The Mitochondrial Pathway of Anesthetic Isoflurane-induced Apoptosis

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The common inhalation anesthetic isoflurane has been shown to induce apoptosis, which then leads to accumulation of β-amyloid protein, the hallmark feature of Alzheimer disease neuropathogenesis. The underlying molecular mechanism of the isoflurane-induced apoptosis is largely unknown. We, therefore, set out to assess whether isoflurane can induce apoptosis by regulating Bcl-2 family proteins, enhancing reactive oxygen species (ROS) accumulation, and activating the mitochondrial pathway of apoptosis. We performed these studies in cultured cells, primary neurons, and mice. Here we show for the first time that treatment with 2% isoflurane for 6 h can increase pro-apoptotic factor Bax levels, decrease anti-apoptotic factor Bcl-2 levels, increase ROS accumulation, facilitate cytochrome c release from the mitochondria to the cytosol, induce activation of caspase-9 and caspase-3, and finally cause apoptosis as compared with the control condition. We have further found that isoflurane can increase the mRNA levels of Bax and reduce the mRNA levels of Bcl-2. The isoflurane-induced ROS accumulation can be attenuated by the intracellular calcium chelator BAPTA. Finally, the anesthetic desflurane does not induce activation of mitochondrial pathway of apoptosis. These results suggest that isoflurane may induce apoptosis through Bcl-2 family proteins- and ROS-associated mitochondrial pathway of apoptosis. These findings, which have identified at least partially the molecular mechanism by which isoflurane induces apoptosis, will promote more studies aimed at studying the potential neurotoxic effects of anesthetics.

An estimated 200 million patients worldwide undergo anesthesia and surgery each year. Some clinical studies suggest that

Anesthesia and surgery may be associated with Alzheimer disease (AD) (1–3), although different findings also exist (4, 5). Several recent studies have reported that isoflurane, one of the most commonly used inhalation anesthetics, may potentially contribute to AD neuropathogenesis by inducing apoptotic cell death and increasing β-amyloid protein oligomerization and accumulation in vitro and in vivo (6–13). However, the upstream mechanism by which isoflurane induces apoptosis remains largely to be determined.

Apoptosis is a programmed cell death which can be triggered by environmental and/or developmentally associated signals (14). The central components of the apoptosis process are a group of proteolytic enzymes called caspases, which can be activated by various types of stimulation (15). The extrinsic, death receptor pathway involves activation of caspase-8, which then cleaves caspase-3, leading to apoptosis (for review, see Ref. 16). The intrinsic, mitochondrial pathway is regulated by Bcl-2 family proteins, including the anti-apoptotic factor Bcl-2 and the pro-apoptotic factor Bax (for review, see Ref. 17) and involves cytochrome c release from the mitochondria to the cytosol. The released cytochrome c then activates caspase-9, which consequently induces caspase-3 activation, leading to apoptosis (for review, see Ref. 16). The other signaling pathways that lead to apoptosis are the caspase-2-dependent pathway and the caspase-independent pathway (for review, see Ref. 16). Previous studies have suggested that isoflurane may induce apoptosis by elevating cytosolic calcium levels (Refs. 13 and 18; for review, see Ref. 19). However, the subsequent targets, e.g. signaling pathways that lead to apoptosis, after the isoflurane-induced elevation of cytosolic calcium levels, are unknown. Finally, accumulation of reactive oxygen species (ROS) has been suggested to be associated with mitochondrial damage, leading to apoptosis (Ref. 20; for review, see Ref. 21).

In the present studies we set out to determine the effects of isoflurane on protein and mRNA levels of Bax and Bcl-2, mitochondria-associated ROS accumulation, release of cyto-

3 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β precursor protein; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma protein 2; Bax, Bcl-2-associated X protein; STS, staurosporine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
Isoflurane and Mitochondrial Pathway of Apoptosis

chrome c from the mitochondria to the cytosol, activation of caspase-9, caspase-8, and caspase-3, and the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells. We also assessed the effects of desflurane, another commonly used inhalation anesthetic, on the mitochondrial pathway of apoptosis. We performed these studies in human neuroglioma cells stably transfected with human amyloid precursor protein (APP) (H4-APP cells) because we have shown that isoflurane can induce apoptosis in the H4-APP cells. We also performed the studies in the primary neurons from naïve mice, the non-tumor cells without overexpression of human APP, and in naïve mice.

EXPERIMENTAL PROCEDURES

H4-APP Cells—We employed H4 human neuroglioma cells, stably transfected to express full-length APP (H4-APP cells) in the experiments. The cell line was cultured in Dulbecco’s modified Eagle’s medium (high glucose) containing 9% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine and was supplemented with 220 µg/ml G418.

Primary Neurons—The protocol was approved by Massachusetts General Hospital Standing Committee on Animals (Boston) on the Use of Animals in Research and Teaching. Naïve (C57BL/6) mice with the gestation stage of day 15 were euthanized with carbon dioxide. We then performed a cesarean section to pull out the embryos and decapitate them in a 100-mm dish of phosphate-buffered saline. We placed the head on the top of a 100-mm dish and dissected out the cortices, removed meninges, and placed the neurons into another 100-mm dish of phosphate-buffered saline. The neurons were dissociated by trypsinization and trituration. The dissociated neurons were resuspended in serum-free B27/neurobasal medium and were placed into 6-well plates with a confluent rate of 50%. 7–10 days after the harvest, the neurons were exposed to the isoflurane treatment.

Treatments for H4-APP Cells and Primary Neurons—Isoflurane or desflurane was delivered from an anesthesia machine to a sealed plastic box in a 37 °C incubator containing 6-well plates seeded with 1 million cells or 0.25 million neurons in 1.5 ml of cell or neuron culture media. A Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) was used to continuously monitor the concentration of anesthetics, oxygen, and carbon dioxide. We used a Spin-X UF 500 concentrator (Corning, Lowell, MA), which enhanced the concentration of cytochrome c in cytosol for Western blot analysis.

Mice Anesthesia—The mice anesthesia was similar to that described by Xie et al. (10). The animal protocol was approved by Massachusetts General Hospital Standing Committee on Animals on the Use of Animals in Research and Teaching. C57/BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were randomly assigned to either an anesthesia or a control group. Mice randomized to the anesthesia group received 1.4% isoflurane in 100% oxygen for 2 h in an anesthetizing chamber, whereas the control group received 100% oxygen at an identical flow rate for 2 h in an identical chamber. The mice breathed spontaneously, and the concentration of anesthetics, oxygen, and carbon dioxide was measured continuously (Datex, Tewksbury, MA). The temperature of the anesthetizing chamber was controlled to maintain rectal temperature of the animals at 37 ± 0.5 °C. Mean arterial blood pressure was measured noninvasively using two tail cuffs (Kent Scientific Corp., Torrington, CT) in the anesthetized mice. The isoflurane anesthesia did not significantly affect blood pressure and blood gas of the mice (10). Anesthesia was terminated by discontinuing isoflurane and placing animals in a chamber containing 100% oxygen until 20 min after return of the righting reflex. They were then returned to individual home cages until sacrifice.

Brain Tissue Lysis and Protein Amount Quantification—The harvested brain tissues were homogenized on ice using an immunoprecipitation buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1 µg/ml aprotenin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). The lysates were collected, centrifuged at 13,000 rpm for 15 min, and quantified for total proteins by a bicinchoninic acid protein assay kit (Pierce).
Western Blot Analysis—The harvested H4-APP cells, primary neurons, and brain tissues were subjected to Western blot analyses as described by Xie et al. (23). Bax antibody (1:1000 dilution; Cell Signaling Technology, Inc. Beverly, MA) was used to recognize Bax (20 kDa). Bcl-2 antibody (1:1000 dilution; Cell Signaling Technology) was used to recognize Bcl-2 (28 kDa). Rabbit polyclonal cytochrome c antibody (1:1000 dilution; Cell Signaling Technology) was used to recognize cytochrome c (14 kDa). Rabbit polyclonal caspase-9 antibody (1:1000 dilution; Cell Signaling Technology) was used to recognize two caspase-9 fragments (17 and 37 kDa). Rabbit polyclonal cleaved caspase-8 antibody (1:1000 dilution; Cell Signaling Technology) was used to recognize two caspase-8 fragments (18 and 41 kDa). A caspase-3 antibody (1:1000 dilution; Cell Signaling Technology) was used to recognize full-length caspase-3 (35–40 kDa) and caspase-3 fragment (17–20 kDa) resulting from cleavage at aspartate position 175. Antibody anti-β-actin (1:10,000, Sigma) was used to detect β-actin (42 kDa). Each band in the Western blot represents an independent experiment. We have averaged results from three to eight independent experiments. The quantification of Western blots was performed as described by Xie et al. (7). Briefly, the intensity of signals was analyzed by using the National Institutes of Health image program (National Institutes of Health Image 1.62, Bethesda, MD). We quantified the Western blots using two steps. First, we used levels of β-actin to normalize (e.g. determining ratio of full-length caspase-3 amount to β-actin amount) levels of Bax, Bcl-2, cytochrome c, caspase-9, caspase-8, and caspase-3 to control for any loading differences in total protein amounts. Second, we presented changes in levels of Bax, Bcl-2, cytochrome c, caspase-9, caspase-8, and caspase-3 in treated cells or mice as percentages of those in cells or mice with the control condition.

Reverse Transcription Polymerase Chain Reaction—Real-time reverse transcriptase (RT) PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA). Bax and Bcl-2 mRNA levels were determined and standardized with glyceraldehyde-3-phosphate dehydrogenase as an internal control. Primers of mouse Bax (QT00102536), Bcl-2 (QT00156282), and glyceraldehyde-3-phosphate dehydrogenase (QT01658692) were purchased from Qiagen.

Mitochondrial Immunocytochemistry Staining—Organelle Lights™ intracellular-targeted fluorescent proteins (Invitrogen) was used in the experiments according to the protocol provided by the company. Briefly, 1 million H4-APP cells were placed in a 100-mm dish. We added the diluted Organelle Lights™ transduction solution (Component A) to the plate and incubated the cells at 25 °C in the dark for 2 h with gentle rocking. Next, we aspirated the Organelle Lights™ transduction solution, added the enhancer (Component B), and incubated the cells for another 2 h in 37 °C. We finally removed the enhancer, added the culture medium, and incubated cells at 37 °C for 16 h. The cells were then used for isoflurane treatment, mitochondrial immunocytochemistry staining with fluorescence microscopy (100×), and ROS accumulation staining (see below).

ROS Accumulation and Quantification—OxiSelect™ ROS assay kit (Cell Biolabs, Inc. San Diego, CA) was used in the experiments according to the protocol provided by the company. Briefly, cultured H4-APP cells were placed in a clear 96-well cell culture plate overnight in the incubator. We then added the 2',7'-dichlorofluorescein diacetate/media solution to the cells. The 2',7'-dichlorofluorescein diacetate-loaded H4-APP cells were then exposed to 2% isoflurane for 6 h. Finally, the treated cells were analyzed by using the fluorescence microscopy (100×) to detect the ROS images. Parts of the treated cells were lysed by adding 100 μl of cell lysis buffer and were mixed thoroughly and incubated for 5 min at room temperature. We then transferred 150 μl of the mixture to each well of a 96-well plate that is suitable for fluorescence measurement. Finally, we read the fluorescence with a fluorometric plate reader at 480/530 nm.

Cytochrome c Immunocytochemistry Studies—ApoTrack™ cytochrome c Apoptosis ICC Antibody kit (Mitosciences, Eugene, OR) was used in the experiments according to the protocol provided by the company. Specifically, H4-APP cells were placed on coverslips overnight in the incubator. At the end of the isoflurane treatment, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were then placed in the antigen retrieval buffer and were heated to 95 °C for 10 min. After being washed with phosphate-buffered saline three times, the coverslips were then incubated with 0.1% Triton X-100 at 4 °C for 10 min. We blocked the nonspecific reaction with 10% normal goat serum for 1 h at room temperature. We incubated the cells on the coverslips with anti-cytochrome c monoclonal antibody (1:200, Mitosciences) and anti-complex Vα monoclonal antibody (for mitochondrial staining, 1:200, Mitosciences) overnight at 4 °C. Then the cells on coverslips were incubated with secondary antibodies for 1 h at room temperature away from light. Finally, the coverslips were incubated with 10 μg/ml Hoechst 33342 in a humidified dark chamber for 10 min, and were analyzed in mounting medium under a 100× objective lens fluorescence microscope.

TUNEL Staining—Tetramethyl-rhodamine red kit (Roche Diagnostics) was used for TUNEL staining. Specifically, cells or neurons were grown on coverslips overnight in the incubator. The cells or neurons were fixed in 4% paraformaldehyde for 30 min after the isoflurane treatment or control condition. After being washed with phosphate-buffered saline, the coverslips were then incubated with 0.1% Triton X-100 at 4 °C for 10 min. We blocked the nonspecific reaction with 10% normal goat serum for 1 h at room temperature. We incubated the cells on the coverslips with anti-cytochrome c monoclonal antibody (1:200, Mitosciences) and anti-complex Vα monoclonal antibody (for mitochondrial staining, 1:200, Mitosciences) overnight at 4 °C. Then the cells on coverslips were incubated with secondary antibodies for 1 h at room temperature away from light. Finally, the coverslips were incubated with 10 μg/ml Hoechst 33342 in a humidified dark chamber for 10 min, and were analyzed in mounting medium under a 100× objective lens fluorescence microscope.
Isoflurane and Mitochondrial Pathway of Apoptosis

Statistics—Given the presence of background caspase activation and apoptosis in the cells and neurons, we did not use absolute values to describe changes in caspase activation and apoptosis. Instead, apoptosis and caspase activation were presented as a percentage or fold of those of the control group. 100% caspase activation or 1-fold apoptosis refers to control levels for purposes of comparison to experimental conditions. We presented changes in levels of Bax, Bcl-2, ROS, cytochrome c, caspase activation, and apoptosis in treated cells, neurons, or mice as a percentage or fold of those in cells, neurons, or mice after the control condition. Data were expressed as the mean ± S.D. The number of samples varied from 3 to 8, and the samples were normally distributed. Analysis of variance with repeated measurements or t tests was used to compare the difference from the control group. p values less than 0.05 (* or #) and 0.01 (**) were considered statistically significant. The significance testing was two-tailed, and we have used SAS software (Cary, NC) to analyse the data.

RESULTS

Isoflurane Increases Bax Levels in H4-APP Cells and Primary Neurons from Naïve Mice—The common inhalation anesthetic isoflurane has been shown to induce caspase activation and apoptosis (7–12, 24, 25). The molecular mechanism underlying these effects, however, is largely unknown. Bcl-2 family proteins (e.g. pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2), ROS accumulation, and the mitochondrial pathway are all involved in apoptosis (for review, see Refs. 16, 17 and 21). Therefore, we set out to determine the effects of isoflurane on levels of Bax, Bcl-2, and ROS, cytochrome c release, activation of caspase-9 and caspase-3, and apoptosis in H4-APP cells, primary neurons from naïve mice, and brain tissues of naïve mice.

We employed H4-APP cells in the current experiments because we have established the isoflurane-induced apoptosis in the cells. The cells were harvested at the end of the isoflurane treatment and were subjected to Western blot by which Bax antibody was used to detect levels of Bax (20 kDa). Bax immunoblotting showed visible increases in the levels of Bax after the isoflurane treatment as compared with the control condition (Fig. 1A). The quantification of the Western blot revealed that the isoflurane treatment increased the levels of Bax: 100 versus 146%, p = 0.04 (Fig. 1B).

Then, we assessed the effects of isoflurane on Bax levels in the primary neurons from naïve mice. Bax immunoblotting showed visible increases in the levels of Bax after the isoflurane treatment as compared with the control condition in the neurons (Fig. 1C). The quantification of the Western blot revealed that the isoflurane treatment increased the levels of Bax: 100 versus 170%, p = 0.008 (Fig. 1D). Furthermore, we were able to show that the isoflurane treatment increased the mRNA levels of Bax as compared with the control condition (Fig. 1E, p = 0.02) in the primary neurons from naïve mice. These results suggest that isoflurane may increase the Bax protein levels via enhancing the generation of Bax.

Isoflurane Decreases Bcl-2 Levels in H4-APP Cells, Primary Neurons, and Brain Tissues from Naïve Mice—Next, Bcl-2 immunoblotting showed visible decreases in the levels of Bcl-2 after the isoflurane treatment as compared with the control condition (Fig. 2A) in H4-APP cells. The quantification of the Western blot showed that the isoflurane treatment decreased the levels of Bcl-2: 100 versus 57%, p = 0.017 (Fig. 2B). The isoflurane treatment also decreased the Bcl-2 levels in the primary neurons from naïve mice: 100 versus 61%, p = 0.027 (Fig. 2, C and D). Furthermore, the isoflurane treatment decreased the mRNA levels of Bcl-2 in the primary neurons from naïve mice (Fig. 2E, p = 0.049). These results suggest that isoflurane may decrease the Bcl-2 protein levels via reducing the generation of Bcl-2.

To determine the in vivo relevance of these in vitro findings, we also assessed the effects of isoflurane on Bcl-2 levels in brain tissues of naïve mice. Naïve mice were subjected to anesthesia with 1.4% isoflurane for 2 h as described before (10). Quantitative Western blot revealed that anesthesia with 1.4% isoflurane for 2 h led to decreases in Bcl-2 levels in vivo (Fig. 2, F and G, 100 versus 63%, p = 0.004).

Because Bax and Bcl-2 have been suggested to be associated with mitochondrial damage (Refs. 26 and 27; for review, see Ref. 16), these results suggest that isoflurane may increase pro-apoptotic factor Bax levels and reduce anti-apoptotic factor Bcl-2 levels to induce apoptosis through the mitochondrial pathway.

Isoflurane Increases ROS Levels in H4-APP Cells—Next, we assessed the effects of isoflurane on ROS levels in H4-APP cells. The H4-APP cells were treated with 2% isoflurane for 6 h, and the cells were harvested at the end of the treatment and subjected to immunocytochemistry and fluorescence studies by which ROS levels were detected. ROS immunocytochemistry imaging showed visible increases in ROS levels in the cells treated with the isoflurane as compared with the control condition (column 2 of Fig. 3A). Moreover, the counterstained mitochondrial image (column 3 of Fig. 3A) confirmed that the increased ROS was indeed inside the mitochondria. As can be seen in Fig. 3A, the orange color, but not red color, in the merged images (column 3) indicated that ROS was inside the mitochondria. The treatment with isoflurane (row b) led to accumulation of ROS inside the mitochondria (orange color) as compared with the control condition (row a, red color). Next, we were able to show that the isoflurane treatment increased ROS levels, detected by fluorescence assay, in the lysed whole cells as compared with the control condition (Fig. 3B): 100 versus 153%, p = 0.006.

Given that isoflurane may induce apoptosis by elevating the calcium levels in the cytosol (Refs. 13 and 18; for review, see Ref. 19), we next asked whether the isoflurane-induced ROS accumulation is dependent on the cytosol calcium levels. Previous studies have shown that intracellular calcium chelator BAPTA can attenuate the isoflurane-induced caspase-3 activation in H4-APP cells (18). We therefore set out to determine the effects of BAPTA on the isoflurane-induced ROS accumulation by fluorescence assay in the H4-APP cells. We were able to show that 10 μM BAPTA treatment attenuated the isoflurane-induced ROS accumulation: 288 versus 211%, p = 0.025 (Fig. 3C). Taken together, these results suggest that the clinically relevant isoflurane treatment (2% for 6 h) may increase ROS accumulation inside the mitochondria by increasing the cytosolic calcium levels.
Isoflurane Induces Cytochrome c Release from the Mitochondria to the Cytosol in H4-APP Cells—Given that isoflurane may cause mitochondrial damage, we next asked whether the isoflurane treatment can facilitate cytochrome c release from the mitochondria to the cytosol. As can be seen in Fig. 4A, column 1 is the immunocytochemistry imaging of mitochondria (red), column 2 is the immunocytochemistry imaging of cytochrome c (green) both inside and outside mitochondria, column 3 is the 4′,6-diamidino-2-phenylindole (a fluorescent stain that binds strongly to DNA) immunocytochemistry imaging of nuclei (blue), and column 4 is the merged image. The orange color in the merged images (column 4) indicated the existence of cytochrome c inside the mitochondria; the green dots in the merged images indic
Isoflurane decreases Bcl-2 levels in H4-APP cells, primary neurons from naïve mice, and brain tissues of naïve mice. A, treatment with 2% isoflurane for 6 h (lanes 5–8) decreased Bcl-2 levels as compared with the control conditions (lanes 1–4) in the H4-APP cells. There was no significant difference in the amounts of β-actin under the control condition- or isoflurane-treated cells. B, quantification of the Western blot shows that isoflurane treatment (black bar) decreased Bcl-2 levels compared with the control conditions (white bar), normalized to β-actin levels (*, \( p = 0.017 \)). C, treatment with 2% isoflurane for 6 h (lanes 3 and 4) decreased Bcl-2 levels as compared with the control conditions (lanes 1 and 2) in the primary neurons from naïve mice. There was no significant difference in amounts of β-actin in the control condition- or isoflurane-treated neurons. D, quantification of the Western blot shows that the isoflurane treatment (black bar) decreased Bcl-2 levels compared with the control condition (white bar), normalized to β-actin levels (*, \( p = 0.027 \)). E, treatment with 2% isoflurane for 6 h (black bar) decreased the mRNA levels of Bcl-2 as compared with the control condition (white bar) in the primary neurons from naïve mice (*, \( p = 0.049 \)). F, treatment with 1.4% isoflurane for 2 h (lanes 4–6) decreased Bcl-2 levels as compared with the control conditions (lanes 1–3) in brain tissues of naïve mice. There was no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated naïve mice. G, quantification of the Western blot shows that isoflurane treatment (black bar) decreased Bcl-2 levels compared with the control condition (white bar), normalized to β-actin levels (**, \( p = 0.004 \)).
cated the existence of cytochrome c outside the mitochondria. Row a shows cells following the control condition, row b shows cells treated with STS, the positive control of the experiment, and row c shows cells treated with 2% isoflurane for 6 h. As can be seen in the merged images (column 4), the treatment with STS (row b) or isoflurane (row c) led to appearance of cytochrome c outside the mitochondria (green dots indicated by the arrows) as compared with the control condition (row a).

Furthermore, we used cellular fraction and quantitative Western blot methods to assess the levels of cytochrome c in the cytosol after the isoflurane treatment. Cytochrome c immunoblotting showed visible increases in protein levels of cytochrome c after the treatment with 2% isoflurane for 6 h as com-

**FIGURE 3. Isoflurane induces ROS accumulation in the mitochondria of H4-APP Cells.** A, column 1 is the image of mitochondria (red), column 2 is the image of ROS (green) both inside and outside of the mitochondria, and column 3 is the merged image. The orange color, but not red color, in the merged images (column 3) indicates that ROS is inside the mitochondria. Row a shows cells following the control condition, and the row b shows cells treated with 2% isoflurane for 6 h. The treatment with isoflurane (row b) led to accumulation of ROS inside the mitochondria (orange color) as compared with the control condition (row a, red color). B, a fluorescence assay shows that isoflurane treatment (black bar) increases ROS accumulation compared with the control condition (white bar) (**, \( p = 0.006 \)). C, treatment with BAPTA plus isoflurane (net bar) induced a lesser degree of ROS accumulation compared with a treatment with DMSO plus isoflurane (black bar) (*, \( p = 0.025 \)).
FIGURE 4. Isoflurane facilitates the release of cytochrome c from the mitochondria to the cytosol in H4-APP cells. A, column 1 is the image of mitochondria (red), column 2 is the image of cytochrome c (green) both inside and outside mitochondria, column 3 is the image of nuclei (blue), and column 4 is the merged image. The orange color in the merged images (column 4) indicates the existence of cytochrome c inside the mitochondria, and the green dots in the merged images indicate the existence of cytochrome c outside the mitochondria. Row a shows cells following the control condition, row b show cells treated with STS, the positive control of the experiment, and the row c show cells treated with 2% isoflurane for 6 h. The treatment with STS (row b) or isoflurane (row c) leads to the appearance of cytochrome c outside of the mitochondria (green dots, indicated by the arrows) as compared with the control condition (row a). B, treatment with 2% isoflurane for 6 h (lanes 5–8) increased cytochrome c levels in cytosol as compared with the control condition (lanes 1–4) in H4-APP cells. There was no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated H4-APP cells. C, quantification of the Western blot shows that isoflurane treatment (black bar) increased cytochrome c levels in cytosol compared with the control condition (white bar), normalized to β-actin levels (*, p = 0.01).
pared with the control condition (Fig. 4B). Quantification of the Western blot revealed that the isoflurane treatment led to a 188% increase in cytochrome c levels as compared with the control condition (Fig. 4C) \((p = 0.01)\). Collectively, these results suggest that isoflurane can regulate Bax and Bcl-2 levels and increase ROS accumulation inside the mitochondria, which causes mitochondrial damage, leading to cytochrome c release from the mitochondria to the cytosol.

**Isoflurane Induces Activation of Caspase-9 and Caspase-3, but Not Caspase-8, in H4-APP Cells—Caspase-9, which can be activated by cytochrome c, is a part of the mitochondrial pathway of apoptosis. Because isoflurane can facilitate cytochrome c release from the mitochondria to the cytosol, we assessed the effects of isoflurane on caspase-9 activation in H4-APP cells. The cells were harvested at the end of the isoflurane treatment and were subjected to Western blot by which caspase-9 antibody was used to detect the levels of caspase-9 fragments (17 and 37 kDa). Caspase-9 immunoblotting showed visible increases in the levels of caspase-9 fragments after the isoflurane treatment as compared with the control condition (Fig. 5A). The quantification of the Western blot revealed that the isoflurane treatment increased the levels of the 17-kDa caspase-9 fragment (100% versus 387%, \(p = 0.001\)) and the 37-kDa caspase-9 fragment (100% versus 218%, \(p = 0.0009\)).**

These results further suggest that isoflurane can induce apoptosis through the mitochondrial pathway as evidenced that the isoflurane treatment can induce activation of caspase-9, one of the components of mitochondrial pathway.

Given that isoflurane may induce apoptosis through the mitochondrial pathway, we next asked whether isoflurane can also induce activation of caspase-8, one of the components of extrinsic, death receptor pathway. We have found that whereas the positive control (serum deprivation) in the experiment increased levels of 18-and 41-kDa fragments of caspase-8 (caspase-8 activation), the isoflurane treatment did not induce caspase-8 activation as compared with the control condition in the present experiment (Fig. 5B). Collectively, these results suggest that isoflurane may induce apoptosis through the mitochondrial pathway but not the death receptor pathway.

Finally, we asked whether the isoflurane treatment can induce caspase-3 activation, one of the final steps in the caspase cascade of apoptosis (15), in non-tumor cells without overexpression of human APP. We, therefore, set out to assess the effects of isoflurane on caspase-3 activation in primary neurons from naïve mice. We were able to show that the treatment with 2% isoflurane for 6 h induced caspase-3 activation as compared with the control condition in primary neurons from naïve mice (Fig. 5C): 100 versus 202%, \(p = 0.005\). These results suggest that isoflurane can induce caspase-3 activation not only in H4-APP cells (7) and H4 naïve cells (9) but also in primary neurons from naïve mice, leading to apoptosis.

**Isoflurane Increases the Number of TUNEL-positive Cells—** Given that caspase-3 activation alone may not represent apoptotic cell damage (28), we also assessed the effects of treatment of 2% isoflurane for 6 h on cellular apoptosis by TUNEL study. As can be seen in Fig. 6A, column 1 is Hoechst staining of nuclei (blue), column 2 is the TUNEL staining (red), and column 3 is the merged image (purple). Row a shows cells following the control condition, and row b shows cells following the isoflurane treatment. We were able to show that the isoflurane treatment increased TUNEL-positive cells (apoptosis) as compared with the control condition: row b versus row a in columns 3 (Fig. 6A). The quantification of the TUNEL staining revealed that the isoflurane treatment increased the number of the TUNEL-positive H4-APP cells: 1-fold versus 5.75-fold, \(p = 0.002\) (Fig. 6B). The isoflurane also increased TUNEL-positive cells in primary neurons from naïve mice (data not shown). These results suggest that isoflurane can induce caspase activation as well as apoptotic cytotoxicity.

**Desflurane Does Not Induce the Activation of the Mitochondrial Pathway of Apoptosis—** Our previous studies have shown that desflurane, another commonly used inhalation anesthetic,
Isoflurane does not induce caspase activation and apoptosis in H4-APP cells (22). However, the other commonly used inhalation, isoflurane (7), and sevoflurane (29), at equipotent concentrations, can induce caspase activation and apoptosis. Therefore, we have postulated that desflurane, different from isoflurane and sevoflurane, may not be able to cause mitochondrial stress and, thus, will not initiate the mitochondrial pathway to lead to apoptosis. In the present studies, we assessed the effects of the treatment with 12% desflurane for 6 h on the mitochondrial pathway of apoptosis, and we found that the desflurane treatment did not alter levels of Bax (Fig. 7A), Bcl-2 (Fig. 7B), ROS accumulation detected by ROS staining (data not shown), ROS accumulation detected by fluorescence assay (Fig. 7C), cytochrome c levels (Fig. 7D), and caspase-9 activation (Fig. 7E). Collectively, these results suggest that whereas isoflurane can induce caspase activation and apoptosis through the mitochondrial pathway, desflurane does not induce mitochondrial stress, thus, not leading to caspase activation and apoptosis.

**DISCUSSION**

The commonly used inhalation anesthetic isoflurane has previously been shown to induce caspase-3 activation and apoptosis (7–13). The underlying molecular mechanism of the isoflurane-induced apoptosis, however, is largely unknown. Bcl-2 protein family members, including pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2, can regulate apoptosis by modulating out mitochondrial membrane permeability (Refs. 26 and 27; for review, see Ref. 16). We, therefore, assessed the effects of isoflurane on levels of Bax and Bcl-2 in vitro and in vivo. We have found that the isoflurane treatment can increase Bax levels in H4-APP cells and primary neurons from naïve mice (Fig. 1, A–D) and decrease Bcl-2 levels in H4-APP cells and primary neurons from naïve mice (Fig. 2, A–D) and in brain tissues of naïve mice (Fig. 2, F and G). These findings suggest that isoflurane can regulate Bax and Bcl-2 levels, leading to mitochondrial damage, which will initiate the mitochondrial pathway of apoptosis. Moreover, we have found that the isoflurane treatment can increase the mRNA levels of Bax (Fig. 1E) and decrease the mRNA levels of Bcl-2 (Fig. 2E) in the primary neurons from naïve mice. These findings suggest that isoflurane may affect the genes of Bax and Bcl-2 to alter the generations of Bax and Bcl-2, leading to increases in Bax levels and decreases in Bcl-2 levels.

Isoflurane has been shown to reduce endoplasmic reticulum calcium levels and to elevate calcium levels in cytosol and mitochondria (Refs. 13 and 18; for review, see Ref. 19). In addition, it has been suggested that high cytosolic calcium levels can permit mitochondrial uniporter to more efficiently transfer the released calcium from endoplasmic reticulum into mitochondria, which can lead overload of calcium in mitochondria, leading to collapse of mitochondrial membrane potential and induction of apoptosis (for review, see Ref. 19 and 30). Furthermore, accumulation of ROS has been suggested to be associated with mitochondrial damage, leading to apoptosis (Ref 20; for review, see Ref. 21). Therefore, we set out to determine the effects of isoflurane on mitochondrial damage by assessing ROS levels. We have found that isoflurane can increase ROS levels inside mitochondria (Fig. 3, A and B), which can be attenuated by the intracellular calcium chelator BAPTA (Fig. 3C). Taken together, these results suggest that isoflurane may elevate cytosol calcium levels, which increase ROS accumulation inside mitochondria, leading to mitochondrial damage.

The damaged mitochondria may then release cytochrome c to cytosol, which is a part of the mitochondrial pathway (for...
review, see Ref. 16). We, therefore, assessed the effects of isoflurane on cytochrome \( c \) release from mitochondria to cytosol, and cytochrome \( c \) levels in cytosol. We have found that the isoflurane treatment can facilitate the cytochrome \( c \) release from mitochondria to cytosol (Fig. 4A). Furthermore, we have found that the isoflurane treatment can specifically increase cytochrome \( c \) levels in cytosol as compared with the control condition (Fig. 4, B and C). Collectively, these findings suggest that isoflurane may regulate levels of Bax, Bcl-2, and ROS to cause mitochondrial damage, which then facilitate the release of cytochrome \( c \) from the mitochondria to the cytosol, ultimately leading to apoptosis.

Interestingly, we did not see a significant reduction in cytochrome \( c \) levels inside mitochondria after the isoflurane treatment as compared with the control condition in current experiments (data not shown). This could be due to the fact that the treatment of 2% isoflurane for 6 h can facilitate the release of only a small portion of the total cytochrome \( c \) inside the mitochondria, leading to an insignificant reduction in the amount of cytochrome \( c \) in the mitochondria. In the future studies, we will determine the dose- and time-dependent effects of isoflurane on the amount of cytochrome \( c \) in the mitochondria to further test this hypothesis.

Furthermore, we have found that the isoflurane treatment can induce activation of caspase-9, a component of the mitochondrial pathway after cytochrome \( c \) (Fig. 5A). Moreover, we were able to show that the isoflurane treatment did not induce activation of caspase-8, a component of the death receptor pathway, in the present experiments (Fig. 5B). We have further illustrated that isoflurane can induce caspase-3 activation in primary neurons from naïve mice (Fig. 5C), the non-tumor cells without overexpression of human APP, which is consistent with our previous studies in H4-APP cells, H4 naïve cells, and brain tissues of naïve mice (7–10). Collectively, these findings suggest that isoflurane can regulate Bax and Bcl-2 levels, increase ROS accumulation, and damage mitochondria to induce apoptosis via the mitochondrial pathway. In future studies we will determine whether isoflurane can also induce apoptosis via other signaling pathways, including the caspase-2-dependent pathway and caspase-independent pathway.

McLaughlin et al. (28) have shown that caspase-3 activation alone may not represent apoptotic cell damage; our previous studies have also shown that the treatment of 70% nitrous oxide can induce caspase-3 activation without causing apoptosis (31). Therefore, we finally assessed the effects of isoflurane on apoptosis by TUNEL studies. We have found that the isoflurane treatment can increase TUNEL-positive H4-APP cells (Fig. 6) and TUNEL-positive primary neurons from naïve mice (data not shown). Taken together, these findings suggest that isoflurane can reduce Bcl-2 levels, increase Bax levels, and increase ROS accumulation to cause mitochondrial damage, leading to apoptosis.
Isoflurane and Mitochondrial Pathway of Apoptosis

FIGURE 8. Hypothetical pathway by which isoflurane induces apoptosis. Isoflurane increases pro-apoptotic factor Bax levels, decreases anti-apoptotic factor Bcl-2 levels, and enhances ROS accumulation to induce mitochondrial damage. The damaged mitochondria release cytochrome c, which activates caspase-9. The activated caspase-9 then induces caspase-3 activation, leading to apoptosis.

Previous studies have shown that the commonly used inhalation anesthetics isoflurane (7–10, 12) and sevoflurane (12, 29), but not desflurane (12, 22), can induce caspase activation and apoptosis. The reason why desflurane does not induce caspase activation and apoptosis is unknown. Other studies have shown that isoflurane at equipotent concentrations can induce a greater elevation in cytosolic and mitochondrial calcium levels and more reductions in endoplasmic reticulum calcium levels than does either sevoflurane or desflurane (32). Moreover, Loop et al. (12) have shown that another common inhalation anesthetic sevoflurane can cause disruption of mitochondrial membrane potential and release of cytochrome c from the mitochondria to the cytosol in human T lymphocytes. In the present studies we have found that desflurane does not induce activation of the mitochondrial pathway of apoptosis (Fig. 7). Taken together, it is conceivable that in contrast to isoflurane and sevoflurane, desflurane may not be able to elevate calcium levels in cytosol and mitochondria sufficiently enough to increase ROS accumulation inside mitochondria and to cause mitochondrial damage and, thus, will not initiate the mitochondrial pathway to lead to apoptosis.

Even though the findings in the current and other studies suggest that isoflurane may induce apoptosis and cause neurotoxic effects including AD neuropathogenesis, these experiments were performed only in cultured cells and animals. The determination of the in vivo relevance of isoflurane on AD neuropathogenesis in humans will be necessary before we can conclude that the inhalation anesthetic isoflurane facilitates or exacerbates neurotoxicity including AD neuropathogenesis.

In conclusion, we have found that isoflurane, but not desflurane, can increase levels of pro-apoptotic factor Bax, decrease levels of anti-apoptotic factor Bcl-2, and increase ROS accumulation inside mitochondria, leading to induction of apoptosis through the mitochondrial pathway. These results suggest that isoflurane can induce apoptosis through Bcl-2 family proteins and ROS-associated mitochondrial pathway, which have identified at least partially the molecular mechanism by which isoflurane induces apoptosis. Given that the isoflurane-induced apoptosis can lead to accumulation of β-amyloid protein (7, 9, 10), the hallmark feature of AD neuropathogenesis, the findings from the current studies will likely promote more studies aimed at determining the up-stream mechanism of the anesthetics-induced apoptosis, designing safer anesthetics and searching for prevention and treatment of the anesthesia-induced neurotoxicity. Ultimately, these efforts will lead to better anesthesia care to patients, especially elderly patients, and AD patients.

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