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Effect of sevoflurane treatment on microglia activation, NF-kB and MAPK activities

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ABSTRACT

Microglia activation has been implicated in neurodegenerative disease. Sevoflurane is fluorinated methyl isopropyl ether with anti-inflammatory activity. In this study, we evaluated the potential effects of sevoflurane on lipopolysaccharides (LPS)-induced microglia activation. We treated primary microglia cells with sevoflurane prior to LPS treatment and tested the microglia migration, the productions of pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-6 and interleukin-8. We also explored the effects of sevoflurane on NF-κB and p38 MAPK activation. Finally, we examined the effect of sevoflurane on cytokines production in rat brain. Sevoflurane significantly reduced LPS-induced microglial migration. Sevoflurane significantly decreased the production of pro-inflammatory cytokines both *in vitro* and *in vivo*. Sevoflurane attenuated activations of NF-κB and MAPK signaling pathways. Sevoflurane treatment decreased microglia activation by suppressing NF-kB and MAPK signaling pathways.

1. Introduction

Microglial cells are a type of neuroglia located in brain and spinal cord. Microglia is the resident macrophage in the brain and plays an important role in neuroinflammation (Perry and Teeling, 2013). Accumulating evidences has demonstrated that activated microglia produces multiple neurotoxic factors which drive progressive neuron damage (Lull and Block, 2010). It has been described that in neuropathological conditions including Alzheimer`s disease (AD), Parkinson`s disease (PD), multiple sclerosis and cerebral ischemia, microglia is over-reactive and results in neural cell death and deterioration of the on-going disease process (Matt and Johnson, 2016).

Activation of microglia results in dramatic change in morphology of microglia. In addition, activated microglia secrets a variety of factors including pro-inflammatory cytokines, reactive oxygen species and nitric oxide. These factors are thought to be responsible for neuroglia-mediated neurotoxicity (Banati et al., 1993). It has been demonstrated that these factors are produced in glial cell after exposure to lipopolysaccharides (LPS) (Liu et al., 2001).

Sevoflurane (1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy) propane), is a highly fluorinated methyl isopropyl ether, which was used as an inhalational anesthetic for induction and maintenance of general anesthesia (Patel and Goa, 1996). Sevoflurane has been reported to exaggerate cognitive decline in rat with chronic intermittent hypoxia (Dong et al., 2018). It has been also described that sevoflurane prevents lung inflammation in rat and mice (Song et al., 2013; Wang et al., 2017; Xiong et al., 2013). The anti-inflammatory activity of sevoflurane in lung suggested the potential anti-inflammatory effect in other tissues and organs.

In the present study, we aim to examine the potential effect of sevoflurane on LPS-induced microglial activation and inflammation.

2. Materials and methods

2.1. Primary microglial culture and treatment

Primary microglial cells were isolated following the protocol previously described (Pei et al., 2017). Briefly, fresh cerebral cortices were isolated from newborn mice pups and dissected with sterile scissors. Then 0.25% trypsin-EDTA solution was used for sample digestion for 20 min at 37 °C. After digestion, equal amount of complete culture medium DMEM-F-12 supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 μg/ml) was added to stop the digestion. Then DNase was added to digest the sticky DNA released from dead cells. After centrifuging at 200 *g* for 5 min, the pellets were suspended in culture medium and suspended by pipetting. Cells after

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passing through a 100 μm pore mesh were seeded in flasks and cultured at 37 °C with 5% CO_2. In 5–7 days, astrocytes at the bottom of the flask formed a confluent cell layer and microglia grew on top of the astrocytic layer. To collect microglia, vigorously tap the flasks on the bench top and collect the floating cells in culture media. After centrifugation, cell pellets were re-suspended in mixed glial-conditioned medium and seeded in 6-well plates. After 2 h, aspirate medium and replace with fresh culture medium. The microglial cells are ready to use the next day. For sevoflurane treatment, the cells were placed in an air-tight container which was matched with inflow and outflow connectors. The inlet port was connected to the sevoflurane vaporizer to make up for volatilization loss of sevoflurane and the outlet port was connected to a gas analyzer which measured sevoflurane concentration (3.3%). After 10 min continuous sevoflurane balancing, the container was sealed to incubate for 30 min at 37 °C (Li et al., 2015). For activation, the microglial cells were treated with 100 ng/ml LPS after sevoflurane treatment for 24 h as described previously (Kim et al., 2016).

2.2. Cell invasion assay

The cell invasion assay was performed by using Transwell chamber (Corning, NY, USA) as described previously (Gao et al., 2017). Briefly, microglia cells were harvested and re-suspended in serum-free medium and laid on the top of polycarbonate Transwell filter pre-coated with Matrigel (BD, USA). The lower compartment was filled with complete culture medium containing 10% FBS. The cells were cultured for 24 h and then the noninvasive free cells were removed with cotton swab from the top chamber. The invaded cells in Matrigel were fixed and stained with crystal violet for visualization.

2.3. RT-PCR

The total RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNAs were synthesized using a reverse transcription kit (Applied Biosystem, Waltham, MA, USA). Real time quantitative PCR reactions were set up in triplicate with SYBR® Green Master Mix (Biorad, Hercules, CA, USA) and run on a 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher, Waltham, MA USA). The following primers were used in the current study: tumor necrosis factor (TNF)-α: forward: 5' -CCCTCACACTCAG ATCATCTTCT -3', reverse: 5' -GCTACGACGTGGGCTAC AG -3'. Interleukin (IL)-6: forward: 5' -TAGTCCTTCCTACCCCAATTTCC -3', reverse: 5' -TTGGTCCTTA GCCACTCCTTC - 3'. IL-8: forward: 5' -AAT TCTCGAGTCGCGAATGGCTGCTCAAGGC T G -3', Reverse primer 5' -ATTACGGCCGTCGCGATTAGGCATCACTGCCTG -3'. β-actin: forward: 5' -TCACCAACTGGGACG -3', reverse: 5' -GCATACAGGGACAACA -3'. The amounts of TNF-α, IL-6, IL-8 mRNA expressions were normalized to β-actin mRNA value.

2.4. ELISA

The levels of TNF-α, IL-6, IL-8 in cell culture supernatant and in whole brain homogenates were detected using commercial ELISA kits from R&D systems (Minneapolis, USA), following manufacturer's instructions.

2.5. Immunofluorescence assay

3 h after LPS treatment, cells were fixed in 4% paraformaldehyde for 1 h at room temperature. After washing with phosphate-buffered saline (PBS), the fixed cells were permeabilized with 1% Triton X-100 in PBS for 5 min at room temperature. After washing with PBS 3 times, the permeablized cells were incubated with 1:100 dilution of mouse anti-NF-κB (p65) antibody (Santa Cruz, USA) for 1 h at 37 °C and washed with PBS for 3 times. The cells were then incubated with Goat anti-mouse IgG labeled with Alexa Fluor 568 antibody (Invitrogen, Waltham, MA USA) for 1 h at room temperature and washed with PBS for 3 times. The cells were then stained with 1 μM of Hoechst (Invitrogen, Waltham, MA USA) staining solution for 10 min at room temperature and washed with PBS.

2.6. Western blotting

Cellular and whole brain proteins were extracted using Total Protein Extraction Kit (Millipore, Billerica, MA, USA) following manufacture's protocol. In some experiments, the nuclear protein was prepared using a nuclear extraction kit (Thermo Scientific, USA) following manufacture's protocol. 30 μg protein was loaded on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and then transferred to PVDF membrane (Bio-rad, Hercules, CA, USA). After blocking, the blots were incubated with IκBα, p-IκBα, p-p65, p38, p-p38, MEF-2, nucleplon and β-actin antibodies (dilution 1:1000) (Abcam, Cambridge, MA, USA) in 5% non-fat skim milk. The membranes were washed and probed with corresponding horseradish peroxidase conjugated secondary antibody. Chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA USA) was used to detect the bands. The western blot results were quantitated and analyzed using GS-900™ Calibrated Densitometer and software Image Lab (Bio-Rad, Hercules, CA, USA) following manufacturer's instructions.

2.7. Animal experiment

Clean grade Sprague–Dawley (SD) rats were randomly divided into three groups including control group, LPS group and sevoflurane group. Control group rats were treated with PBS. Rats in LPS group were injected intraperitoneally (i.p.) with 2 mg/kg LPS as described previously (Prince et al., 2017). Rats in sevoflurane group were injected with LPS and then moved to anesthesia box after 4 h since modeled, with sevoflurane inhalation (concentration 3.3%) for l h, during spontaneous breathing as described previously (Song et al., 2013). This study was approved by the Ethics Committee of Guizhou Provincial People's Hospital.

2.8. Statistical analysis

The values given are the means ± SEM. One-way ANOVA analysis, followed by a Tukey's post hoc test was used for comparison. Statistical difference was considered as significant only if $p < 0.05$.

3. Results

3.1. Sevoflurane decreased LPS-induced microglial migration

LPS had been shown to activate microglial activation and initiate inflammatory response, which could be destructive (Dheen et al., 2007). First, we tested the effects of Sevoflurane on LPS-induced microglial migration by using transwell assay. As shown in Fig. 1A, under normal condition, there was few microglial migrated across the transwell. Once microglial were activated by LPS, there were obviously more cells migrated to the bottom (Fig. 1B). In contrast, sevoflurane treatment decreased the migration as there were less cells detected in the bottom (Fig. 1C). The sevoflurane mediated inhibition of migration was significant after quantitation (Fig. 1D). Therefore, our data indicated that sevoflurane inhibited LPS-induced microglial migration, suggestion a potential inhibitory role of sevoflurane on microglial activation.

3.2. Sevoflurane attenuated LPS-induced pro-inflammatory cytokines production in microglial

We continued to explore the effects of sevoflurane on LPS-induced pro-inflammatory cytokine production in microglial. It was found that sevoflurane treatment at 1.1% concentration failed to decrease the

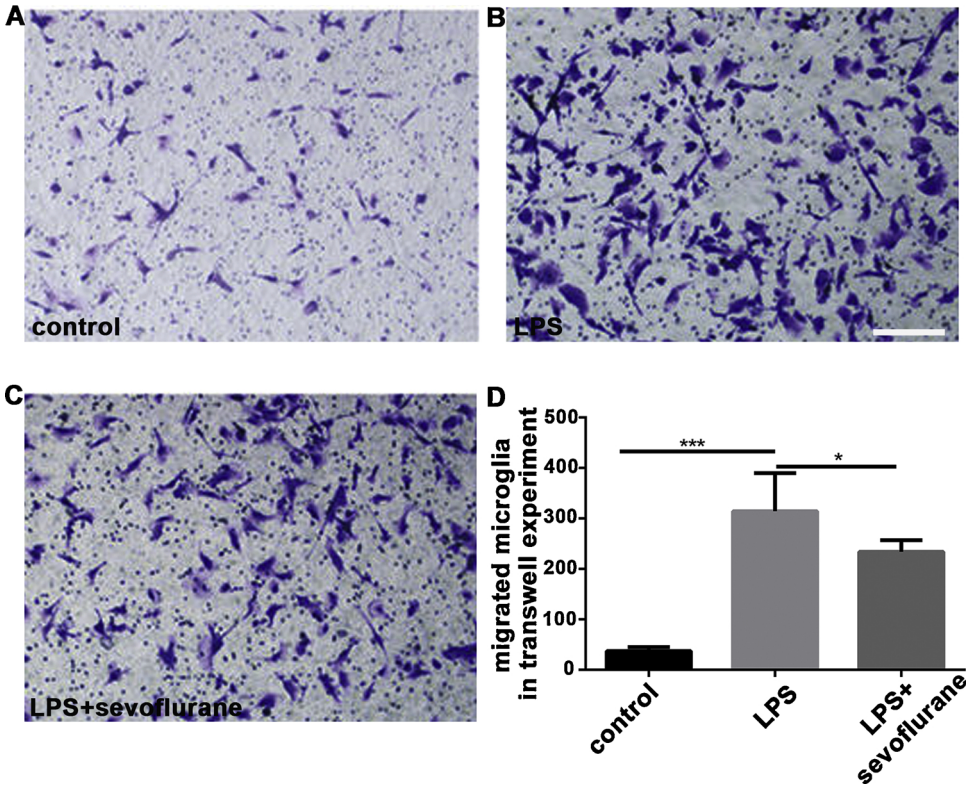

Fig. 1. Effects of sevoflurane treatment on cell migration in LPS-stimulated microglia. The microglia were treated with the sevoflurane (3.3%) for 10 min before LPS treatment (100 ng/mL). A transwell migration assay was performed to examine microglia cell migration. (A) In control group, there is a few numbers of microglia that migrated to the chamber. (B) LPS treatment significantly increased the number of cells that migrated to the chamber, which could be partly inhibited by sevoflurane (C). The data represent the mean ± SD from three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way ANOVA).

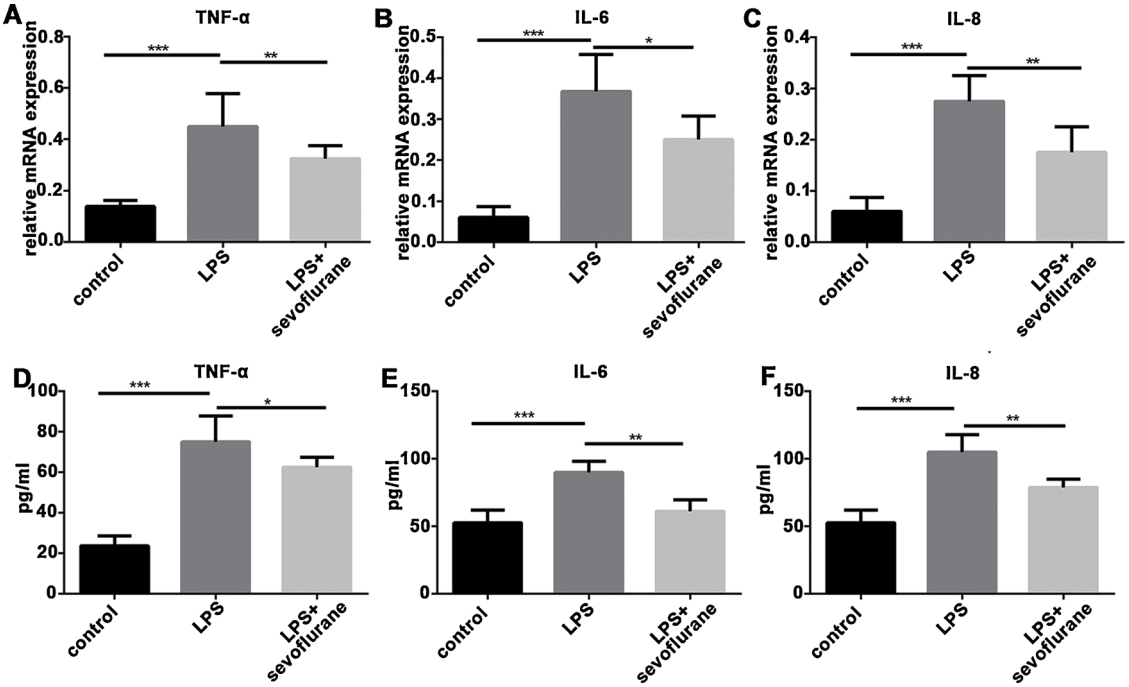

Fig. 2. Sevoflurane treatment (3.3%) decreases the levels of inflammatory mediators induced by LPS in microglia. (A–C) The relative mRNA expression levels of TNF-α, IL-1, IL-6 and IL-10 in microglia were determined by real-time PCR in each group. (D–F) The protein expression levels of TNF-α, IL-6 and IL-8 levels in supernatant of cultured microglia were determined by ELISA. The data represent the mean ± SD from three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way ANOVA).

levels of inflammatory mediators induced by LPS in microglia, while sevoflurane at the concentrations of 2.2%, 3.3% and 4.4% could attenuate LPS-induced pro-inflammatory cytokine production (Supplementary Fig. S1A–C). Of note, there was no significant difference in the inhibition of cytokine production between the groups under 3.3% and 4.4% sevoflurane treatment. Consistent to previous reports, LPS significantly induced mRNA expressions of pro-inflammatory cytokines TNF-α (Fig. 2A), IL-6 (Fig. 2B) and IL-8 (Fig. 2C) in microglial. Correspondingly, the protein levels of TNF-α (Fig. 2D), IL-6 (Fig. 2E) and IL-8 (Fig. 2F) in cell culture supernatants were significantly increased after LPS treatment. In contrast, sevoflurane treatment significantly inhibited the LPS induced expressions of TNF-α, IL-6 and IL-

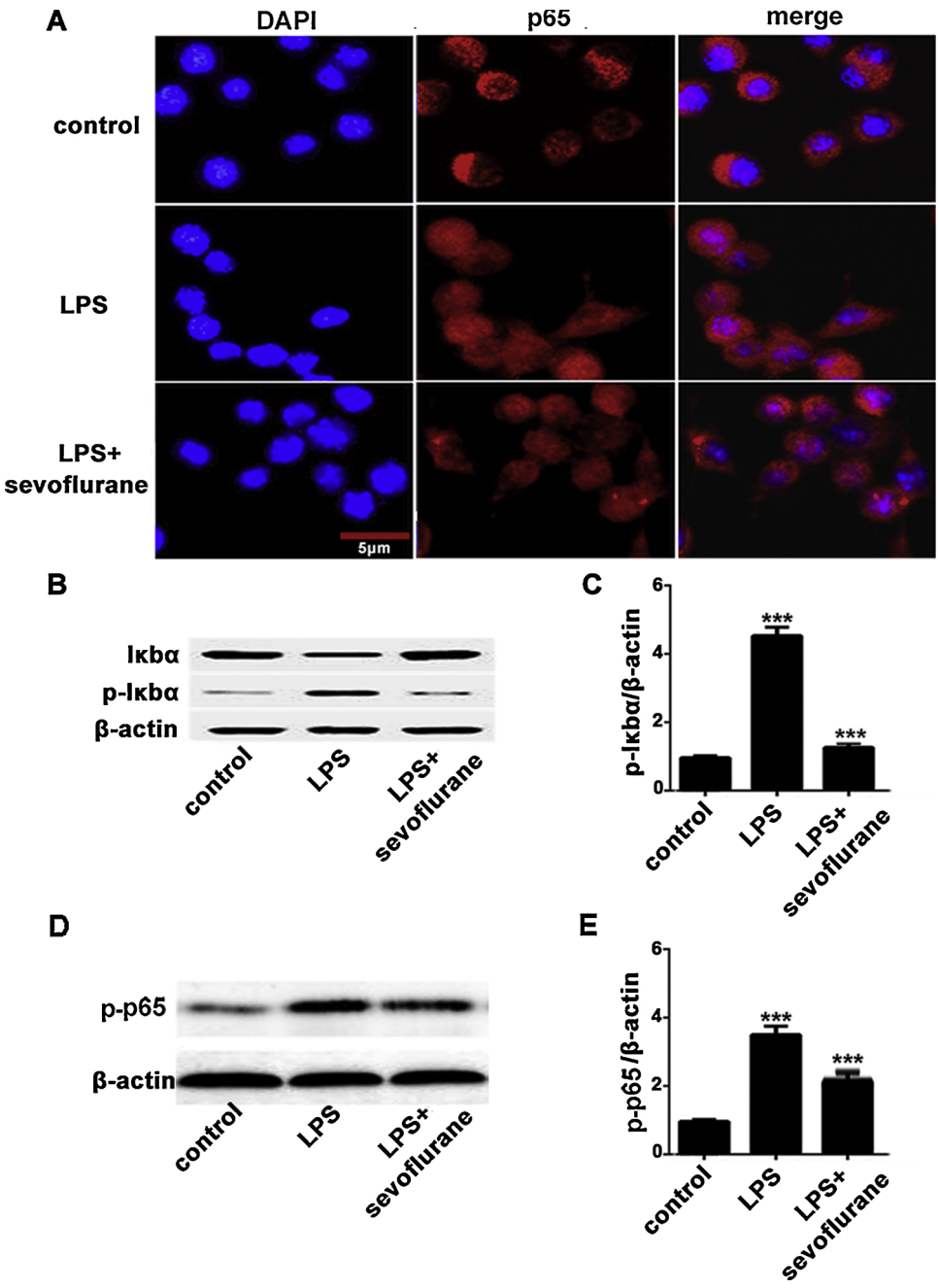

Fig. 3. Sevoflurane treatment significantly inhibits LPS-induced NF-κB activation. (A) The microglia were treated with LPS (100 ng/mL) in the absence or presence of sevoflurane. The nuclear translocation of the NF-κB p65 subunit was determined using immunofluorescence assay. (B) The cell extracts were prepared and analyzed by Western blot using IκBα, p-IκBα and β-actin antibodies. (C) The densitometry analysis of p- IκBα is presented. (D) The cell extracts were prepared and analyzed by Western blot using p-p65 and β-actin antibodies. (E) The densitometry analysis of p-p65 is presented. The data represent the mean ± SD from three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way ANOVA).

8. Therefore, our data indicated that sevoflurane attenuated LPS-induced pro-inflammatory cytokine productions in microglial.

3.3. Sevoflurane reduced LPS-induced NF-κB activation in microglial

As we detected the inhibition of LPS-induced microglial activation by sevoflurane, we further explored the underlying mechanism. It had been well-studied that LPS treatment induced activation of NF-κB signaling pathway (Sharif et al., 2007). We continued to detect the effects of sevoflurane on NF-κB activation. First, we examined the nuclear translocation of NF-κB. As shown in Fig. 3A, under normal condition, the NF-κB p65 distributed mainly in cell cytosol. After LPS treatment, the majority of NF-κB p65 translocated into host cell nuclei and co-colocalized with nucleus. In contrast, sevoflurane prevented nuclear translocation of NF-κB p65 induced by LPS, suggesting sevoflurane prevented NF-κB p65 activation. Activation of NF-κB required IκB phosphorylation and degradation (Viatour et al., 2005). We detected increased p-IκBα level and p-p65, as well as decreased total IκBα level in LPS-treated microglial (Fig. 3B–E). In contrast, sevoflurane decreased the levels of p-IκBα and p-p65 in LPS-treated microglial (Fig. 3B–E). Furthermore, it was found that sevoflurane reduced the activated microglial cells and attenuated microglial phagocytosis (Supplementary Fig. S2A–D).

3.4. Sevoflurane attenuated LPS-induced MAPK activation in microglial

LPS also activated MAPK signaling pathway, which was one of the important signaling pathways during inflammation (Neuder et al., 2009). Therefore, we examined the effects of sevoflurane on LPS-induced p38 MAPK activation. LPS treatment significantly induced phosphorylation of p38 while sevoflurane significantly prevented LPS-induced phosphorylation of p38 (Fig. 4A&B). MEF-2 was a downstream transcriptional factor which was activated by p38 MAPK during inflammation (Han et al., 1997). Corresponding to the inhibition of p38

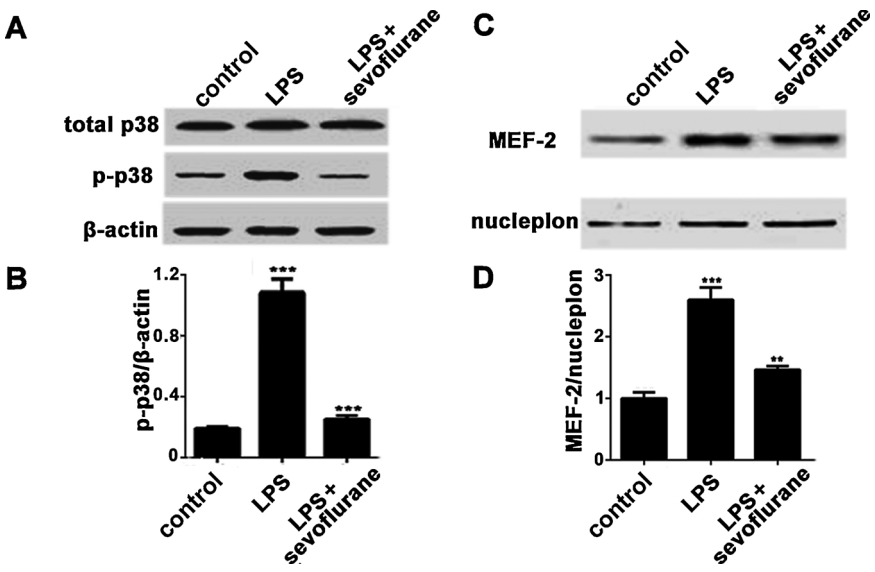

Fig. 4. Inhibitory effects of sevoflurane on the LPS-induced activation of p38 and the expression of MEF-2 in the microglia. (A, B) The analysis of the p38 and p-p38 is presented in the lower panel. (C, D) The cells were pretreated with sevoflurane for 10 min before LPS (100 ng/mL) treatment. The MEF-2 protein was examined by western blotting. The quantification data are shown in the lower panel. The data represent the mean ± SD from three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way ANOVA).

phosphorylation, we detected significantly decreased levels of nuclear MEF-2 (Fig. 4C&D) in sevoflurane treated microglial, indicating the inhibition of MAPK activation by sevoflurane. In addition, LPS activated JNK signaling as LPS treatment significantly increased level of phosphorylated-JNK (Supplementary Fig. 3A&B). In contrast, sevoflurane attenuated the LPS-induced phosphorylation of JNK, indicating that sevoflurane prevented LPS-induced JNK activation. Furthermore, we treated the microglia with p38 agonist and inhibitor, respectively, in the presence of sevoflurane. It was found that p38 agonist could partially reverse sevoflurane-induced decreased production of pro-inflammatory cytokines in LPS-treated microglia (Supplementary Fig. 4A–C). Also, p38 inhibitor failed to further inhibit sevoflurane-induced inhibition of NF-kB activation (Supplementary Fig. 4D and E). These results provide direct evidences that MAPK and NF-kB (as a downstream target of p38) signaling is involved in the effects of sevoflurane.

3.5. Sevoflurane decreased LPS-induced pro-inflammatory cytokine production in brain

As sevoflurane successfully prevented LPS-induced pro-inflammatory cytokines production *in vitro,* we continued to examine whether the inhibitory effects exist *in vivo.* After administration of sevoflurane, both mRNA and protein levels of TNF-α (Fig. 5A&D), IL-6 (Fig. 5B&E) and IL-8 (Fig. 2C&F) were measured. LPS alone significantly induced production of TNF-α, IL-6 and IL-8 while administration of sevoflurane significantly decreased LPS-induced production of TNF-α, IL-6 and IL-8 in brain. Therefore, our data indicated that the inhibitory effect of sevoflurane also existed *in vivo*.

4. Discussion

In current study, we demonstrated that sevoflurane exhibited inhibitory effect on microglial activation, including decreasing LPS-induced microglial migration and pro-inflammatory cytokines production. Microglial is the primary immune cell that responsible for detecting invading pathogens and neuronal injuries. In response to injury or immunological stimulation, microglia become activated and the activation of microglia has been reported to be associated with pathogenesis of various neurodegenerative diseases. Activated microglia produces a variety of pro-inflammatory including TNF-α, IL-6 and IL-8, nitric oxide and reactive oxygen intermediates. These factors impact on neurons to induce neurodegeneration.

In current study, by using LPS treatment, we demonstrated that LPS treatment (100 ng/ml) induced primary microglial migration and production of pro-inflammatory cytokines. We also observed that sevoflurane significantly inhibited both mRNA and protein expressions of TNF-α, IL-6 and IL-8 induced by LPS. The anti-inflammatory effect of sevoflurane has been described previously. Hofstetter and colleagues described that sevoflurane significantly reduced TNF-α levels in LPS-treated rats (Hofstetter et al., 2007). Lee et al. demonstrated that sevoflurane reduced pro-inflammatory cytokines production and reduced necrosis and inflammation in proximal tubules (Lee et al., 2006). Watanabe and colleagues reported that sevoflurane suppressed IL-6 and IL-8 production in pulmonary epithelial cells (Watanabe et al., 2013). Our results were consistent with these previous reports.

NF-κB is a transcriptional factor that regulates the expressions of various pro-inflammatory cytokines (Baldwin, 1996). Activation of NF-κB results in nuclear translocation of NF-κB, which is controlled by the targeted phosphorylation and subsequent degradation of IκB. In this study, we identified that sevoflurane prevented LPS-induced IκB phosphorylation and degradation, which leads to diminish the nuclear translocation of p65 subunit of NF-κB. The inhibition of NF-κB activation by sevoflurane has been described previously. Sun et al demonstrated that sevoflurane inhibited NF-κB activation in LPS-induced acute inflammatory lung injury (Sun et al., 2015).

The MAPK signaling pathway participates in a variety of cellular activities including cell proliferation and differentiation. Phosphorylation of MAPK activates the production of inflammatory molecules (Guimaraes et al., 2013). Deviation from the strict control of MAPK signaling pathways has been implicated in the development of neurodegenerative diseases such as AD and PD. Persistent activation of p38 has been suggested to mediate neuronal apoptosis in AD and PD (Kim and Choi, 2010). In this study, we demonstrated that LPS induced activation of p38 signaling pathway in microglial while sevoflurane significantly inhibited LPS-induced p38 phosphorylation. MEF-2 is the downstream signaling transcription factor mediating p38 MAPK signaling (Han et al., 1997). The activation of p38 signaling pathway by LPS resulted in enhanced protein level of MEF-2 in the nuclear extract in LPS-treated microglial cells. In contrast, sevoflurane significantly inhibited nuclear expression of MEF-2, suggesting the inhibition of p38 MAPK signaling pathway by sevoflurane.

Interestingly, a recent publication described that sevoflurane activated inflammation and cell death in rats and altered cognitive function. It seems that there was contradiction of sevoflurane activity on inflammation. One reasonable explanation could be the different model used. In current study, we treated animal with LPS and then administrated sevoflurane. It is possible that in an environment with

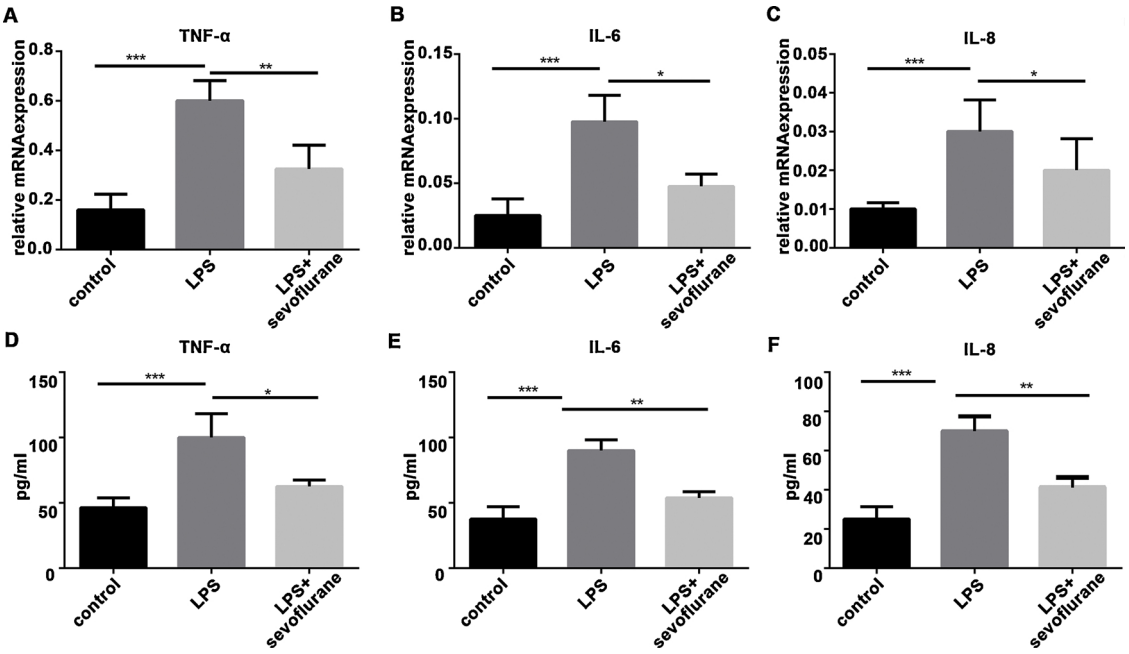

Fig. 5. Sevoflurane treatment decreases the cytokines expression induced by LPS in the brain. Q-PCR and ELISA experiment was used to examine the effect of sevoflurane on cytokine expression. Rats was treated with LPS (2 mg/kg) in the absence or presence of sevoflurane. (A, B, C) Q-PCR assay showed that LPS significantly increased the expression TNF-α, IL-6, IL-8 and sevoflurane treatment significantly reduced the expression of TNF-α, IL-6 and IL-8 in the brain. ELISA experiment also showed that sevoflurane treatment decreased the expression of TNF-α (D), IL-6 (E) and IL-8 (F) caused by LPS treatment. The data represent the mean ± SD from three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way ANOVA).

inflammation, sevoflurane exhibited anti-inflammatory functions as described previously (Song et al., 2013). In contrast, in steady environment, sevoflurane may exhibit the pro-inflammatory activity.

In this study, besides the *in vitro* cell model, we also utilized rat model and demonstrated that sevoflurane inhibited pro-inflammatory cytokines production *in vivo*. Our results are consistent to other study which showed the biological activity of sevoflurane in animal models. Until now the mechanism of how sevoflurane modulated inflammation remains unknown. It has been described that sevoflurane regulated miRNA expression which regulate inflammation (Otsuki et al., 2015). Efforts should be put in exploring the underlying mechanism.

5. Conclusion

Our study demonstrated the anti-inflammatory activities of sevoflurane on LPS-treated microglial by decreasing production of pro-inflammatory cytokines. We also explored the underlying mechanisms and found that sevoflurane prevented activations of both NF-κB and MAPK signaling pathway.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.07.004.

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