Published in final edited form as:

Neuroscience. 2008 June 26; 154(3): 1002–1008.

Isoflurane preconditioning reduces mouse microglial activation and injury induced by lipopolysaccharide and interferon- γ

Xuebing Xu^{*,^}, Jie Ae Kim^{*,#}, and Zhiyi Zuo^{*}

^{*} Department of Anesthesiology, University of Virginia, Charlottesville, U.S.A.

[^] Department of Anesthesiology, the First People's Hospital of Guangzhou, Guangzhou, China

[#] Department of Anesthesiology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Korea

Abstract

Activation and injury of microglial cells are involved in a broad range of brain diseases including stroke, brain infection and neurodegenerative diseases. However, there is very little information regarding how to reduce microglial reaction and preserve these cells to provide neuroprotection. Here, we showed that the incubation of C8-B4 mouse microglial cells with lipopolysaccharide (LPS) plus interferon- γ (IFN γ) for 24 hr decreased the viability of these cells. Pretreatment of these cells with 1%, 2% or 3% isoflurane, a commonly used volatile anesthetic, for 1 hr at 30 min before the exposure to LPS plus IFN γ attenuated the reduction of cell viability (preconditioning effect). LPS plus IFN γ also activated these microglial cells to express inducible nitric oxide synthase (iNOS) and to induce accumulation of nitrite, a stable oxidation product of nitric oxide, in the incubation medium. Isoflurane preconditioning attenuated these LPS plus IFN γ effects on the iNOS expression and nitrite accumulation. Aminoguanidine, an iNOS inhibitor, attenuated the LPS plus IFN γ -induced glutamate release and decrease of microglial viability. Isoflurane preconditioning also reduced LPS plus IFN γ -induced glutamate release. Exogenous glutamate decreased microglial viability. Finally, the isoflurane preconditioning-induced protection was abolished by chelerythrine, a protein kinase C inhibitor. These results suggest that LPS plus IFN γ activates the iNOS-nitric oxide-glutamate pathway to induce microglial injury and that this activation is attenuated by isoflurane preconditioning. Protein kinase C may be involved in the isoflurane preconditioning effects.

Keywords

Glutamate; inducible nitric oxide; isoflurane; microglia; neuroprotection; preconditioning

Address correspondence to: Dr. Zhiyi Zuo, Department of Anesthesiology, University of Virginia Health System, 1 Hospital Drive, PO Box 800710, Charlottesville, Virginia 22908-0710, USA. Tel: 434-924-2283, Fax: 434-924-2105, e-mail: zz3c@virginia.edu.

Section Editor: Constantino Sotelo

The research work was performed in and should be attributed to the Department of Anesthesiology, University of Virginia, Charlottesville, VA22908, U.S.A.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Inflammatory process is involved in a broad range of pathologies and diseases of the central nervous system including brain trauma, stroke, brain infection and neurodegenerative diseases (Eddleston and Mucke, 1993, Kreutzberg, 1996). One of the brain cells, microglial cells, is macrophage-like cells in the brain and plays a critical role in brain inflammatory reactions (Kreutzberg, 1996). Microglia can be activated by various agents, such as bacterial products, virus, prion and β -amyloid, to produce cytokines and inducible nitric oxide (iNOS) that can then induce neuronal death (Brown and Bal-Price, 2003). However, despite the prominent role of microglia in the inflammation and host defense, there is very little information regarding how to reduce microglial activation and preserve these cells to provide neuroprotection under various pathological conditions in the literature.

Preconditioning is a phenomenon in which a prior exposure to a stimulus induces protection against the detrimental effects of a subsequent insult (Barry and Zuo, 2005). Multiple stimuli including short episodes of ischemia and hypoxia have been shown to induce preconditioning effects in the brain (Fu et al., 1994, Nandagopal et al., 2001). However, the use of ischemia and hypoxia to induce neuroprotection may be limited because these stimuli are capable of producing injury with only minor changes in their intensity or duration. We and others have shown that isoflurane, a commonly used and relatively safe volatile anesthetic, can also induce preconditioning effects in the brain (Kapinya et al., 2002, Zheng and Zuo, 2003, Zheng and Zuo, 2004, Zheng and Zuo, 2005). These previous studies show that isoflurane preconditioning reduces ischemia- or glutamate-induced neuronal death under *in vivo* or *in vitro* conditions. In this study, we test the hypothesis that isoflurane can induce a preconditioning effect in microglial cells. We used mouse microglial cultures and stimulated these cells by lipopolysaccharide (LPS) plus interferon- γ (IFN γ). LPS is a component of the outer membrane of gram-negative bacteria and has been a common agent used to induce experimental endotoxemia and inflammation (Fuentes et al., 2006, Reutershan et al., 2006). IFN γ enhances these LPS effects (Zhuang and Wogan, 1997, Lopez-Collazo et al., 1998). The combination of these two agents is very potent to stimulate microglial cells (Bal-Price and Brown, 2001). Since many of the isoflurane pharmacologic effects in various cells have been shown to be mediated by protein kinase C (PKC) (Su and Vo, 2002, Huang and Zuo, 2005) and PKC may be involved in the volatile anesthetic preconditioning-induced cardioprotection (Zaugg et al., 2003), we also determined the role of PKC in the isoflurane preconditioning-induced protection in microglial cells.

Materials and methods

Materials

C8-B4 cells (CRL-2540TM), a microglial clone isolated from 8-day-old mouse cerebellum, were purchased from the American Type Culture Collection (Manassas, VA). Heat inactivated fetal bovine serum (FBS), L-glutamine, Griess Reagent Kit(G7962) and recombinant rat IFN γ produced from *E. coli* were purchased from Invitrogen Corporation (Carlsbad, California). Rabbit polyclonal anti-iNOS antibody 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). Isoflurane was purchased from Abbott Laboratories (North Chicago, IL). Chelerythrine chloride was obtained from Biomol (Plymouth Meeting, PA). D,L-threo- β -benzyloxyaspartate (TBOA) was purchased from Toronto Research Chemicals Inc (North York, ON, Canada). LPS (*Escherichia coli* 0111:B4), aminoguanidine bicarbonate salt and other general chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

C8-B4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 10% heat inactivated FBS, 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 three days. The cells were plated at a density of $3.5 \times 10^4 - 5 \times 10^4$ /well on 96-well tissue culture plates for viability experiments by MTT assay. Cells were grown to ~90% confluence in 6 well plates or 100 mm dishes for experiments other than viability assay.

Isoflurane exposure and application of chemicals

The first experiment was designed to determine the sensitivity of C8-B4 cells to LPS and IFN γ and the concentrations of LPS and IFN γ used in the subsequent experiments. Cells were incubated with various concentrations (10 – 1000 ng/ml) of LPS and 10 or 50 U/ml IFN γ for 24 hr. The cells were then harvested for viability assay.

Isoflurane pretreatment of the cells was performed in an airtight chamber as we described earlier (Huang and Zuo, 2003, Huang and Zuo, 2005). Briefly, cells were placed in the airtight chamber at 3 hr after the cells were plated. The chamber was then gassed with 95% air-5% CO₂ 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 DatexTM infrared analyzer (Capnomac, Helsinki, Finland) and reached the target concentrations at ~3 min after the onset of gassing. The chamber was sealed and the incubation was for 45 min at 37°C (the total isoflurane exposure time is 1 hr). 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 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr at 37°C. In some experiments, cells were incubated with or without 10 μ M aminoguanidine, an iNOS inhibitor, or 10 μ M TBOA, a potent competitive inhibitor of glutamate/aspartate transporters (also called excitatory amino acid transporters, EAAT), in the presence or absence of 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr. These cells were not pretreated with isoflurane. To determine the involvement of PKC in the isoflurane preconditioning, the PKC inhibitor chelerythrine (10 μ M) was added just before the isoflurane application. After the isoflurane exposure, the incubation medium was replaced with fresh medium without chelerythrine before the cells were exposed to 10 ng/ml LPS plus 10 U/ml IFN γ . To determine glutamate-induced toxicity in microglial cells, C8-B4 cells were incubated with 5 mM glutamate for 24 hr. These cells were not treated with isoflurane or LPS plus IFN γ .

In a separate experiment, the isoflurane concentrations in the incubation solutions were determined by gas chromatography as we described before (Zuo and Johns, 1995). The aqueous isoflurane concentrations at 37°C were ~209, 415 and 620 μ M, respectively, when 1%, 2%, and 3% isoflurane was delivered and the samples were taken at the end of the 1-hr isoflurane incubation. There was no detectable isoflurane in the incubation solution at the end of the 30-min isoflurane-free period.

Cell viability

Cell viability was determined by the MTT assay according to manufacturer's procedure and as we described before (Zuo et al., 2006). Briefly, 10 μ l of the MTT labeling reagent was added to each well that contained 100 μ l culture medium and cells, and mix gently. Four hours later, 100 μ l of the solubilization solution was added into each well and mix thoroughly by repeated pipetting. The plate was kept at 37°C for 1 hr. 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, USA). 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.

Western blot analysis

Western blot analysis was performed as we detailed before (Zuo and Johns, 1997). Cells were homogenized in 25 mM Tris HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) α -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. The homogenates were centrifuged at 1000 g for 10 min at 4°C. The supernatants were used for Western blotting and loaded at 20 μ g proteins 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, USA).

Nitrite concentration measurement

Microglial cells at ~ 90% confluence in Corning® 6 well culture plates were pretreated with or without 2% isoflurane for 1 hr and then exposed to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr at 37°C. The culture medium was collected at the end of the incubation. The concentrations of nitrite, a stable oxidation product of nitric oxide (NO), in the culture medium were measured with 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, Hercules, CA, USA).

Analysis of glutamate levels by high performance liquid chromatography (HPLC)

Microglial cells at ~ 90% confluence were treated with or without 2% isoflurane for 1 hr before the exposure to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr at 37°C. In some experiments, 10 μ M aminoguanidine and 10 μ M TBOA were added to cells just before the application of LPS plus IFN γ . These cells were not treated with isoflurane. The culture medium was collected at the end of the incubation for the measurement of glutamate concentrations by HPLC (Shimadzu, Kyoto, Japan) using the electrochemical detector Coulochem III (ESA, Inc., Chelmsford, MA) after a precolumn derivatization with OPA/ β ME solution (1,2-phthalic dicarboxaldehyde, Acros, New Jersey/ β -mercapto-ethanol, Sigma) as we described before (Wang et al., 2007). The reversed-phase column Supelcosil LC-8-DB, 5 μ m, 5 cm $\times$ 4.6 cm (Supelco Inc., Bellefonte, PA) was eluted isocratically with 0.1 M Na₂HPO₄ mobile phase in 35% methanol (pH = 6.4 adjusted with phosphoric acid) at 1 ml/min. Standard stock solution of glutamate (1 mg/ml in 50% methanol, kept refrigerated) was diluted with 0.05 M HClO₄ (1:1) before analysis. Stock OPA/ β ME solution was prepared by dissolving 27 mg OPA in 1 ml of HPLC grade methanol and then adding 5 μ l β ME and 9 ml of 0.1 M sodium tetraborate. The solution was kept in amber glass at room temperature for up to 5 days. On the day of analysis, 1 ml of OPA/ β ME stock solution was diluted with 3 ml of 0.1 M sodium tetraborate solution to prepare OPA/ β ME working solution. Using auto-injector precolumn derivatization program, 10 μ l of samples and 5 μ l of OPA/ β ME working solution were injected to column for analysis. Electrochemical detector with analytical cell model 5011 was set as follows: electrode 1: -0.40 V, electrode 2: + 0.60 V, guard cell: + 0.65 V, filter: 5 second and current range: 200 nA. The cell culture media samples were analyzed after the standard curve of glutamate has been established.

Data analysis

Results are presented as means $\pm$ S.D. from at least three separate experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student-Newman-Keul's test for *post hoc* comparison. A P < 0.05 was considered statistically significant.

Results

Isoflurane preconditioning reduced LPS plus IFN γ -induced microglial injury

Incubation of C8-B4 microglial cells with various concentrations of LPS and IFN γ caused a dose-dependent microglial injury (Fig. 1). The incubation with 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr reduced cell viability assessed by MTT assay to ~ 60% of control cells (Figs. 1 and 2). We used this condition for the subsequent experiments. Pretreatment of the cells with 1%, 2% or 3% isoflurane significantly attenuated the LPS plus IFN γ -induced microglial injury (Fig. 2B), suggesting that isoflurane induces a preconditioning effect in these cells.

LPS plus IFN γ increased iNOS expression and glutamate release that were attenuated by isoflurane preconditioning

C8-B4 microglial cells did not express iNOS under normal culture conditions (Fig. 3A). There was no detectable nitrite in the culture medium of control cells. The exposure of these cells to LPS plus IFN γ for 24 hr induced a large amount of iNOS expression (Fig. 3A). Consistent with this result, the exposure of LPS plus IFN γ also increased the accumulation of nitrite in the incubation medium (Fig. 3B). Aminoguanidine, an iNOS inhibitor, reduced the LPS plus IFN γ -induced cell injury (Fig. 4A) and did not affect cell viability under control condition (MTT metabolism with aminoguanidine alone was $105 \pm 3\%$ of the control, $n = 4$, $P > 0.05$), suggesting that iNOS contributes to the LPS plus IFN γ -induced microglial injury. LPS plus IFN γ also induced glutamate release from microglial cells ($39.0 \pm 23.5 \mu\text{M}$ in the incubation medium), which was inhibited by aminoguanidine (Fig. 4B). These results suggest the involvement of iNOS-nitric oxide (NO) in the LPS plus IFN γ -induced glutamate release. The released glutamate may contribute to the LPS plus IFN γ -induced microglial cell injury because exogenous glutamate decreased microglial cell viability (Fig. 2A) and TBOA, an EAAT inhibitor, enhanced the LPS plus IFN γ -induced glutamate release and microglial injury (Fig. 4).

Isoflurane pretreatment dose-dependently decreased the LPS plus IFN γ -induced iNOS expression and nitrite accumulation in the incubation medium (Fig. 3). Pretreatment with 2% isoflurane also reduced LPS plus IFN γ -induced glutamate release from the microglial cells (Fig. 4B).

Isoflurane preconditioning-induced protection may be mediated by PKC

Chelerythrine, a PKC inhibitor, did not affect the viability of microglial cells after the application of LPS plus IFN γ only (Fig. 5) and under the control condition (MTT metabolism with chelerythrine alone was $104 \pm 3\%$ of the control, $n = 4$, $P > 0.05$). However, chelerythrine abolished the 2% isoflurane preconditioning-induced protection in microglial cells stimulated by LPS plus IFN γ (Fig. 5). These results suggest a role of PKC in the isoflurane preconditioning-induced protection in microglial cells.

Discussion

Isoflurane is a volatile anesthetic that is often used in neurosurgical patients. One minimum alveolar concentration that is the concentration to inhibit 50% subjects to respond to surgical stimulus is 1.15% isoflurane in human and 1.4% isoflurane in rodents (Orliaguet et al., 2001, Sonner et al., 2003). We and others have shown that isoflurane at clinically relevant concentrations can precondition neurons against ischemia (Kapinya et al., 2002, Zheng and Zuo, 2003, Zheng and Zuo, 2004, Zheng and Zuo, 2005). However, it is not known yet whether isoflurane preconditioning can protect other brain cells. In this study, we showed that preconditioning with 1%, 2% or 3% isoflurane significantly attenuated the LPS plus IFN γ -induced microglial cell activation and injury (Figs. 2 and 3). Clinically, anesthetic

concentrations higher than 2 minimum alveolar concentrations (2.3% isoflurane in human and 2.8% isoflurane in rodents) are very rarely used. However, isoflurane concentrations in brain tissues are predicated to be 2.24 times that in alveolar gas at 37°C because the partition coefficients for isoflurane at 37°C for blood:gas and brain:blood are 1.4 and 1.6, respectively (Eger, 2000)(of note, the anesthetic concentrations in the extracellular fluid of brain tissues presumably is similar to that in other electrolyte solutions in equilibrium). Thus, our results suggest that isoflurane at clinically relevant concentrations induces a preconditioning effect in the microglial cells. Since microglia play a critical role in inflammation and many brain diseases including stroke (Eddleston and Mucke, 1993, Kreutzberg, 1996), our results suggest an additional mechanism for isoflurane preconditioning to protect neurons in those diseases.

LPS plus IFN γ was shown to activate rat primary culture of microglial cells to release NO (Bal-Price and Brown, 2001). Our study showed that LPS plus IFN γ induced iNOS expression in the C8-B4 microglial cells and increased nitrite accumulation in the incubation medium. This induced expression of iNOS contributes to the cell injury because aminoguanidine, an iNOS inhibitor, attenuated the LPS plus IFN γ -induced decrease of microglial viability. LPS plus IFN γ also induced glutamate release and this release was decreased by aminoguanidine, suggesting that iNOS contributes to the glutamate release. Consistent with our results, a previous study showed that addition of LPS plus IFN γ -stimulated microglial cells or NO to neuronal cultures induced glutamate release and the subsequent glutamate excitotoxicity to result in neuronal death (Bal-Price and Brown, 2001). In our study, exogenously added glutamate reduced microglial cell viability under control condition and TBOA enhanced the LPS plus IFN γ -induced extracellular glutamate accumulation and microglial injury. These results suggest that the released glutamate contributes to the LPS plus IFN γ -induced microglial injury. Thus, the activation of iNOS-NO-glutamate pathway plays a critical role in LPS plus IFN γ -induced cell injury. Since microglial cells can be activated to express iNOS under various pathological conditions and diseases, the activation of iNOS-NO-glutamate pathway may be a shared mechanism to cause cell injury in those diseases.

It has been proposed that one of the mechanisms for NO to induce glutamate release is via inhibition of cell respiration to result in cell energy deprivation (Bal-Price and Brown, 2001). Energy deprivation can then cause reversed transport of glutamate via EAATs. EAATs are cell membrane proteins in neurons and glial cells. EAATs uptake glutamate from extracellular space under physiological conditions (Danbolt, 2001). This process is driven by the normal transmembrane gradients of Na⁺, K⁺ and H⁺. These gradients will not be maintained when cell energy is deprived and glutamate will be transported according to glutamate chemical gradient, from intracellular to extracellular space, via EAATs. This reversed transport of glutamate will increase extracellular glutamate concentrations. However, our results do not suggest the role of reversed transport of glutamate via EAATs in the LPS plus IFN γ -induced glutamate release because TBOA enhanced the glutamate accumulation. TBOA is a nontransportable EAAT inhibitor (Koch et al., 1999) and should reduce the reversed transport of glutamate via EAATs. Thus, mechanisms other than reversed transport of glutamate via EAATs may contribute to the LPS plus IFN γ -induced glutamate release from microglial cells. Our results also suggest that EAATs in the microglial cells stimulated by LPS plus IFN γ have normal uptake functions because the inhibition of EAATs by TBOA enhanced the glutamate accumulation in these cells. Consistent with our data, it has been shown that LPS-induced glutamate release involves the Xc exchange system, an amino acid transporter that antiports glutamate for cystine (Piani and Fontana, 1994, Barger et al., 2007). Many studies including our study suggest the role of NOS and NO in this LPS-induced glutamate release (Bal-Price and Brown, 2001, Nakamura et al., 2003). However, a recent study has showed that NO may not be involved in the LPS-activated, Xc exchange system-mediated glutamate release, although the same study has also shown that this glutamate release requires the oxidative burst and lipid peroxidation (Barger et al., 2007).

Our study showed that isoflurane preconditioning reduced LPS plus IFN γ -induced microglial injury. Isoflurane preconditioning also attenuated the LPS plus IFN γ -induced iNOS expression in the microglial cells and nitrite and glutamate accumulation in the incubation medium. Since activation of iNOS-NO-glutamate pathway may mediate the microglial injury caused by LPS plus IFN γ , our results suggest that attenuation of the activation of this pathway may be the mechanism for isoflurane preconditioning to induce the protection.

Our results showed that chelerythrine, a PKC inhibitor, abolished the 2% isoflurane pretreatment-induced protection in microglial cells, suggesting a role of PKC in the isoflurane preconditioning effects on these cells. Consistent with our results, PKC may play a role in the isoflurane preconditioning effect against cytokines-induced cell injury in smooth muscle cells and endothelial cells (de Klaver et al., 2002). In a recent study, we showed that PKC might be involved in isoflurane preconditioning-induced protection in macrophages (Xu et al., 2007). It has been shown that volatile anesthetics may directly activate PKC (Hemmings et al., 1995) and many of isoflurane pharmacological effects on various cells and tissues may be mediated by PKC (Su and Vo, 2002, Huang and Zuo, 2005). In addition, although PKC activation can increase LPS- and cytokines-induced iNOS expression in macrophages (Salonen et al., 2006), activation of certain PKC isozymes, such as PKC ϵ , also can lead to the inhibition of iNOS expression in macrophages and astroglial cells stimulated by LPS, IFN γ or other cytokines (Muhl and Pfeilschifter, 1994, Paul et al., 1997, Kopnisky and Sumners, 2000). Thus, these previous findings are consistent with our identified mechanisms that isoflurane preconditioning reduces LPS plus IFN γ -induced iNOS over-expression to provide protection in microglial cells and that activation of PKC may play a role in this effect.

We used LPS plus IFN γ to induce microglial injury in this study. It has been shown that LPS at small doses can induce preconditioning effects against ischemia-induced cell death (Orio et al., 2007, Rosenzweig et al., 2007). Similarly, exposure to a high concentration of isoflurane for a long period of time induces cell death (Wei et al., 2005); while exposure to low concentrations of isoflurane for a short period of time induces preconditioning effects as shown in this study. This double-edged sword nature may be inherent in many preconditioning stimuli/agents.

Microglial cells have been considered the major immune cells in the brain. They respond to various endogenous and exogenous stimuli. Recent data show that they carry out active tissue scanning and their functions are diverse and dynamic: their activation can be detrimental or protective dependent on the stimuli and the microenvironment (Schwartz et al., 2006, Hanisch and Kettenmann, 2007). For example, low concentrations of IFN γ -stimulated microglial cells support neurogenesis and remove extracellular glutamate (Shaked et al., 2005, Butovsky et al., 2006a, Butovsky et al., 2006b). Treatment of microglial cells with LPS and/or high concentrations of IFN γ do not support cell renewal (Butovsky et al., 2006a, Butovsky et al., 2006b). Thus, our findings that isoflurane preconditioning reduces microglial cell over-activation and injury after LPS and IFN γ stimulation may have a broad implication.

Our study is focused on isoflurane preconditioning-induced protection. This protection will require the application of isoflurane before the insult. Although predication of insult occurrence is difficult or impossible in many clinical situations, clinical scenarios that can benefit preconditioning-induced protection exist. For example, patients receive isoflurane anesthesia for surgery. These patients may develop infection or brain ischemia in the intraoperative or postoperative periods. Preconditioning by isoflurane or other stimuli could be practical and beneficial to these patients.

In conclusion, our results suggest that LPS plus IFN γ activates the iNOS-NO-glutamate pathway to induce microglial injury. Isoflurane preconditioning attenuated the activation of

this pathway to provide protection. The activation of PKC during isoflurane exposure contributes to the mechanisms of isoflurane preconditioning-induced protection. These results provide initial evidence on how to protect microglial cells that play an important role in the inflammatory reactions involved in many brain diseases, such as stroke, infection and neurodegenerative diseases.

Acknowledgements

This study was supported by grants (R01 GM065211 and R01 NS045983 to Z Zuo) from the National Institute of Health, Bethesda, Maryland, by a grant from the International Anesthesia Research Society (Frontiers in Anesthesia Award to Z Zuo), by a Grant-in-Aid from the American Heart Association Mid-Atlantic Affiliate (0755450U to Z Zuo) and by a grant from the National Nature Science Foundation of China (30500481 to X Xu).

Abbreviations

FBS	fetal bovine serum
HPLC	high performance liquid chromatography
IFNγ	interferon- γ
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
PKC	protein kinase C
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TBOA	D,L- <i>threo</i> - β -benzyloxyaspartate

References

- Bal-Price A, Brown GC. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J Neurosci* 2001;21:6480–6491. [PubMed: 11517237]
- Barger SW, Goodwin ME, Porter MM, Beggs ML. Glutamate release from activated microglia requires the oxidative burst and lipid peroxidation. *J Neurochem* 2007;101:1205–1213. [PubMed: 17403030]
- Barry U, Zuo Z. Opioids: Old drugs for potential new applications. *Curr Pharm Des* 2005;11:1343–1350. [PubMed: 15853689]
- Brown GC, Bal-Price A. Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol Neurobiol* 2003;27:325–355. [PubMed: 12845153]
- Butovsky O, Landa G, Kunis G, Ziv Y, Avidan H, Greenberg N, Schwartz A, Smirnov I, Pollack A, Jung S, Schwartz M. Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J Clin Invest* 2006a;116:905–915. [PubMed: 16557302]
- Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, Martino G, Schwartz M. Microglia activated by IL-4 or IFN- γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci* 2006b;31:149–160. [PubMed: 16297637]
- Danbolt NC. Glutamate uptake. *Prog Neurobiol* 2001;65:1–105. [PubMed: 11369436]

- 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(1):24–32. [PubMed: 12131100]
- Eddleston M, Mucke L. Molecular profile of reactive astrocytes--implications for their role in neurologic disease. *Neuroscience* 1993;54:15–36. [PubMed: 8515840]
- Eger, EI. Uptake and Distribution. In: Miller, RD., editor. *Anesthesia*. 1. Churchill Livingstone; Philadelphia: 2000. p. 74-95.
- Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Gidday JM. Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neurosci Lett* 1994;168:221–224. [PubMed: 8028780]
- 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;13:281–288. [PubMed: 16467339]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007;10:1387–1394. [PubMed: 17965659]
- Hemmings HJ, Adamo A, Hoffma M. Biochemical characterization of the stimulatory effects of halothane and propofol on purified brain protein kinase C. *Anesth Analg* 1995;81:1216–1222. [PubMed: 7486107]
- Huang Y, Zuo Z. Isoflurane enhances the expression and activity of glutamate transporter type 3 in C6 glioma cells. *Anesthesiology* 2003;99:1346–1353. [PubMed: 14639147]
- 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;67:1522–1533. [PubMed: 15709112]
- Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke K, Isaev NK, Dirnagl U. Tolerance Against Ischemic Neuronal Injury Can Be Induced by Volatile Anesthetics and Is Inducible NO Synthase Dependent. *Stroke* 2002;33:1889–1898. [PubMed: 12105371]
- Koch HP, Chamberlin AR, Bridges RJ. Nontransportable inhibitors attenuate reversal of glutamate uptake in synaptosomes following a metabolic insult. *Mol Pharmacol* 1999;55:1044–1048. [PubMed: 10347246]
- Kopnisky KL, Sumners C. Angiotensin II-induced decrease in expression of inducible nitric oxide synthase in rat astroglial cultures: role of protein kinase C. *J Neurochem* 2000;74:613–620. [PubMed: 10646512]
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 1996;19:312–318. [PubMed: 8843599]
- 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;18:461–467. [PubMed: 9712361]
- 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):607–612. [PubMed: 7526844]
- Nakamura Y, Ohmaki M, Murakami K, Yoneda Y. Involvement of protein kinase C in glutamate release from cultured microglia. *Brain Res* 2003;962:122–128. [PubMed: 12543462]
- Nandagopal K, Dawson TM, Dawson VL. Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *J Pharmacol Exp Ther* 2001;297:474–478. [PubMed: 11303032]
- Orio M, Kunz A, Kawano T, Anrather J, Zhou P, Iadecola C. Lipopolysaccharide induces early tolerance to excitotoxicity via nitric oxide and cGMP. *Stroke* 2007;38:2812–2817. [PubMed: 17761917]
- Orliaguet G, Vivien B, Langer O, Bouhemad B, Coriat P, Riou B. Minimum alveolar concentration of volatile anesthetics in rats during postnatal maturation. *Anesthesiology* 2001;95:734–739. [PubMed: 11575548]

- 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;120:940–946. [PubMed: 9138702]
- Piani D, Fontana A. Involvement of the cystine transport system xc⁻ in the macrophage-induced glutamate-dependent cytotoxicity to neurons. *J Immunol* 1994;152:3578–3585. [PubMed: 8144936]
- Reutershan J, Chang D, Hayes JK, Ley K. Protective effects of isoflurane pretreatment in endotoxin-induced lung injury. *Anesthesiology* 2006;104:511–517. [PubMed: 16508399]
- Rosenzweig HL, Minami M, Lessov NS, Coste SC, Stevens SL, Henshall DC, Meller R, Simon RP, Stenzel-Poore MP. Endotoxin preconditioning protects against the cytotoxic effects of TNF α after stroke: a novel role for TNF α in LPS-ischemic tolerance. *J Cereb Blood Flow Metab* 2007;27:1663–1674. [PubMed: 17327883]
- Salonen T, Sareila O, Jalonon 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;147:790–799. [PubMed: 16432499]
- Schwartz M, Butovsky O, Bruck W, Hanisch UK. Microglial phenotype: is the commitment reversible? *Trends Neurosci* 2006;29:68–74. [PubMed: 16406093]
- Shaked I, Tchoresh D, Gersner R, Meiri G, Mordechai S, Xiao X, Hart RP, Schwartz M. Protective autoimmunity: interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. *J Neurochem* 2005;92:997–1009. [PubMed: 15715651]
- Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, Rampil IJ, Trudell J, Vissel B, Eger EI 2nd. Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. *Anesth Analg* 2003;97:718–740. [PubMed: 12933393]
- Su JY, Vo AC. Role of PKC in isoflurane-induced biphasic contraction in skinned pulmonary arterial strips. *Anesthesiology* 2002;96:155–161. [PubMed: 11753016]
- Wang C, Lee J, Jung H, Zuo Z. Pretreatment with volatile anesthetics, but not with the nonimmobilizer 1,2-dichlorohexafluorocyclobutane, reduced cell injury in rat cerebellar slices after an *in vitro* simulated ischemia. *Brain Res* 2007;1152:201–208. [PubMed: 17434151]
- Wei H, Kang B, Wei W, Liang G, Meng QC, Li Y, Eckenhoof RG. Isoflurane and sevoflurane affect cell survival and BCL-2/BAX ratio differently. *Brain Res* 2005;1037:139–147. [PubMed: 15777762]
- Xu X, Fen J, Zuo Z. Isoflurane preconditioning reduces the rat NR8383 macrophage injury induced by lipopolysaccharide and interferon- γ . *Anesthesiology*. 2007In press
- Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC. Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth* 2003;91:551–565. [PubMed: 14504159]
- Zheng S, Zuo Z. Isoflurane preconditioning reduces Purkinje cell death in an *in vitro* model of rat cerebellar ischemia. *Neuroscience* 2003;118:99–106. [PubMed: 12676141]
- Zheng S, Zuo Z. Isoflurane preconditioning induces neuroprotection against ischemia via activation of p38 mitogen-activated protein kinase. *Mol Pharmacol* 2004;65:1172–1180. [PubMed: 15102945]
- Zheng S, Zuo Z. Isoflurane preconditioning decreases glutamate receptor overactivation-induced Purkinje neuronal injury in rat cerebellar slices. *Brain Res* 2005;1054:143–151. [PubMed: 16081051]
- Zhuang JC, Wogan GN. Growth and viability of macrophages continuously stimulated to produce nitric oxide. *Proc Natl Acad Sci U S A* 1997;94:11875–11880. [PubMed: 9342330]
- 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. [PubMed: 7631962]
- Zuo Z, Johns RA. Inhalational anesthetics up-regulate constitutive and lipopolysaccharide-induced inducible nitric oxide synthase expression and activity. *Mol Pharmacol* 1997;52:606–612. [PubMed: 9380023]
- 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. [PubMed: 16806162]

Fig. 1. Lipopolysaccharide (LPS) plus interferon- γ (IFN γ) dose-dependently decreased cell viability
 The mouse C8-B4 microglial cells were exposed to or were not exposed to various concentrations of LPS and IFN γ for 24 hr. The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are means $\pm$ S.D. (n = 12). * P < 0.05 compared with the control. [^] P < 0.05 compared with the corresponding cells exposed to the same concentrations of LPS plus 10 U/ml IFN γ .

Fig. 2. Isoflurane pretreatment reduced the lipopolysaccharide (LPS) plus interferon- γ (IFN γ)-induced decrease of cell viability

Panel A: The mouse C8-B4 microglial cells were exposed to or were not exposed to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr or 5 mM glutamate for 24 hr. **Panel B:** The mouse C8-B4 microglial cells were pretreated with or without various concentrations of isoflurane. They were then exposed to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr at 30 min after the isoflurane pretreatment. The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are means $\pm$ S.D. (n = 14 -18). * P < 0.05 compared with the control. [^] P < 0.05 compared with the corresponding cells exposed to LPS plus IFN γ only.

Fig. 3. Isoflurane pretreatment reduced the lipopolysaccharide (LPS) plus interferon- γ (IFN γ)-increased inducible nitric oxide synthase (iNOS) expression and nitrite production

The mouse C8-B4 microglial cells were pretreated with or without various concentrations of isoflurane. They were then exposed to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr 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 of iNOS expression (**Panel A**). The culture medium was collected for nitrite measurement (**Panel B**). Results are means $\pm$ S.D. (n = 8 - 9). * P < 0.05 compared with the corresponding cells exposed to LPS plus IFN γ only. Iso: isoflurane.

Fig. 4. The effects of D,L-threo- β -benzyloxyaspartate (TBOA), aminoguanidine (AG) and isoflurane pretreatment on the lipopolysaccharide (LPS) plus interferon- γ (IFN γ)-induced decrease of cell viability (*panel A*) and glutamate release (*panel B*)

The mouse C8-B4 microglial cells were pretreated with or without 2% isoflurane. They were then exposed to 10 ng/ml LPS plus 10 U/ml IFN γ for 24 hr at 30 min after the isoflurane pretreatment. Some cells were exposed to 10 ng/ml LPS plus 10 U/ml IFN γ in the presence of 10 μ M TBOA or 10 μ M AG for 24 hr. The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The glutamate concentrations in the culture medium were measured by high performance liquid chromatography. Results are means $\pm$ S.D. (n = 6 - 31). * P < 0.05 compared with the control. [^] P < 0.05 compared with the corresponding cells exposed to LPS plus IFN γ only. Con: control.

Fig. 5. Attenuation of isoflurane preconditioning-induced protection by protein kinase C inhibition
 The mouse C8-B4 microglial cells were pretreated with or without 2% isoflurane in the presence or absence of 10 μ M chelerythrine. They were then exposed to 10 ng/ml lipopolysaccharide (LPS) plus 10 U/ml interferon- γ (IFN γ) for 24 hr at 30 min after the isoflurane pretreatment. The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are means $\pm$ S.D. (n = 14). * P < 0.05 compared with the control. ^ 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 . Che: chelerythrine; Con: control; Iso: 2% isoflurane pretreatment.