Isoflurane and sevoflurane increase interleukin-6 levels through the nuclear factor-kappa B pathway in neuroglioma cells

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Editor’s key points

- Isoflurane can produce cognitive dysfunction in rodents, possibly involving pro-inflammatory signalling pathways.
- The role of nuclear factor-kappa B (NF-κB) in anaesthetic-induced increases in the inflammatory cytokine interleukin (IL)-6 was investigated in vitro.
- Activation of inflammatory signalling by isoflurane and sevoflurane increased IL-6, which might contribute to neuroinflammation and cognitive dysfunction.

Background. Isoflurane can increase pro-inflammatory cytokine interleukin (IL)-6 levels. However, the up-stream mechanism remains unknown. Nuclear factor-kappa B (NF-κB) promotes the generation of pro-inflammatory cytokines. We examined the effects of isoflurane and sevoflurane on the NF-κB signalling pathway and its association with IL-6 levels in cultured cells.

Methods. H4 human neuroglioma cells (H4 cells), and mouse primary neurones and microglia were treated with 2% isoflurane or 4.1% sevoflurane for 6 h, for analysis of IL-6 and NF-κB. Pyrrolidine dithiocarbamate (an NF-κB inhibitor) or 2-deoxy-D-glucose (2-DG) (an inhibitor of glucose glycolysis) was applied 1 h before anaesthetic treatment.

Results. Isoflurane or sevoflurane treatment increased the levels of IL-6 [isoflurane: 410% (54); sevoflurane: 290% (24)], the nuclear levels of NF-κB [isoflurane: 170% (36); sevoflurane: 320% (30)], and the transcription activity of NF-κB in H4 cells. Moreover, isoflurane enhanced the transcription activity of NF-κB in mouse microglia, but not primary neurones. Finally, pyrrolidine dithiocarbamate and 2-DG attenuated isoflurane-induced increases in IL-6 and NF-κB, and the transcription activity of NF-κB.

Conclusions. These studies in H4 cells suggest that the NF-κB signalling pathway could contribute to isoflurane or sevoflurane-induced neuroinflammation. This could lead to the targeted intervention of anaesthetic-induced neuroinflammation.

Keywords: anaesthetic; interleukin-6; NF-κB

Accepted for publication: 10 March 2013

Postoperative cognitive dysfunction (POCD) is a common complication in senior patients, and is associated with increased cost, morbidity, and mortality. Age is a known risk factor for POCD, but its pathogenesis remains largely unknown. This lack of knowledge has become a barrier that impedes further studies of POCD, and thus a lack of treatment or prevention for POCD.

The commonly used inhalation anaesthetic isoflurane has been shown to induce cognitive impairment in rodents, and might also be associated with a higher incidence of POCD in humans. Isoflurane can increase levels of interleukin (IL)-6, and IL-6 has been associated with learning and memory impairment in animals and cognitive dysfunction, and mild cognitive impairment (MCI) and delirium in medical patients. It is conceivable that isoflurane induces neurobehavioural deficits through increasing brain IL-6 levels. However, the up-stream mechanism by which isoflurane increases IL-6 levels remains largely to be determined. This gap in knowledge prevents further mechanistic studies and potential interventions of isoflurane-induced neurobehavioural deficits.

Isoflurane can increase cytosolic calcium levels in H4 human neuroglioma cells (H4 cells) and other tumour cell lines. The elevation of cytosolic calcium can activate the nuclear factor-kappa B (NF-κB) signalling pathway, which is associated with increased levels of pro-inflammatory cytokines. Pyrrolidine dithiocarbamate (PDTC) can inhibit BNF-κB signalling pathway by attenuating the entrance...
of activated NF-κB into the nucleus and binding of NF-κB to the promoter region of multiple genes. 28–30 2-Deoxy-o-glucose (2-DG), an inhibitor of glycolysis, can attenuate isoflurane-induced elevation of cytosolic calcium. 18 We, therefore, assessed the effects of isoflurane on the levels of IL-6 and NF-κB in H4 cells and determined whether the effects of isoflurane on IL-6 and NF-κB could be attenuated by PDTC and 2-DG. We also assessed the effects of isoflurane on the transcription activity of NF-κB in mouse primary neurons and microglia.

Methods

H4 human neuroglioma cells

H4 cells have been extensively used in the Alzheimer's disease research as an in vitro cellular model, 2 17 31 and these in vitro findings have been confirmed in primary neurons and brain tissue of mice. 32 Cells were cultured in Dulbecco's Modified Eagle Medium (high glucose) containing 10% (v/v) heat-inactivated fetal calf serum, 100 U ml −1 penicillin, 100 μg ml −1 streptomycin, and 2 mM glutamine. Oxygen (21%), 5% CO 2, and 2% isoflurane or 4.1% sevoflurane can be considered as optimal CO 2, O 2, and isoflurane concentrations. We treated the cells with 2% isoflurane or 4.1% sevoflurane for 6 h in serum free media, which has been shown to increase cytosolic calcium, caspase-3 activation, Aβ accumulation, and mitochondrial dysfunction. 7 In some studies, the cells were treated with 10 μM PDTC (Sigma, St. Louis, MO, USA) or 10 mM 2-DG (Sigma) 1 h before treatment with 2% isoflurane. The control conditions for isoflurane and PDTC or 2-DG was 5% CO 2 plus 21% O 2 and saline.

Mouse primary neurons and microglia

The protocol was approved by the Massachusetts General Hospital Standing Committee on the Use of Animals in Research and Teaching. The harvest of neurons was performed as described. 34 After 7–10 days in culture, the cells were treated with 2% isoflurane for 6 h as described. 34

Immunoblotting

Immunoblot analysis was performed as described. 7 Briefly, cell pellets were detergent-extracted on ice using immunoprecipitation buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P-40) plus protease inhibitors (1 μg ml −1 aprotinin, 1 μg ml −1 leupeptin, and 1 μg ml −1 pepstatin A). The lysates were collected, centrifuged at 12,000 × g for 10 min, and proteins determined with a bicinechoninic acid protein assay kit (Pierce, Iselin, NJ, USA). Antibodies to NF-κB (1:1000; sc-109 Santa Cruz, CA, USA), IL-6 (1:1000; ab6672, Abcam, Cambridge, MA, USA), or β-actin (1:5000, Sigma) were used to detect NF-κB, IL-6 and β-actin, respectively.

Real-time polymerase chain reaction

The effects of isoflurane on IL-6 mRNA were determined by real-time polymerase chain reaction (RT-PCR) in H4 cells as described. 32 RNA was isolated using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA), with concentration determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Primers of human IL-6 (ID: QT00083720) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ID: QT01192646) were purchased from Qiagen. RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). IL-6 mRNA levels were determined and standardized with GAPDH as internal controls.

Nuclear extraction

A nuclear extraction kit (SK-0001, Signosis, Inc., Sunnyvale, CA, USA) was used for the preparation of nuclear extracts from H4 cells. Cells were washed three times with phosphate buffered saline. Then, buffer I working reagent was added into the cells. The culture dish was put into an icebox and rocked at 200 rpm for 10 min on a shaking platform. Cells were released from the dish using a sterile scraper and transferred to a 1.5 ml microcentrifuge tube to centrifuge at 12,000 × g for 5 min at 4 °C. The supernatant was discarded completely and buffer II working reagent was added to the pellets and mixed gently. The tube was put into an icebox and shaken at 200 rpm on a platform for 2 h. The sample was centrifuged at 12,000 × g for 5 min at 4 °C, and the nuclear extract was obtained in the supernatant.

Electrophoretic-mobility shift assay

An electrophoretic-mobility shift assay (EMSA kit (GS-0030, Signosis) was used to assess the transcription binding activity of NF-κB. Nuclear extract (5 μg) was incubated with 1 μl poly d(I-C), 2.0 μl 5X Binding Buffer and 1.0 μl of transcription factor (TF) probe in a 0.5 ml microcentrifuge tube (PCR tube) at 20–23 °C for 30 min in a PCR machine. For the cold probe control, 1.0 μl of cold TF probe was added into this reaction. Samples were loaded onto a 6.5% non-denaturing polyacrylamide gel, which was run at 100 V and transferred at 60 V for 1 h at 4 °C. The membrane was imaged using a chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA).

Statistics

Data are expressed as mean (SD). Normality test showed that the data were normally disturbed (data not shown). Student’s t-test, and one-way and two-way analysis of variance (ANOVA) were used to compare differences from the control group, followed by the Bonferroni post hoc test where appropriate. P<0.05 (∗ or #) and P<0.01 (∗∗ or ##) were considered statistically significant. The significance testing was two-tailed, and the Prism 6 software (La Jolla, CA, USA) was used to analyse the data.
Isoflurane increases levels of IL-6 in the H4 cells

H4 cells were treated with 2% isoflurane for 6 h, harvested, and subjected to immunoblot analysis. IL-6 immunoblotting revealed that isoflurane (lanes 4–6 in Fig. 1) increased IL-6 compared with the control group (lanes 1–3 in Fig. 1). There was no significant difference in β-actin between isoflurane-treated and control cells. These results suggest that isoflurane is able to increase the levels of IL-6 in H4 cells.

Isoflurane increases nuclear levels of NF-κB and its transcription binding activity in H4 cells and mouse microglia

Activated NF-κB translocates to the nucleus where it binds to the promoter region of multiple genes, including cytokine genes,21–26 leading to expression of cytokine mRNA. Thus, we assessed the effects of isoflurane on the nuclear levels of NF-κB in H4 cells. H4 cells were harvested at the end of isoflurane treatment, or nuclear extract prepared, and subjected to immunoblotting. Immunoblotting of NF-κB showed that isoflurane (lanes 4–6, Fig. 2A) increased nuclear levels of NF-κB compared with the control group (lanes 1–3, Fig. 2A). There was no significant difference in β-actin between isoflurane-treated and control H4 cells. Next, we assessed the effects of the isoflurane treatment on total NF-κB levels in H4 cells. Isoflurane did not significantly alter total NF-κB levels (Fig. 2A). These findings suggest that isoflurane facilitates nuclear translocation of NF-κB.

Finally, we determined the effect of isoflurane on transcription binding activity of NF-κB in nuclear extracts prepared from H4 cells using EMSA.21 In Figure 2C, lane 1 is the probe, lane 2 is treatment with lipopolysaccharide, a positive control that enhances transcription binding activity of NF-κB,17 lane 3 is control, and lane 4 is isoflurane treatment. Isoflurane enhanced the transcription binding activity of NF-κB compared with the control group (Fig. 2C). We then assessed whether isoflurane could enhance the transcription binding activity of NF-κB in mouse primary neutrons and microglia. Treatment with 2% isoflurane enhanced the transcription binding activity of NF-κB in microglia (Fig. 2D), but not primary neutrons (Fig. 2E). The NF-κB inhibitor PDTC attenuated isoflurane-induced enhancement of the transcription binding activity of NF-κB in microglia (Fig. 2D).

PDTC attenuates isoflurane-induced increase in IL-6 levels in H4 cells

We assessed whether the NF-κB inhibitor PDTC might attenuate the isoflurane-induced increase in IL-6 in H4 cells. Two-way ANOVA demonstrated that there was a significant interaction of group (control and isoflurane) and treatment (saline and PDTC) \((F=24.78, P=0.0001)\). RT-PCR showed that isoflurane increased mRNA levels of IL-6 in H4 cells (Fig. 3A): 4.8 (1.8)-fold \(P<0.01\) (one-way ANOVA with Bonferroni correction). PDTC alone did not significantly alter IL-6 mRNA levels, but PDTC attenuated the isoflurane-induced increase in IL-6 mRNA levels in H4 cells: 1.1 (0.7) vs 4.8 (1.8) (Fig. 3A). PDTC also attenuated the isoflurane-induced increase in protein levels of IL-6 \((F=17, P=0.0045, \text{two-way ANOVA})\): 410% (54) vs 76% (44) (Fig. 3B and C). These data suggest that isoflurane increases the mRNA and protein levels of IL-6 via NF-κB signalling.

PDTC attenuates isoflurane-induced activation of NF-κB signalling

Immunoblotting of NF-κB showed that isoflurane increased nuclear levels of NF-κB in H4 cells (Fig. 4A). Two-way ANOVA showed a significant interaction between the group (control and isoflurane) and treatment (saline and PDTC): \(F=7.55, P=0.0055\) (Fig. 4A). PDTC attenuated the isoflurane-induced increase in nuclear NF-κB levels: 130% (34) vs 170% (36) (Fig. 4A). PDTC did not significantly alter total NF-κB levels (Fig. 4C and D). Finally, PDTC attenuated the isoflurane-induced enhancement of transcription binding activity of NF-κB (Fig. 4E).

Sevoflurane increases levels of IL-6, nuclear levels of NF-κB and the transcription binding activity of NF-κB in H4 cells

Given the findings that isoflurane might increase mRNA and protein levels of IL-6 through NF-κB signalling pathway, we determined whether sevoflurane has similar effects as sevoflurane induces caspase-3 activation and Aβ accumulation in H4 cells.38 IL-6 immunoblotting revealed that 4.1% sevoflurane for 6 h (lanes 4–6 in Fig. 5A) increased IL-6 compared with the control group (lanes 1–3 in Fig. 5A). There was no significant difference in the levels of β-actin between sevoflurane-treated and control cells. Quantification of the immunoblots showed that sevoflurane increased protein levels of IL-6 compared with control; 320% (30), \(P=0.0001\) (Fig. 5A).

Immunoblotting of NF-κB showed that sevoflurane (lanes 4–6, Fig. 6A) increased nuclear levels of NF-κB in H4 cells compared with the control group (lanes 1–3, Fig. 6A). There was no significant difference in levels of β-actin. Quantification of the immunoblots showed that sevoflurane increased nuclear levels of NF-κB in H4 cells: 290% (23), \(P=0.0001\) (Fig. 6A). Sevoflurane did not significantly alter total NF-κB
levels (Fig. 6c and d). These findings suggest that sevoflurane facilitates NF-κB translocation to the nucleus, rather than affecting total levels. Sevoflurane enhanced transcription binding activity of NF-κB compared with the control group (Fig. 6E). These data suggest that sevoflurane, like isoflurane, increases IL-6 levels via NF-κB activation of IL-6 gene, which was attenuated by PDTC.

2-DG reduces isoflurane-induced increases in IL-6 and nuclear NF-κB in H4 cells

2-DG attenuates isoflurane-induced elevation of cytosolic calcium, thus, we assessed whether 2-DG could attenuate the isoflurane-induced increases in IL-6 and nuclear NF-κB in H4 cells. Quantitative immunoblotting showed that 2-DG reduced the isoflurane-induced increase in IL-6 (Fig. 7A). Two-way ANOVA demonstrated a significant interaction of group (control and isoflurane) and treatment (saline and 2-DG): $F=4.382, P=0.044$ (Fig. 7A). Specifically, 2-DG attenuated the isoflurane-induced increase in IL-6 levels: 180% (23) vs 120% (34).

2-DG also attenuated the isoflurane-induced increase in nuclear NF-κB levels ($F=18.17, P=0.0002$, two-way ANOVA): 200% (18) vs 120% (38) (Fig. 8A and B). Finally, there was no significant interaction between group (control and isoflurane) and treatment (saline and 2-DG) on total NF-κB ($F=0.0048, P=0.95$) (Fig. 8C and D). Taken together, these findings suggest that 2-DG reduces the isoflurane-induced increase in IL-6 by decreasing nuclear NF-κB levels.
Discussion

The commonly used inhalation anaesthetic isoflurane can induce neuroinflammation (e.g. increasing IL-6 levels in the brain tissues of mice), which could contribute to the isoflurane-associated decline in cognitive function in rodents and possibly humans. However, it is largely unknown how isoflurane increases IL-6 levels. We, therefore, established a cellular system to determine the mechanism by which isoflurane increases IL-6 using H4 cells. Treatment with isoflurane increased IL-6 in H4 cells. These data, together with our previous findings that isoflurane increases cytosolic calcium, mitochondrial dysfunction, caspase-3 activation, and Aβ accumulation in H4 cells, suggested that we could use these cells to perform mechanistic studies. We found that isoflurane increased NF-κB in nucleus, but not in whole cells. NF-κB belongs to a family of inducible dimeric transcription factors that recognizes a consensus DNA sequence and regulates many target genes, especially genes involved in inflammation, injury, and stress. NF-κB is usually present in the cytoplasm as a p65 and p50 heterodimer. Activated NF-κB enters the nucleus and promotes transcription of its target genes, including the pro-inflammatory cytokine IL-6. The finding that isoflurane increased nuclear levels of NF-κB but not total NF-κB levels suggests that isoflurane might facilitate translocation of NF-κB to the nucleus. Moreover, isoflurane enhances the transcription activity of NF-κB. These findings suggest that isoflurane induces activation of NF-κB signalling by facilitating translocation of NF-κB into the nucleus and transcription.

Interestingly, the isoflurane-induced increase in transcription activity of NF-κB was cell-type dependent, occurring in mouse microglia, but not neurones. These findings suggest that isoflurane specifically targets microglia to induce neuroinflammation. The ‘neuroinflammation AD hypothesis’ suggests that microglia-associated neuroinflammation impairs axonal transport by inducing mitochondrial dysfunction, and isoflurane can induce mitochondrial dysfunction. It would be interesting to know whether isoflurane can induce microglia-dependent neuroinflammation, which then leads to impairment of axonal transport through mitochondrial dysfunction. Moreover, it is important to systematically compare the effects of different anaesthetics (e.g. isoflurane, sevoflurane, desflurane, propofol, and ketamine)

Fig 3 PDTC attenuates isoflurane-induced increases in the mRNA and protein levels of IL-6 in H4 cells. (a) Isoflurane increases IL-6 mRNA level in the H4 cells, and PDTC attenuates this increase. (b) Isoflurane increases protein levels of IL-6, and PDTC attenuates this increase. (c) Quantification of the immunoblots shows that PDTC attenuates the isoflurane-induced increase in the protein levels of IL-6. IL, interleukin; PDTC, pyrrolidine dithiocarbamate (n=9).
on neuroinflammation, its up-stream mechanisms such as NF-κB signalling, and down-stream consequences such as impairment of axonal transport in future studies.

These findings cannot specifically determine the cause–effect relationships between isoflurane-induced increases in IL-6 and isoflurane-induced activation of NF-κB signalling. The findings that the NF-κB pathway inhibitor PDTC inhibited both isoflurane-induced increase in IL-6 and activation of NF-κB signalling further suggest that isoflurane increases IL-6 levels via activation of NF-κB signalling. Finally, we found that 2-DG attenuated the isoflurane-induced increase in IL-6 levels by inhibiting activation of NF-κB signalling.

Sevoflurane, another commonly used inhalation anaesthetic, also increased the levels of IL-6 and nuclear NF-κB, but not total NF-κB. These findings suggest that sevoflurane, like isoflurane, increases IL-6 protein levels via NF-κB-mediated activation of IL-6 expression. Future research should include in vivo relevance experiments, including studies to determine whether PDTC and 2-DG can also attenuate isoflurane or sevoflurane-induced cognitive impairment in vivo.

**Fig 4** PDTC attenuates the isoflurane-induced changes in NF-κB in H4 cells. (a) Isoflurane increases nuclear levels of NF-κB, and PDTC attenuates the increase. (b) Quantification of the immunoblots shows that PDTC attenuates the isoflurane-induced increase in nuclear NF-κB levels. (c) PDTC does not alter total NF-κB levels. (d) Quantification of the immunoblots shows that PDTC does not alter total NF-κB levels. (e) PDTC inhibits the isoflurane-induced activation of the transcription binding activity of NF-κB. PTDC, pyrrolidine dithiocarbamate; NF-κB, nuclear factor-kappa B (n=9); LPS, lipopolysaccharide.
**Fig 5** Sevoflurane increases IL-6 levels in H4 cells. (a) Sevoflurane treatment (lanes 4–6) increases IL-6 levels. (b) Quantification of the immunoblots shows that sevoflurane increases IL-6 levels. IL, interleukin (n=9).

**Fig 6** Sevoflurane increases nuclear NF-κB levels and the transcription binding activity of NF-κB in H4 cells. (a) Sevoflurane treatment (lanes 4–6) increases NF-κB levels in nuclear extracts. (b) Quantification of the immunoblots shows that sevoflurane increases NF-κB levels in nuclear extracts. (c) Sevoflurane does not change total NF-κB levels. (d) Quantification of the immunoblots shows that sevoflurane does not change total NF-κB levels. (e) Sevoflurane enhances the transcription binding activity of NF-κB, and PDTC attenuates the sevoflurane-induced enhancement of the transcription binding activity of NF-κB. NF-κB, nuclear factor-kappa B; LPS, lipopolysaccharide; PDTC, pyrrolidine dithiocarbamate (n=9).
These studies have several limitations. First, we did not determine isoflurane effects on the levels of other pro-inflammatory cytokines (e.g. tumour necrosis factor-α), which itself can activate NF-κB signalling. We, therefore, chose IL-6 to establish a cellular system to investigate anaesthetic effects on pro-inflammatory cytokines. Secondly, we did not study the
effects of isoflurane on NF-κB signalling in brain tissue in vivo. It is technically difficult to determine the effects of isoflurane on the transcription binding activity of NF-κB using EMSA in brain tissue. Nevertheless, our findings illustrate an up-stream mechanism by which isoflurane might increase levels of the pro-inflammatory cytokine IL-6. Future studies are required to determine the in vivo relevance of these in vitro findings. Thirdly, cytokines are normally generated and released from glial cells. We did not assess the effects of isoflurane on IL-6 levels and the nuclear level of NF-κB in microglia. However, our studies illustrate that isoflurane enhanced transcription activity of NF-κB in cultured mouse microglia, but not neurons. Finally, our studies did not systematically assess the time- and dose-dependent effects of isoflurane and sevoflurane on NF-κB signalling. The main goal of the current studies was to establish a cellular model to determine up-stream mechanisms by which anaesthetics increase pro-inflammatory cytokines. These findings should facilitate future studies of the potential effects of anaesthetics on neuroinflammation and the underlying mechanisms.

In conclusion, we have established a cellular system to study the mechanisms by which anaesthetics affect the pro-inflammatory cytokine IL-6. Either isoflurane or sevoflurane can increase IL-6 levels via activation of NF-κB signalling, which can be attenuated by inhibition of the NF-κB signalling pathway or glycolysis. This suggests potential therapeutic approaches to prevent or treat anaesthesia neurotoxicity.

**Authors’ contributions**
Conceived and designed the experiments: L.Z.; J.Z., Y.Z., and Z.X. Performed the experiments: L.Z., J.Z., L.Y., and Y.D. Analysed the data: J.Z., Y.D. Wrote the paper: Z.X. and Y.Z.

**Declaration of interest**
None declared.

**Funding**
This research was supported by R21 AG038994, R01 GM088801 and R01 AG041274 from National Institutes of Health, Bethesda, MD, USA; investigator-initiated research grant from Alzheimer’s Association, Chicago, IL, USA; and Cure Alzheimer’s Fund, Wellesley, MA, USA to Z.X. Isoflurane and sevoflurane were generously provided by the Department of Anaesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. These studies are attributed to the Department of Anaesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School.

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Handling editor: H. C. Hemmings