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 ELISA. The control condition consisted of 5% CO₂, 95% 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 neurodegenerative disorders, including AD (reviewed in Raina et al.). Several studies have also suggested that perioperative factors, such as hypoxia, hypcapnia, and anesthetics may contribute to AD neuropathogenesis. Isoflurane, a commonly used inhalation anesthetic, has previously been reported to promote Aβ oligomerization and cytotoxicity in pheochromocytoma 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 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 37°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

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treated with 250 nM N-[N-(3,5-difluorophenacetyl-L-alan-
yl)]-S-phenylglycine t-butyl ester (DAPT), 100 μM benz-
yloxyxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD),
15 μM Aβ, 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 immu-
noprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM
NaCl, 2 mM EDTA, 0.5% Nonidet P-40) plus protease inhib-
itors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pep-
statin 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. 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 per-
formed as described by Xie et al.

Quantitation of Aβ Using Sandwich Enzyme-linked
Immunosorbert Assay

Secreted Aβ was measured with a sandwich enzyme-
linked immunosorbert 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. Specifically, 96-well plates were coated with mouse
monoclonal antibodies specific to Aβ40 (Ab266) or Aβ42
(21F12). After blocking with albumin, wells were incu-
bated overnight at 4°C with test samples of conditioned
cell culture media, and then an anti-Aβ (α-Aβ-HR1) con-
jugated to horseradish peroxidase was added. Plates
were then developed with 3,3′,5,5′-tetramethylbenzene-
dine reagent and well absorbance measured at 450 nm.
Aβ levels in test samples were determined by compari-
sion with signal from unconditioned media spiked with
known quantities of Aβ40 and Aβ42.

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 accord-
ing 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 absor-
bance at a wavelength of 570 nm. We present the
changes in the absorbance, as the number of cell viabil-
ity, 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 experimen-
tal conditions. We evaluated caspase-3 activation, cell
viability, APP processing, and Aβ levels in the experi-
mental 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 consid-
ered statistically significant.

Results

2% Isoflurane Induces Apoptosis and Increases
Levels of Secreted Aβ Levels

We first assessed whether control conditions, consist-
ing 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 signifi-
cant 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 incuba-
tor 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, we assessed the effects of isoflurane on
caspase-3 activation by quantitative Western blot analy-
yses. 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 frag-
ment (17–19 kd) to FL caspase-3 (35 kd). Immunoblot-
ing 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 indicate 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...
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 Aβ10 (193 vs. 50 pg/ml) and Aβ12 (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 roughly 57% (fig. 2B; \( P < 0.01 \)) decrease in levels of APP-CTFs after isoflurane treatment. Two percent isoflurane significantly increased the levels of both Aβ10 (193 vs. 50 pg/ml) and Aβ12 (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.

**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 dimethyl 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; caspase activation.
Interestingly, 2% isoflurane plus DAPT led to an even greater (50%) increase in caspase-3 activation than did treatment with 2% isoflurane plus DMSO (311% vs. 207%; figs. 4A and B; *P < 0.01). In addition, whereas isoflurane plus DMSO led to a roughly 45% decrease in both APP-CTFs, treatment with 2% isoflurane plus DAPT increased levels of APP-C83 (225%) and APP-C99 (174%) as compared with control conditions (figs. 4A and B; *P < 0.01). As expected, DAPT treatment also decreased Aβ levels (data not shown). These findings indicate that neither γ-secretase cleavage of APP nor increased secretion of Aβ is necessary for isoflurane-induced caspase-3 activation and increases amyloid precursor protein C-terminal fragment (APP-CTF) levels.

**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 isoflur-
rane has been previously reported to protect against apoptosis, the opposite has also been reported. The difference could be due to the use of different cell lines, e.g., rat cardiac cells 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 (e.g., 0.5 and 1% isoflurane) and durations (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 Aβ in the culture media with either DAPT, a γ-secretase inhibitor, or the anti-Aβ 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 γ-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. 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 Aβ production. We have observed that isoflurane can induce caspase-3 activation and apoptosis without affecting APP processing and Aβ generation in H4 naïve cells, and we also found that DAPT treatment increases neither isoflurane-induced apoptosis nor levels of APP-CTFs in H4 naïve 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 Aβ production. The exact mechanism by which isoflurane induces apoptosis remains to be determined.

The clinical significance of these results is unknown. Aβ 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 Aβ burden include Down syndrome, β-amyloid angiopathy, mutations in presenilin (a rare condition that is a risk factor for early-onset AD), and expression of apolipoprotein E-ε4, a vulnerability gene for late-onset AD that is present in at least one copy in roughly 25% of the population. Although our results would suggest that such individuals might be at increased risk for Aβ accumulation after isoflurane anesthesia, there is currently no evidence that isoflurane increases Aβ levels 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 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 Aβ in brain.

Nonetheless, our results have implications for understanding mechanisms of postoperative cognitive dysfunction and raise the possibility that isoflurane has adverse effects on APP and Aβ 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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