation.^{4,8}

Materials and Methods

Materials

C8-B4 cells (CRL-2540), a microglial clone isolated from 8-day-old mouse cerebellum, were purchased from the American Type Culture Collection (Manassas, VA). Lipopolysaccharide from *Escherichia coli* 0111:B4 and other chemicals except for those described below were obtained from Sigma-Aldrich (St. Louis, MO). Heat inactivated fetal bovine serum, Griess Reagent Kit (G7921), and recombinant rat interferon γ (IFN γ) produced from *E. coli* were purchased from Invitrogen Corporation (Carlsbad, CA). Rabbit polyclonal anti-iNOS immunoglobulin G whose epitope is at the C-terminus of iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; catalogue number: sc-650). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay kit was obtained from Chemicon International, Inc. (Temecula, CA). Isoflu-

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rane was purchased from Abbott Laboratories (North Chicago, IL). Chelerythrine chloride and bisindolylmaleimide IX (R8220) were obtained from Biomol (Plymouth Meeting, PA). Lactate dehydrogenase (LDH) activity assay kit was from Clontech Laboratory (La Jolla, CA).

Cell Culture. As we described before,⁴ C8-B4 cells were cultured in Dulbecco Modified Eagle's Medium containing 4 mM L-glutamine, 4,500 mg/l glucose, 1 mM sodium pyruvate, and 1,500 mg/l sodium bicarbonate supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 pg/ml streptomycin in a humidified atmosphere of 95% air–5% CO₂ at 37°C. The medium was changed every 3 days. The cells were plated at a density of 4.5×10^4 cells/ml on 96-well tissue culture plates for viability experiments by MTT assay and LDH release experiments and plated at a density of 2×10^4 cells/ml in 6-well plates for other experiments.

Isoflurane Exposure and Application of Chemicals. Three hours after the cells were plated, they were exposed to 10 ng/ml lipopolysaccharide and 10 U/ml IFN γ for 24 h. The amounts of lipopolysaccharide and IFN γ applied were based on our previous experiment.⁴ Lipopolysaccharide concentration was increased to 1,000 ng/ml (IFN γ was still at 10 U/ml) in the LDH-release experiments because our preliminary study showed that this combination of lipopolysaccharide and IFN γ induced a significant release of LDH.

Isoflurane exposure was performed in an airtight chamber as we described previously.^{4,11} The chamber was gassed with 95% air–5% CO₂ through or not through an isoflurane vaporizer for 10 min at 37°C. The isoflurane concentrations in the gases from outlet of the chambers were monitored with a Datex infrared analyzer (Capnomac, Helsinki, Finland) and reached the target concentrations at 3 min after the onset of gassing. Isoflurane was applied for 1 h immediately after the addition of lipopolysaccharide and IFN γ to the incubation medium except for the time window experiment that isoflurane was applied at 0, 2, 4, 8, 16, and 23 h after the addition of lipopolysaccharide and IFN γ to the incubation medium. Two percent isoflurane was used except for the isoflurane dose-response experiment in which 1, 2, or 3% isoflurane was applied. At the end of the 1-h incubation time, isoflurane concentrations in the gases from outlet of the chamber were confirmed to be at the target concentrations. The cells were then removed from the chamber and were kept under isoflurane-free conditions for the rest times of incubation with lipopolysaccharide and IFN γ . The cells and/or incubation solution were harvested for viability assay, LDH activity assay, or nitrite measurement at 24 h after the initiation of lipopolysaccharide and IFN γ stimulation. As we described before,⁷ the aqueous isoflurane concentrations at 37°C were 209, 415, and 620 μ M, respectively, as measured by gas chromatography when 1, 2, and 3% isoflurane was

delivered, and the samples were taken for measurements at the end of the 1-h isoflurane incubation.

Aminoguanidine (10 μ M), an iNOS inhibitor, was added to some cells for 24 h during lipopolysaccharide and IFN γ application. These cells were not treated with isoflurane. Chelerythrine chloride (2 μ M) or bisindolylmaleimide IX (10 μ M), protein kinase C (PKC) inhibitors, were added to the incubation medium during isoflurane application. In these cases, the incubation solutions were replaced with fresh medium not containing these reagents after isoflurane application and before the re-exposure to lipopolysaccharide and IFN γ . Similar procedure (incubation medium changing) was applied to other experimental groups in this set of experiments.

Cell Viability Assay. Cells plated on 96-well plates were gently mixed with 10 μ l of MTT solution and incubated for 3 h at 37°C. After formation of black fuzzy crystals, 100 μ l of isopropanol with 0.4 N HCl was added and thoroughly mixed in each well. One hour later, the absorbance of samples was measured at 570 nm with the reference wavelength of 650 nm by using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The results of MTT measurements from the controls without any treatments were set as 1. The values from various treatments were then calculated as percentages of the controls.

Nitrite Concentration Measurement. To detect the concentrations of nitrite, a stable oxidation product of nitric oxide, equal volumes of *N*-(1-naphthyl)ethylenediamine and sulfanilic acid were mixed to form Griess reagent immediately before experiments. The culture media collected from 6-well plates with the cells treated with various conditions were centrifuged. Griess reagent (20 μ l), the culture media (150 μ l), and deionized water (130 μ l) were mixed in 96-well plates and incubated in the dark for 30 min at room temperature. The absorbance of samples was measured at 570 nm against a standard curve in a spectrophotometry (Bio-Rad Laboratories).

In Vivo Animal Studies. Two-month old C57Bl/6 male mice received intraperitoneal injection of 4 mg/kg lipopolysaccharide or saline (control mice). They were then placed immediately or at 2 h after the lipopolysaccharide injection in an airtight chamber that was continuously gassed with air containing 1.5% isoflurane for 30 min. This isoflurane exposure condition was chosen because our preliminary study showed that a higher concentration (2% isoflurane) or a longer incubation (1 h) significantly altered the arterial blood gases (most animals had a PaCO₂ greater than 60 mmHg and a PaO₂ less than 50 mmHg at the end of the exposure). The animals were killed at 6 h after the lipopolysaccharide injection, and their brain cortices were harvested for Western blotting.

Western Blot Analysis. Cells or cerebral cortices of animals after various conditions were harvested and homogenized in 25 mM TRIS hydrochloride, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) α -mer-

captoethanol, 1 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1 μM pepstatin A.² The homogenates were centrifuged at 14,000 g for 10 min at 4°C. The supernatants were used for Western blotting, and 40 μg of proteins were loaded to each lane. After incubation with the anti-iNOS or anti- β -actin antibody, the 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). The results of iNOS protein bands were normalized by the data of the corresponding β -actin. The results of cells treated with various conditions were then normalized by the data of cells exposed to lipopolysaccharide and IFN γ only. The results of animals treated with various conditions were then normalized by the mean value of control animals. These different normalization procedures were used for cell culture and animal studies because microglial cell cultures without exposure to lipopolysaccharide and IFN γ did not express iNOS.

LDH Activity Assay. Incubation solution of the microglial cells treated with various conditions was centrifuged at 1,000 g for 10 min, and the cell-free supernatant was transferred to 96-well plates. The 100 μl of supernatant was incubated with the same amount of reaction mixture from the LDH detection kit. LDH activity was determined by a colorimetric assay. The absorbance of samples was measured at 492 nm, with the reference wavelength of 655 nm in a spectrophotometry (Bio-Rad Laboratories). Background absorbance from the cell-free buffer solution was subtracted from all absorbance measurements. After removal of the incubation solution from cells, 1% triton X-100 lysing solution was applied to the cells. The percentage of LDH released to incubation solution in total LDH was calculated: $100 \times \text{LDH in the incubation solution} / (\text{LDH in the incubation solution} + \text{intracellular LDH released by triton X-100})$.

Data Analysis. Each experimental condition was repeated multiple times (n for each condition is described in the figure legends) by using at least three different batches of cells. At least seven animals were used for each experimental condition in the *in vivo* study. Data are expressed as mean \pm SD. Statistical analyses were performed by one-way analysis of variance followed by the Tukey test for *post hoc* analysis or by Student *t* test for comparisons between lipopolysaccharide plus IFN γ alone and the corresponding control in the time course studies involving MTT or LDH release assay. $P < 0.05$ was considered as statistically significant.

Results

Delayed Treatment with Isoflurane-induced Time- and Concentration-dependent Protection

Lipopolysaccharide and IFN γ application reduced the cell viability to $70 \pm 6\%$ of control as measured by MTT

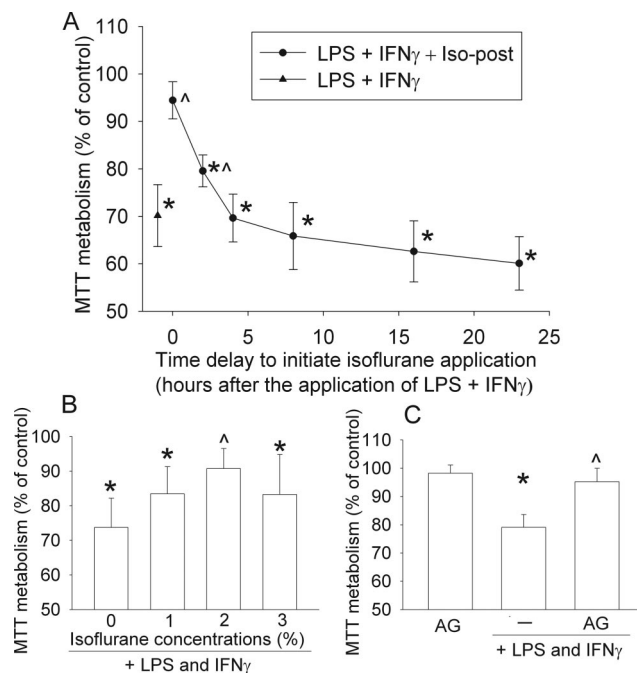


Fig. 1. Protective effects of isoflurane and aminoguanidine (AG) on cell viability. (A) Time window of delayed isoflurane treatment. The mouse C8-B4 microglial cells were incubated with 10 ng/ml lipopolysaccharide (LPS) and 10 U/ml interferon γ (IFN γ) for 24 h. Cells were exposed to 2% isoflurane for 1 h at 0, 2, 4, 8, 16, and 23 h after the initiation of the lipopolysaccharide and IFN γ stimulation. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are mean \pm SD ($n = 10$ –15). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to lipopolysaccharide plus IFN γ only. Iso-post = 2% isoflurane posttreatment. (B) Dose-response of isoflurane effects on cell viability. The mouse C8-B4 microglial cells were incubated with 10 ng/ml lipopolysaccharide and 10 U/ml IFN γ for 24 h. Cells were exposed to 1, 2, or 3% isoflurane for 1 h immediately after the initiation of the lipopolysaccharide and IFN γ stimulation. Results are mean \pm SD ($n = 8$). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to lipopolysaccharide plus IFN γ only. (C) AG effect. The mouse C8-B4 microglial cells were incubated with or without 10 ng/ml lipopolysaccharide and 10 U/ml IFN γ in the presence or absence of 10 μM AG for 24 h. Results are mean \pm SD ($n = 9$). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to lipopolysaccharide plus IFN γ only.

assay. Two percent isoflurane applied at 0 and 2 h after the lipopolysaccharide and IFN γ application enhanced the cell viability to $94 \pm 4\%$ and $79 \pm 4\%$, respectively (both $P < 0.05$ compared with lipopolysaccharide and IFN γ alone). However, delayed application of isoflurane at 4 h or longer after the addition of lipopolysaccharide and IFN γ did not improve the cell viability (fig. 1A). Concentration-response study showed that isoflurane at 2%, but not at 1% or 3%, applied immediately after the initiation of lipopolysaccharide, and IFN γ stimulation improved cell viability (fig. 1B). Consistent with our previous study,⁴ the lipopolysaccharide and IFN γ -induced reduction of cell viability was abolished by aminoguanidine (fig. 1C), an iNOS inhibitor, suggesting the role of iNOS in this lipopolysaccharide and IFN γ effect.

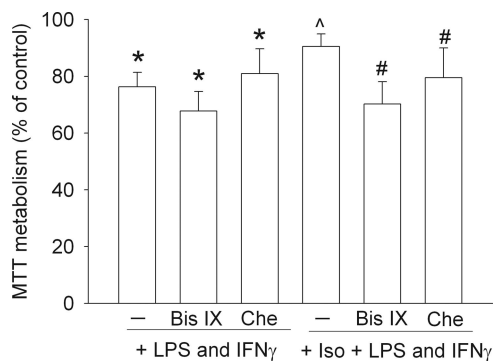


Fig. 2. Inhibition of isoflurane (Iso) protection by protein kinase C inhibition. The mouse C8-B4 microglial cells were incubated with 10 ng/ml lipopolysaccharide (LPS) and 10 U/ml interferon γ (IFN γ) for 24 h. Cells were exposed to 2% isoflurane for 1 h immediately after the initiation of the lipopolysaccharide and IFN γ stimulation. Chelerythrine chloride (Che, 2 μ M) or bisindolylmaleimide IX (Bis IX, 10 μ M) were present during isoflurane exposure. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are mean \pm SD (n = 15). * P < 0.05 compared to control. [^] P < 0.05 compared to lipopolysaccharide plus IFN γ only. # P < 0.05 compared with isoflurane and lipopolysaccharide plus IFN γ .

PKC May Mediate the Delayed Isoflurane Treatment-caused Attenuation of Lipopolysaccharide Plus IFN γ -induced Decrease of Microglial Viability

Although chelerythrine chloride and bisindolylmaleimide IX, two PKC inhibitors, did not affect cell viability in the presence of lipopolysaccharide and IFN γ , these PKC inhibitors abolished the protective effects of isoflurane on microglial viability after the application of lipopolysaccharide and IFN γ (fig. 2).

PKC May Mediate the Delayed Isoflurane Treatment-caused Attenuation of Lipopolysaccharide Plus IFN γ -induced iNOS Expression

The microglial cell cultures did not express iNOS under normal culture conditions (fig. 3A). Lipopolysaccharide and IFN γ induced a large amount of iNOS expression. This iNOS expression was inhibited by isoflurane applied immediately or at 2 h, but not at 8 h, after the initiation of the lipopolysaccharide and IFN γ stimulation (fig. 3). Chelerythrine chloride and bisindolylmaleimide IX did not affect the iNOS expression in cells exposed to lipopolysaccharide and IFN γ . However, chelerythrine chloride and bisindolylmaleimide IX abolished the isoflurane-induced inhibition of iNOS expression stimulated by lipopolysaccharide and IFN γ (fig. 3). Consistent with the results/effects of isoflurane on iNOS protein expression, 2% isoflurane reduced the production of nitrite after lipopolysaccharide and IFN γ stimulation (fig. 4).

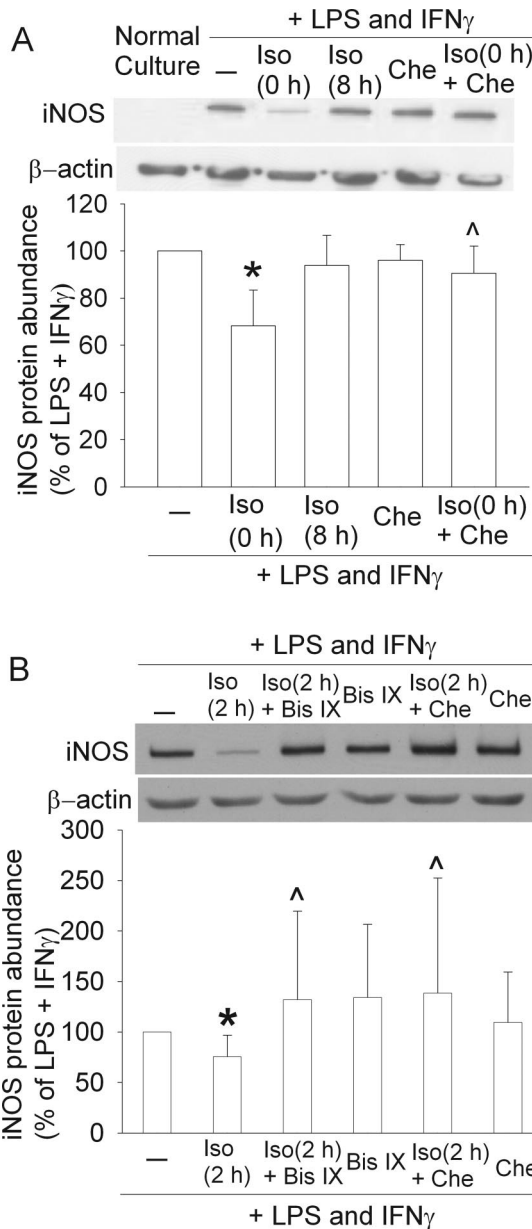


Fig. 3. Effects of isoflurane (Iso) and protein kinase C inhibition on inducible nitric oxide synthase (iNOS) expression in microglial cells. The mouse C8-B4 microglial cells were incubated with 10 ng/ml lipopolysaccharide (LPS) and 10 U/ml interferon γ (IFN γ) for 24 h. Cells were exposed to 2% isoflurane for 1 h immediately [Iso(0 h)] (A), 8 h [Iso(8 h)] (A), or 2 h [Iso(2 h)] (B) after the initiation of the lipopolysaccharide and IFN γ stimulation. Chelerythrine chloride (Che, 2 μ M) or bisindolylmaleimide IX (Bis IX, 10 μ M) was present during isoflurane exposure. These cells and the cells that were not exposed to isoflurane or lipopolysaccharide plus IFN γ (normal culture) were harvested for Western blotting. Results are mean \pm SD (n = 9 for A and 12 for B). * P < 0.05 compared to lipopolysaccharide plus IFN γ only. [^] P < 0.05 compared with isoflurane and lipopolysaccharide plus IFN γ .

Delayed Isoflurane Treatment Also Attenuated Lipopolysaccharide Plus IFN γ -induced iNOS Expression in the Mouse Brain

To determine the effects of isoflurane on lipopolysaccharide-induced iNOS expression in the mouse brain

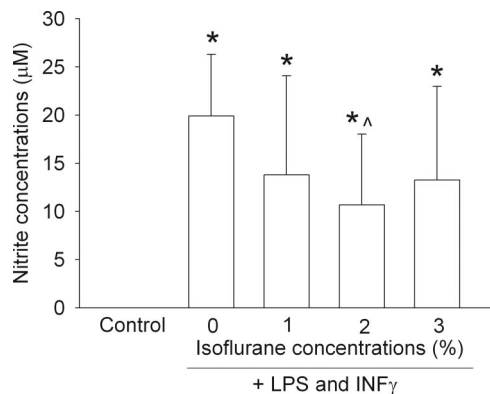


Fig. 4. Dose-response of isoflurane effects on nitrite production. The mouse C8-B4 microglial cells were incubated with 10 ng/ml lipopolysaccharide (LPS) and 10 U/ml of interferon γ ($\text{IFN}\gamma$) for 24 h. Cells were exposed to 1, 2, or 3% isoflurane for 1 h immediately after the initiation of the lipopolysaccharide and $\text{IFN}\gamma$ stimulation. The culture medium was collected for nitrite measurement. Results are mean \pm SD ($n = 15$). * $P < 0.05$ compared to control. $\wedge P < 0.05$ compared to lipopolysaccharide plus $\text{IFN}\gamma$ only.

under *in vivo* condition, we first identified an isoflurane exposure condition that would not severely alter the arterial blood gases in mice without tracheal intubation and mechanic ventilation. The mouse arterial blood pH, Paco_2 and Paco_2 were 7.26 ± 0.05 , 56 ± 13 , and 96 ± 27 ($n = 5$), respectively, at the end of the exposure to 1.5% isoflurane for 30 min. We chose this isoflurane exposure condition for further experiment.

Similar to our *in vitro* experiments, lipopolysaccharide injection significantly increased iNOS expression in the cerebral cortex. This increased iNOS expression was also inhibited by the exposure to 1.5% isoflurane for 30 min applied immediately or 2 h after the lipopolysaccharide injection (fig. 5).

Delayed Isoflurane Treatment Attenuated iNOS-independent Microglial Injury after Lipopolysaccharide Plus $\text{IFN}\gamma$ Application

Lipopolysaccharide and $\text{IFN}\gamma$ caused a significant LDH release from microglial cell cultures (fig. 6A), suggesting that lipopolysaccharide and $\text{IFN}\gamma$ induced microglial injury. Interestingly, this increased LDH release was abolished by isoflurane applied at later time points (8, 16, and 23 h after the addition of lipopolysaccharide and $\text{IFN}\gamma$), but it was not affected by isoflurane applied at early time points (fig. 6A). Further study showed that lipopolysaccharide and $\text{IFN}\gamma$ caused more LDH release than control condition only after application of lipopolysaccharide and $\text{IFN}\gamma$ for longer than 12 h (fig. 6B). In addition, the lipopolysaccharide and $\text{IFN}\gamma$ -induced LDH release was not affected by aminoguanidine (fig. 6C).

Discussion

Similar to our previous study,⁴ the incubation of microglia with lipopolysaccharide and $\text{IFN}\gamma$ reduced cell

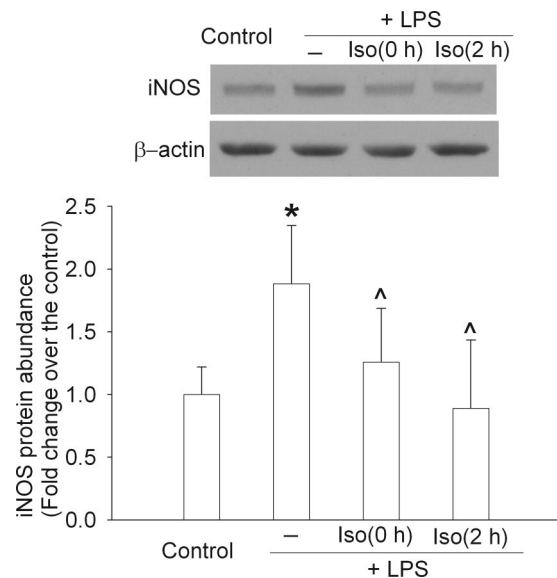


Fig. 5. Effects of isoflurane (Iso) on inducible nitric oxide synthase (iNOS) expression in mice. C57Bl/6 mice received an intraperitoneal injection of 4 mg/kg lipopolysaccharide (LPS) and exposed to 1.5% isoflurane for 30 min immediately [Iso(0 h)] or 2 h [Iso(2 h)] after the injection. Cerebral cortex was harvested at 6 h after lipopolysaccharide injection for Western analysis. Results are mean \pm SD ($n = 7-8$). * $P < 0.05$ compared to control. $\wedge P < 0.05$ compared with lipopolysaccharide alone group.

viability. It has been well-established that lipopolysaccharide and $\text{IFN}\gamma$ can stimulate/activate microglial cells to express iNOS.^{3,4,12} We have shown that this increased iNOS expression contributes to microglial damage caused by lipopolysaccharide and $\text{IFN}\gamma$.⁴ Consistent with our previous findings, we showed that aminoguanidine, an iNOS inhibitor, abolished the decreased viability of microglial cells after lipopolysaccharide and $\text{IFN}\gamma$ application.

The lipopolysaccharide and $\text{IFN}\gamma$ -reduced microglial viability was dose-dependently attenuated by isoflurane that was applied immediately after the application of lipopolysaccharide and $\text{IFN}\gamma$. This protection existed when isoflurane was applied even at 2 h after the application of lipopolysaccharide and $\text{IFN}\gamma$. These results suggest that delayed treatment with isoflurane can induce a protective effect in microglia. Isoflurane exposure immediately or at 2 h after the application of lipopolysaccharide and $\text{IFN}\gamma$ also decreased the lipopolysaccharide and $\text{IFN}\gamma$ -induced iNOS expression and nitrite production. These results, along with the knowledge that iNOS plays a critical role in damaging microglial cells after stimulation with lipopolysaccharide and $\text{IFN}\gamma$,^{4,12} indicate that the mechanisms for protection provided by delayed treatment with isoflurane involve decreasing lipopolysaccharide and $\text{IFN}\gamma$ -induced iNOS expression.

Consistent with these *in vitro* cell culture results, our studies also showed that delayed isoflurane treatment also inhibited lipopolysaccharide-induced iNOS expression in the mouse brain. This finding is significant be-

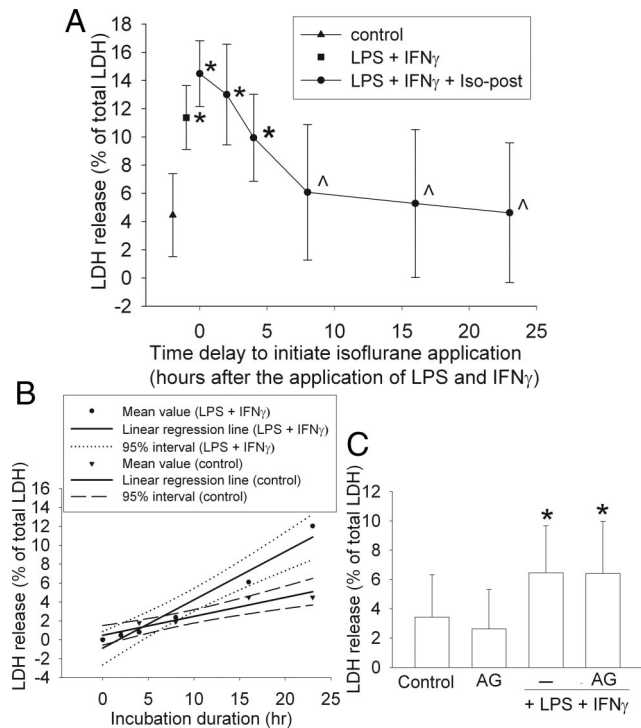


Fig. 6. Protective effects of isoflurane on lipopolysaccharide (LPS) and interferon γ (IFN γ)-induced cell injury. (A) Time window of delayed isoflurane treatment. The mouse C8-B4 microglial cells were incubated with 1000 ng/ml lipopolysaccharide and 10 U/ml IFN γ for 24 h. Cells were exposed to 2% isoflurane for 1 h at 0, 2, 4, 8, 16, and 23 h after the initiation of the lipopolysaccharide and IFN γ stimulation. Cell injury was quantified by lactate dehydrogenase (LDH) release assay. Results are mean \pm SD (n = 20–42). * P < 0.05 compared to control. \wedge P < 0.05 compared to lipopolysaccharide plus IFN γ only. Iso-post = 2% isoflurane posttreatment. (B) Time course of LDH release. The mouse C8-B4 microglial cells were incubated with or without 1,000 ng/ml lipopolysaccharide and 10 U/ml IFN γ for 0, 2, 4, 8, 16, and 23 h. The incubation solution and the cells were harvested for LDH activity assay. The actual mean values for each time point (n = 4–6) from the experiments and the 95% confidence interval are presented. (C) Aminoguanidine (AG) effect. The mouse C8-B4 microglial cells were incubated with or without 1,000 ng/ml lipopolysaccharide and 10 U/ml IFN γ in the presence or absence of 10 μ M AG for 24 h. Results are mean \pm SD (n = 18–24). * P < 0.05 compared to control.

cause volatile anesthetic effects on iNOS expression in the brain under *in vivo* conditions have not been reported. Although it is not known from our study in which cell types isoflurane treatment decreased iNOS expression, it has been shown that microglial cells are the primary cells expressing iNOS in rodent brains after lipopolysaccharide stimulation.^{13,14} This knowledge, along with our results, suggests that delayed isoflurane treatment can also decrease lipopolysaccharide-induced iNOS expression in the microglial cells of mouse brain.

PKC is a group of important intracellular signaling molecules that are involved in a broad range of biologic functions, such as cell survival.^{15,16} It has been shown that many isoflurane pharmacological effects may be mediated by PKC.^{11,17,18} In this study, we showed that

the isoflurane protective effects were inhibited by chelerythrine and bisindolylmaleimide IX. The isoflurane-decreased iNOS expression was also inhibited by chelerythrine and bisindolylmaleimide IX. Chelerythrine and bisindolylmaleimide IX are structurally different. Chelerythrine is a peptide site competitive PKC inhibitor, and bisindolylmaleimide IX is an adenosine triphosphate site competitive PKC inhibitor.¹⁹ Our results suggest that PKC mediates the isoflurane protective effects in the microglial cells. Consistent with our results, volatile anesthetics may directly stimulate PKC.²⁰ Also, although activation of PKC has been shown to enhance lipopolysaccharide- and cytokines-induced iNOS expression,²¹ activation of certain PKC isozymes, such as PKC ϵ , can inhibit lipopolysaccharide- and cytokines-induced iNOS expression in macrophages and astroglial cells.^{4,22–24} Although the exact mechanisms for the regulation of lipopolysaccharide-induced iNOS expression by PKC are not fully understood, it has been shown that PKC regulates activation of signal transducer and activator of transcription 1, which can then modulate iNOS expression.²¹

Isoflurane has been shown to inhibit lipopolysaccharide-induced iNOS expression in J774A.1 cells, a macrophage-like cell line, and this isoflurane effect was attenuated by the calcium ionophore ionomycin.²⁵ These results suggest the role of decreased intracellular free Ca²⁺ in mediating this isoflurane effect. Consistent with this idea, increased intracellular Ca²⁺ has been proposed to mediate lipopolysaccharide- or IFN γ -induced iNOS expression in mouse macrophages,²⁶ although contrary results (increased intracellular Ca²⁺ leads to decreased iNOS expression in mouse macrophages) have also reported.²⁷ Similar to this confusing picture, many studies have reported a decrease of intracellular Ca²⁺ by volatile anesthetics,^{28,29} and other studies showed that volatile anesthetics induced a slight increase of intracellular Ca²⁺.^{30,31} Thus, although lipopolysaccharide has been consistently shown to increase iNOS expression in various cells, including microglial cells, further studies are needed to clarify the role of intracellular Ca²⁺ in iNOS expression and the regulation of volatile anesthetics on this process.

We observed a bell-shaped response: 2% isoflurane, but not 1% and 3% isoflurane, reduced lipopolysaccharide and IFN γ -induced decrease of cell viability and nitrite production. The mechanisms for the lost protective effects at a higher concentration of isoflurane are not known. However, activation of different PKC isozymes may increase or decrease iNOS expression in macrophages stimulated by lipopolysaccharide and IFN γ .^{4,21–24} 3% isoflurane may activate PKC isozymes that can increase iNOS expression. This effect may cancel out the inhibitory effects of isoflurane on iNOS expression *via* other PKC isozymes. Preferential activation of PKC isozymes by various concentrations of isoflurane has been hypothesized to explain the bell-shaped effects of isoflurane on

contraction of skinned pulmonary arterial strips and isoflurane preconditioning-induced protection in macrophages.^{18,32} Also, we exposed microglial cells to various concentrations of isoflurane for 1 h. It is not known whether this length of exposure is optimal for its protective effect, especially for 1% or 3% isoflurane. Finally, prolonged exposure to high concentrations of isoflurane can induce cell injury.³³ Exposure to 3% isoflurane for 1 h in the presence of lipopolysaccharide and IFN γ may be strong enough to cause a detrimental effect, which counteracts its protective effect.

In addition to MTT assay, we used LDH release to measure isoflurane protective effects. An increased LDH release may require plasma membrane damage and signify that cells are injured. Our results showed that isoflurane application at later time points, but not at early time points, abolished lipopolysaccharide and IFN γ -induced LDH release. This finding contrasts sharply with our results from MTT assay, where isoflurane application only at early time points decreased lipopolysaccharide and IFN γ -induced decrease of MTT metabolism. Decreased MTT metabolism suggests a decrease of mitochondrial reduction ability and, therefore, decreased cell viability. Our results showed that the lipopolysaccharide and IFN γ -induced decrease of MTT metabolism was iNOS-dependent and that isoflurane exposure at early time points, but not a later time point, after the application of lipopolysaccharide and IFN γ reduced the lipopolysaccharide and IFN γ -induced iNOS expression. Thus, the attenuation of lipopolysaccharide and IFN γ -induced decrease of MTT metabolism by isoflurane may be the results of its inhibition of iNOS expression. In contrast, the isoflurane-decreased cell injury of the cells exposed to lipopolysaccharide and IFN γ , as measured by LDH release, may not be the result of the effects of isoflurane on iNOS expression because isoflurane applied at early time points reduced lipopolysaccharide and IFN γ -induced iNOS expression but failed to decrease the lipopolysaccharide and IFN γ -induced LDH release. Consistent with this idea, aminoguanidine did not reduce lipopolysaccharide and IFN γ -induced LDH release. In our study, it appears that more LDH was released from cells incubated with lipopolysaccharide and IFN γ for longer than 12 h, and isoflurane application around and during this period significantly attenuated this lipopolysaccharide and IFN γ -induced LDH release. Future studies are needed to understand the mechanisms for isoflurane to reduce the LDH release under this experimental condition.

Microglial cells play a critical role in immune reactions and host defense. They perform active tissue scanning and respond to various endogenous and exogenous stimuli. We used lipopolysaccharide and IFN γ , a common *in vitro* method to simulate endotoxemia and to induce inflammation, to activate microglial cells. Considering the fact that inflammatory process is involved in almost

all of the human diseases acquired later in their lives, our findings that isoflurane applied after the lipopolysaccharide and IFN γ administration can reduce microglial activation and injury may have a broad implication.

In conclusion, we have shown that delayed treatment with isoflurane can induce a protective effect in microglial cells and reduce iNOS expression, a microglial cell activation indicator, during inflammatory process. PKC may mediate these isoflurane-protective effects.

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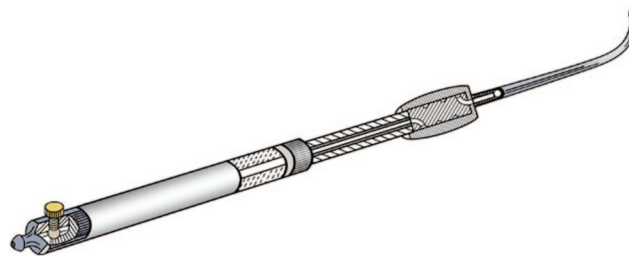
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■ ANESTHESIOLOGY REFLECTIONS

The Lennox “Anaesthetic Wand”



Several years after attending Philadelphia Dental College, Dr. Charles Parker Lennox (c.1833–1898) sided against two of his Union-sympathizing brothers by joining a Confederate regiment that their uncle had mustered in Kentucky. After deserting his commission as a Major in the Confederacy in 1864, Parker fled to Canada and eventually resumed the civilian practice of dentistry in Toronto. In 1891 Parker filed his U.S. Patent application for a “Dental Tool” (pictured above, courtesy of the Wood Library-Museum), which permitted nitrous oxide to pass through a hollow metal wand’s proximal nipple, *via* a chloroform chamber, through a handle, and around a spiral-grooved warming chamber, before jetting out a distal aperture into a dental patient’s mouth. Parker’s strange “anaesthetic wand” likely worked as a combined inhalational and topical analgesic. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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