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ISOFLURANE PRECONDITIONING REDUCES MOUSE MICROGLIAL ACTIVATION AND INJURY INDUCED BY LIPOPOLYSACCHARIDE AND INTERFERON-γ

X. XU,[a,b] J. A. KIM[a,c] AND Z. ZUO[a]*

[a]*Department of Anesthesiology, University of Virginia Health System, University of Virginia, 1 Hospital Drive, PO Box 800710, Charlottesville, VA 22908-0710, USA*

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

[c]*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 (IFNγ) for 24 h decreased the viability of these cells. Pretreatment of these cells with 1%, 2% or 3% isoflurane, a commonly used volatile anesthetic, for 1 h at 30 min before the exposure to LPS plus IFNγ attenuated the reduction of cell viability (preconditioning effect). LPS plus IFNγ 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 IFNγ effects on the iNOS expression and nitrite accumulation. Aminoguanidine, an iNOS inhibitor, attenuated the LPS plus IFNγ-induced glutamate release and decrease of microglial viability. Isoflurane preconditioning also reduced LPS plus IFNγ-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 IFNγ 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.

Key words: glutamate, inducible nitric oxide, isoflurane, microglia, neuroprotection, preconditioning.

Inflammatory process is involved in a broad range of pathologies and diseases of the CNS including brain trauma, stroke, brain infection and neurodegenerative diseases (Eddleston and Mucke, 1993; Kreutzberg, 1996). One of the brain cells, microglial cells, is a macrophage-like cell 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, 2004, 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 (IFNγ). 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). IFNγ 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.

*Corresponding author. Tel: +1-434-924-2283; fax: +1-434-924-2105.
E-mail address: zz3c@virginia.edu (Z. Zuo).
Abbreviations: EAAT, excitatory amino acid transporter; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PKC, protein kinase C; TBOA, D,L-*threo*-β-benzyloxyaspartate.

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EXPERIMENTAL PROCEDURES

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, USA). Heat inactivated fetal bovine serum (FBS), L-glutamine, Griess Reagent Kit (G7962) and recombinant rat IFNγ produced from *E. coli* were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Rabbit polyclonal anti-iNOS antibody whose epitope is at the C-terminus of iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA; 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, USA). Isoflurane was purchased from Abbott Laboratories (North Chicago, IL, USA). Chelerythrine chloride was obtained from Biomol (Plymouth Meeting, PA, USA). 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, USA).

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% CO_2 at 37 °C. The medium was changed every 3 days. The cells were plated at a density of 3.5×10^4-5×10^4/well on 96-well tissue culture plates for viability experiments by MTT assay. Cells were grown to ~90% confluence in six 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 IFNγ and the concentrations of LPS and IFNγ used in the subsequent experiments. Cells were incubated with various concentrations (10–1000 ng/ml) of LPS and 10 or 50 U/ml IFNγ for 24 h. 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 h after the cells were plated. 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 ~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 h). 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 IFNγ for 24 h at 37 °C. In some experiments, cells were incubated with or without 10 μM aminoguanidine, an iNOS inhibitor, or 10 μM 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 IFNγ for 24 h. These cells were not pretreated with isoflurane. To determine the involvement of PKC in the isoflurane preconditioning, the PKC inhibitor chelerythrine (10 μM) 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 IFNγ. To determine glutamate-induced toxicity in microglial cells, C8-B4 cells were incubated with 5 mM glutamate for 24 h. These cells were not treated with isoflurane or LPS plus IFNγ.

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 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

Cell viability was determined by the MTT assay according to manufacturer's procedure and as we described before (Zuo et al., 2006). Briefly, 10 μl of the MTT labeling reagent was added to each well that contained 100 μl culture medium and cells, and mixed gently. Four hours later, 100 μl of the solubilization solution was added into each well and mixed thoroughly by repeated pipetting. The plate was kept at 37 °C for 1 h. 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 $1000\times g$ for 10 min at 4 °C. The supernatants were used for Western blotting and loaded at 20 μg 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® six well culture plates were pretreated with or without 2% isoflurane for 1 h and then exposed to 10 ng/ml LPS plus 10 U/ml IFNγ for 24 h 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).

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

Microglial cells at ~90% confluence were treated with or without 2% isoflurane for 1 h before the exposure to 10 ng/ml LPS plus 10 U/ml IFNγ for 24 h at 37 °C. In some experiments, 10 μM aminoguanidine and 10 μM TBOA were added to cells just before the application of LPS plus IFNγ. 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, USA) after a pre-column derivatization with OPA/βME solution (1,2-phthalic dicarboxaldehyde; Acros, Morris Plains, NJ, USA/β-mercapto-ethanol; Sigma) as we described before (Wang et al., 2007). The reversed-