The Common Inhalation Anesthetic Isoflurane Induces Apoptosis and Increases Amyloid β Protein Levels

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Background: The common inhalation anesthetic isoflurane has previously been reported to enhance the aggregation and cytotoxicity of the Alzheimer disease–associated amyloid β protein (Aβ), the principal peptide component of cerebral β-amyloid deposits.

Methods: H4 human neuroglioma cells stably transfected to express human full-length wild-type amyloid precursor protein (APP) were exposed to 2% isoflurane for 6 h. The cells and conditioned media were harvested at the end of the treatment. Caspase-3 activation, processing of APP, cell viability, and Aβ levels were measured with quantitative Western blotting, cell viability kit, and enzyme-linked immunosorbent assay sandwich. The control condition consisted of 5% CO2 and 21% O2 and balanced nitrogen, which did not affect caspase-3 activation, cell viability, APP processing, or Aβ generation.

Results: Two percent isoflurane caused apoptosis, altered processing of APP, and increased production of Aβ in H4 human neuroglioma cell lines. Isoflurane-induced apoptosis was independent of changes in Aβ and APP holoprotein levels. However, isoflurane-induced apoptosis was potentiated by increased levels of APP C-terminal fragments.

Conclusion: A clinically relevant concentration of isoflurane induces apoptosis, alters APP processing, and increases Aβ production in a human neuroglioma cell line. Because altered processing of APP leading to accumulation of Aβ is a key event in the pathogenesis of Alzheimer disease, these findings may have implications for use of this anesthetic agent in individuals with excessive levels of cerebral Aβ and elderly patients at increased risk for postoperative cognitive dysfunction.

THE excessive accumulation of the β-amyloid protein (Aβ), generated via sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretase, is a key pathogenic event in Alzheimer disease (AD) (reviewed in Tanzi and Bertram†). Increasing evidence indicates that apoptosis is associated with various neurodegenerative disorders, including AD (reviewed in Raina et al.†). Several studies have also suggested that perioperative factors, such as hypoxia, hypocapnia, and anesthetics may contribute to AD neuropathogenesis. Isoflurane, a commonly used inhalation anesthetic, has previously been reported to promote Aβ oligomerization and cytotoxicity in phaeochromocytoma cells. However, the effects of isoflurane on APP processing and Aβ generation have not been previously determined. To investigate a possible role for isoflurane in AD pathogenesis as well as postoperative cognitive dysfunction (POCD), we investigated whether a clinically relevant concentration (2%) of isoflurane can induce apoptosis, alter APP processing, and increase Aβ generation in H4 human neuroglioma cells overexpressing human APP.

Materials and Methods

Cell Lines

We used naive H4 human neuroglioma cells stably transfected to express the APP full-length (FL) (H4-APP-FL cells) and APP-C99 (H4-APP-C99 cells) in the experiments. All cell lines were cultured in Dulbecco’s Modified Eagle Medium (high glucose) containing 9% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Stably transfected H4 cells were in addition supplemented with 200 μg/ml G418.

Cell Treatment

Twenty-one percent O2, 5% CO2, and 2% isoflurane were delivered from an anesthesia machine to a sealed plastic box in a 57°C incubator containing six-well plates seeded with one million cells in 1.5 ml cell culture media. A Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) was used to continuously monitor the delivered carbon dioxide, oxygen, and isoflurane concentrations. We treated the cells with 2% isoflurane for 6 h, during which time the cells were incubated in serum-free media. In the interaction studies, the cells were
treated with 250 nM N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), 100 μM benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD), 15 μM Bcl, 50 ng/ml 6E10, and 50 ng/ml 4G8 1 h before the treatment with 2% isoflurane. Control conditions included 5% CO2 plus 21% O2, which did not affect caspase-3 activation, cell viability, APP processing, or Aβ generation (data not shown).

Cell Lysis and Protein Amount Quantification
Cell pellets were detergent-extracted on ice using 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 aprotinin, 1 μg/ml leupeptin, 1 μg/ml peptatin A). The lysates were collected, centrifuged at 12,000 rpm for 10 min, and quantified for total proteins with use of a BCA protein assay kit (Pierce, Iselin, NJ).

Western Blot Analysis
The cells were harvested at the end of experiments and were subjected to Western blots as described by Xie et al.\textsuperscript{15} Antibodies A8717 (1:1,000; Sigma, St. Louis, MO), 6E10 (1:200; Signet, Dedham, MA), and anti-β-actin (1:2,000; Sigma) were used to visualize APP-FL (110 kd), amyloid precursor protein C-terminal fragments (APP-CTFs; 10–12 kd), APP-C99 (10 kd), and β-actin (42 kd), respectively. A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Inc., Beverly, MA) was used to recognize caspase-3 fragment (17–20 kd) resulting from cleavage at aspartate position 175 and caspase-3 FL (35–40 kd). The quantitation of Western blots was performed as described by Xie et al.\textsuperscript{15}

Quantitation of Aβ Using Sandwich Enzyme-linked Immunosorbent Assay
Secreted Aβ was measured with a sandwich enzyme-linked immunosorbent assay (ELISA) by using an Aβ measurement kit (Bioresource, Camarillo, CA) and by the Aβ ELISA Core Facility at Center for Neurologic Diseases, Harvard Institute of Medicine, Harvard Medical School, Boston, Massachusetts, as described by Xie et al.\textsuperscript{16} Specifically, 96-well plates were coated with mouse monoclonal antibodies specific to Aβ\textsubscript{40} (Ab206) or Aβ\textsubscript{42} (21F12). After blocking with albumin, wells were incubated overnight at 4°C with test samples of conditioned cell culture media, and then an anti-Aβ (α-Aβ-HR1) conjugated to horseradish peroxidase was added. Plates were then developed with 3,3',5,5'-tetramethylbenzidine reagent and well absorbance measured at 450 nm. Aβ levels in test samples were determined by comparison with signal from unconditioned media spiked with known quantities of Aβ\textsubscript{40} and Aβ\textsubscript{42}.

Cell Viability Study
The cell viability was determined by using 3-[4,5-di- methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma). The experiments were performed according to the company protocol. Briefly, we added 150 μl MTT solution to one well, containing 1.5 ml cell culture media, of a six-well plate. We then returned the cell culture to the incubator for 2 h. Finally, we removed the cell culture fluid and added 1.5 ml isopropanol into the wells. We spectrophotometrically measured the absorbance at a wavelength of 570 nm. We present the changes in the absorbance, as the number of cell viability, in the cells treated with isoflurane as the percentage of those in the cells treated with control conditions.

Statistics
Given the presence of background caspase-3 activation and cell death in cells cultured in serum-free media, we did not use absolute values to describe changes in caspase-3 activation and cell viability. Instead, changes in caspase-3 activation and cell viability were presented as a percentage of those of the control group. One hundred percent caspase-3 activation or cell viability refers to control levels for purposes of comparison to experimental conditions. We evaluated caspase-3 activation, cell viability, APP processing, and Aβ levels in the experimental and control cells with a blinded fashion. Data were expressed as mean ± SD. The number of samples varied from 4 to 10, and the samples were normally distributed. We used a two-tailed t test to compare the difference between the experimental groups. P values less than 0.05 (* or #) and 0.01 (** or ##) were considered statistically significant.

Results
2% Isoflurane Induces Apoptosis and Increases Levels of Secreted Aβ Levels
We first assessed whether control conditions, consisting of 5% CO2 plus 21% O2 and balanced nitrogen, can affect caspase-3 activation, cell viability, APP processing, and Aβ levels. We have found that there was no significant difference in caspase-3 activation, cell viability, APP processing, and Aβ levels in the cells exposed to control conditions versus cells exposed to standard cell incubator conditions.

We then exposed H4 human neuroglioma cells stably transfected to express human full-length, wild-type APP (H4-APP-FL cells) to 2% isoflurane for 6 h. Because caspase-3 activation is one of the final steps of cellular apoptosis,\textsuperscript{17} we assessed the effects of isoflurane on caspase-3 activation by quantitative Western blot analyses. Two percent isoflurane induced caspase-3 activation in H4-APP-FL cells (figs. 1A and B) as evidenced by increased ratios of cleaved (activated) caspase-3 fragment (17–19 kd) to FL caspase-3 (35 kd). Immunoblotting for caspase-3 revealed increases in activated caspase-3 fragment and decreases in FL caspase-3 in...
H4-APP-FL cells treated with 2% isoflurane as compared with those treated with control conditions (fig. 1A). Quantitation of the ratio of cleaved (activated) versus FL caspase-3 revealed that the isoflurane treatment led to a 249% increase in caspase-3 activation as compared with control conditions (fig. 1B; \( P < 0.01 \)). Two percent isoflurane treatment also decreased cell viability by 25% in H4-APP-FL cells (fig. 1C; \( P < 0.05 \)). These results suggest that a clinically relevant concentration (2%) of isoflurane can induce apoptosis in H4-APP-FL cells.

We next assessed the effects of 2% isoflurane on APP processing and Aβ generation in H4-APP-FL cells. Two percent isoflurane led to decreases in levels of APP-CTFs in H4-APP-FL cells as compared with those in the cells treated with control conditions (fig. 1D). In contrast, isoflurane treatment led to no significant differences in the amounts indicated that a clinically relevant concentration (2%) of isoflurane can induce apoptosis in H4-APP-FL cells.
of APP-FL (fig. 1D). Quantitation of the ratio of APP-CTFs and APP-FL indicated that isoflurane treatment decreased the ratio of APP-CTFs to APP-FL by 60% as compared with control conditions (fig. 1E; P < 0.05). We next tested whether decrease in levels of APP-CTFs after isoflurane treatment might be due to an increase in γ-secretase cleavage. γ-Secretase cleavage of the β-secretase-derived APP-CTF, APP-C99, would be expected to lead to increased levels of secreted Aβ. Therefore, we next measured levels of secreted Aβ after isoflurane treatment. Two percent isoflurane significantly increased the levels of both Aβ40 (193 vs. 50 pg/ml) and Aβ42 (83 vs. 24 pg/ml) by roughly threefold to fourfold as compared with control conditions (fig. 1F; P < 0.05, P < 0.01). These data suggest that treatment with 2% isoflurane leads to an increase in γ-secretase cleavage of APP-C99, as evidenced by increased levels of secreted Aβ.

We next set out to test whether enhanced caspase activation and apoptosis induced by isoflurane are dependent on increased production of Aβ. For this purpose, we incubated H4-APP-FL cells with anti-Aβ antibodies 6E10 (50 ng/ml) and 4G8 (also 50 ng/ml) for 1 h, followed by treatment with 2% isoflurane for 6 h. The anti-Aβ antibodies 6E10 and 4G8 decreased the amount of Aβ in the culture media (see Materials and Methods for Aβ assay details; data not shown), whereas levels of activated caspase-3 fragment were still increased in cells treated with 2% isoflurane or 2% isoflurane plus 6E10 and 4G8 as compared with control conditions (fig. 2A). Quantitation of the Western blots, based on the ratio of caspase-3 fragment to FL caspase-3, revealed that treatment with 2% isoflurane plus saline induced caspase-3 activation by 289% as compared with control conditions (fig. 2B; P < 0.01). Likewise, treatment with 2% isoflurane plus anti-Aβ antibodies 6E10 and 4G8 still led to a 295% increase in caspase-3 activation as compared with the control (fig. 2B; P < 0.01). Therefore, Aβ in the culture media does not seem to drive isoflurane-induced caspase-3 activation. As a positive control, the caspase inhibitor Z-VAD did attenuate caspase-3 activation induced by 2% isoflurane treatment (fig. 2A) by roughly 57% (fig. 2B; P < 0.01).

We next tested whether isoflurane-induced caspase-3 activation depends on overexpression of FL-APP. For this purpose, we used H4 cells overexpressing APP-C99 (H4-APP-C99 cells). Immunoblotting for caspase-3 revealed a 320% increase in activated caspase-3 fragment in H4-APP-C99 cells treated with 2% isoflurane as compared with control conditions (figs. 3A and B; P < 0.01). These results suggest that the isoflurane-induced apoptosis is not dependent on increased levels of APP-FL.

**DAPT Potentiates Isoflurane-induced Apoptosis**

Given the observations of decreased APP-CTFs and increased levels of secreted Aβ after isoflurane treatment in the H4-APP-FL cells, we next asked whether γ-secretase cleavage was required for isoflurane-induced caspase activation. For this purpose, the γ-secretase inhibitor DAPT, which increases the levels of APP-CTFs and decreases the levels of Aβ, was tested for effects on isoflurane-induced caspase-3 activation. As can be seen in figure 4A, activated caspase-3 fragment levels were increased after treatment with 2% isoflurane plus diethyl sulfoxide (DMSO), as compared with DMSO alone. DAPT alone had no effect on caspase activation but, as expected, did increase APP-CTFs levels (both APP-C83 and APP-C99; fig. 4A). Specifically, 2% isoflurane plus DMSO led to a 207% increase in caspase-3 activation and a roughly 45% decrease in APP-C83 and -C99 levels as compared with control conditions (fig. 4B;
Interestingly, 2% isoflurane plus DAPT led to an even greater (50%) increase in caspase-3 activation (activation) as compared with the control conditions (lanes 1 and 2) in H4-APP-C99 cells. There is no significant difference in the amounts of β-actin in the control conditions or 2% isoflurane-treated H4-APP-C99 cells. (B) Caspase-3 activation assessed by quantifying the ratio of caspase-3 fragment to caspase-3 full-length (FL) in the Western blots. Treatment with 2% isoflurane (black bar) increases caspase-3 activation compared with control conditions (white bar), normalized to β-actin levels. Data are mean ± SD; n = 4 for each experimental group. A t test is used to compare the difference between control conditions and 2% isoflurane treatment: **P < 0.01.

Fig. 3. Isoflurane induces caspase-3 activation in H4 amyloid precursor protein C-99 fragment (H4-APP-C99) cells. (A) Two percent isoflurane treatment (lanes 3 and 4) induces caspase-3 cleavage (activation) as compared with the control conditions (lanes 1 and 2) in H4-APP-C99 cells. There is no significant difference in the amounts of β-actin in the control conditions or 2% isoflurane-treated H4-APP-C99 cells. (B) Caspase-3 activation assessed by quantifying the ratio of caspase-3 fragment to caspase-3 full-length (FL) in the Western blots. Treatment with 2% isoflurane (black bar) increases caspase-3 activation compared with control conditions (white bar), normalized to β-actin levels. Data are mean ± SD; n = 4 for each experimental group. A t test is used to compare the difference between control conditions and 2% isoflurane treatment: **P < 0.01.

Fig. 4. N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-buty l ester (DAPT) potentiates isoflurane-induced caspase-3 activation and increases amyloid precursor protein C-terminal fragment (APP-CTF) levels. (A) Treatment with 2% isoflurane plus dimethyl sulfoxide (DMSO) (lanes 5 and 6) induces caspase-3 activation as compared with control conditions plus DMSO treatment (lanes 1 and 2) or the control conditions plus DAPT treatment (lanes 3 and 4). Treatment with 2% isoflurane plus DAPT (lanes 7 and 8) causes more degree of caspase-3 activation and increases in the levels of APP-CTFs (detected by antibody A8717) and APP-C99 (detected by antibody 6E10) as compared with the 2% isoflurane plus DMSO treatment (lanes 5 and 6). There is no significant difference in the amounts of β-actin in all of the above treatments. (B) Quantitation of the Western blots shows that the 2% isoflurane plus DAPT treatment (net bar) causes more increase in caspase-3 activation and in levels of APP-CTFs and APP-C99 as compared with 2% isoflurane plus DMSO treatment (black bar), normalized to β-actin levels. Data are mean ± SD; n = 6 for each experimental group. A t test is used to compare the difference between control conditions and 2% isoflurane treatment, or between DAPT treatment and DMSO treatment: * P < 0.05, ** P < 0.01, and the difference between 2% isoflurane plus DMSO and 2% isoflurane plus DAPT: # P < 0.05, ## P < 0.01.

Discussion

We have shown for the first time that a clinically relevant concentration of isoflurane can induce apoptosis, alter APP processing (by decreasing APP-CTF levels), and increase Aβ levels in human H4 neuroglioma cells stably transfected with human FL-APP or APP-C99, whereas control conditions, consisting of 5% CO2 plus 21% O2 and balanced nitrogen, did not. Therefore, in addition to inducing apoptosis, isoflurane affects biosynthesis of a protein implicated in the pathogenesis of AD. In agreement with our findings, even though isoflu-
Isoflurane has been previously reported to protect against apoptosis,\textsuperscript{18–24} the opposite has also been reported.\textsuperscript{25–28} The difference could be due to the use of different cell lines, \textit{e.g.}, rat cardiac cells \textit{versus} human neural-derived cells, in these studies. The difference could be also due to the duration and concentration of isoflurane exposure. Isoflurane could protect against apoptosis at low concentrations but induce apoptosis at high concentrations. In future studies, we will assess the effects of isoflurane on apoptosis with different concentrations (\textit{e.g.}, 0.5 and 1\% isoflurane) and durations (\textit{e.g.}, 1, 3, 12, and 24 h).

In the current study, isoflurane-induced caspase-3 activation could not be attenuated by reducing levels of A$\beta$ in the culture media with either DAPT, a $\gamma$-secretase inhibitor, or the anti-A$\beta$ antibodies 6E10 and 4G8. Interestingly, treatment with DAPT actually potentiated isoflurane-induced caspase-3 activation by roughly 1.5 fold, whereas DAPT treatment alone had no effect on caspase-3 activation. Isoflurane treatment alone decreased levels of APP-CTFs, whereas treatment with isoflurane plus DAPT served to increase levels of APP-CTFs by preventing $\gamma$-secretase-mediated proteolysis. Therefore, potentiation of isoflurane-induced caspase-3 activation by DAPT could be related to concurrent increases in APP-CTFs levels. One possible explanation is that caspase cleavage of APP-CTFs contributes to isoflurane-induced apoptosis. This would be consistent with the observation that APP-CTFs levels are decreased after treatment with isoflurane alone, because they likely undergo cleavage by caspase 3. Caspase cleavage of APP-CTFs has previously been reported to generate a short-lived, cytotoxic peptide called C31. C31 consists of the 31 C-terminal residues of APP.\textsuperscript{29,30} The APP antibodies used in this study to detect APP-CTFs would not detect APP-CTFs missing the C31 portion. Therefore, the observed decrease in APP-CTFs levels after the isoflurane treatment might be explained by caspase cleavage of APP-CTFs. In the case of the isoflurane plus DAPT treatment, increased levels of both APP-CTFs and caspase would be generated, potentially resulting in greater amounts of cytotoxic C31 peptide. Further investigation will be necessary to test this intriguing possibility as a mechanism underlying isoflurane-induced apoptosis.

Until now, it has remained unclear whether isoflurane-induced apoptosis is dependent on alterations in APP processing and A$\beta$ production. We have observed that isoflurane can induce caspase-3 activation and apoptosis without affecting APP processing and A$\beta$ generation in H4 naive cells, and we also found that DAPT treatment increases neither isoflurane-induced apoptosis nor levels of APP-CTFs in H4 naive cells (data not shown). These findings suggest that even though DAPT can potentiate isoflurane-induced apoptosis through concurrent increases in APP-CTF levels in H4-APP cells, the isoflurane-induced apoptosis may not be dependent on alterations in APP processing and A$\beta$ production. The exact mechanism by which isoflurane induces apoptosis remains to be determined.

The clinical significance of these results is unknown. A$\beta$ accumulates in the brain during normal aging and reaches especially high levels in AD, where it is believed to be a major pathogenic factor in the development of Alzheimer-type dementia. Other conditions associated with increased A$\beta$ burden include Down syndrome, $\beta$-amyloid angiopathy, mutations in presenilin (a rare condition that is a risk factor for early-onset AD), and expression of apolipoprotein E$\varepsilon$4, a vulnerability gene for late-onset AD that is present in at least one copy in roughly 25\% of the population.\textsuperscript{2,31–34} Although our results would suggest that such individuals might be at increased risk for A$\beta$ accumulation after isoflurane anesthesia, there is currently no evidence that isoflurane increases A$\beta$ levels \textit{in vivo} or that it accelerates development of AD or alters its clinical expression.

One caveat of the current study is that there is currently no satisfactory way to extrapolate apoptosis findings in cultured cells to the \textit{in vivo} aging brain. Future studies will be required to determine the physiologic relevance of the current findings. This could be accomplished by delivering similar concentrations of isoflurane to AD transgenic mice and then assessing whether the treatment causes apoptosis and increased levels of A$\beta$ in brain.

Nonetheless, our results have implications for understanding mechanisms of postoperative cognitive dysfunction\textsuperscript{13} and raise the possibility that isoflurane has adverse effects on APP and A$\beta$ processing in the central nervous system. Additional studies seem warranted given the aging of the population and growing numbers of patients with AD who require surgery and general anesthesia.

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ISOFLURANE, APOPTOSIS, AND A$\beta$

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