Nitrous Oxide Plus Isoflurane Induces Apoptosis and Increases β-Amyloid Protein Levels

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Background: Some anesthetics have been suggested to induce neurotoxicity, including promotion of Alzheimer's disease neuropathogenesis. Nitrous oxide and isoflurane are common anesthetics. The authors set out to assess the effects of nitrous oxide and/or isoflurane on apoptosis and β-amyloid (Aβ) levels in H4 human neuroglioma cells and primary neurons from naïve mice.

Methods: The cells or neurons were exposed to 70% nitrous oxide and/or 1% isoflurane for 6 h. The cells or neurons and conditioned media were harvested at the end of the treatment. Caspase-3 activation, apoptosis, processing of amyloid precursor protein, and Aβ levels were determined.

Results: Treatment with a combination of 70% nitrous oxide and 1% isoflurane for 6 h induced caspase-3 activation and apoptosis in H4 naïve cells and primary neurons from naïve mice. The 70% nitrous oxide plus 1% isoflurane, but neither alone, for 6 h induced caspase-3 activation and apoptosis, and increased levels of β-site amyloid precursor protein-cleaving enzyme and Aβ in H4-amyloid precursor protein cells. In addition, the nitrous oxide plus isoflurane–induced Aβ generation was reduced by a broad caspase inhibitor, Z-VAD. Finally, the nitrous oxide plus isoflurane–induced caspase-3 activation was attenuated by γ-secretase inhibitor L-685,458, but potentiated by exogenously added Aβ.

Conclusion: These results suggest that the common anesthetics nitrous oxide plus isoflurane may promote neurotoxicity by inducing apoptosis and increasing Aβ levels. The generated Aβ may further potentiate apoptosis to form another round of apoptosis and Aβ generation. More studies, especially the *in vivo* confirmation of these *in vitro* findings, are needed.

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ALZHEIMER'S disease (AD), one of the most common forms of dementia, affects 4.5 million Americans and costs $100 billion a year in direct care alone, and its impact will only increase in the coming decades. Excessive production and/or accumulation of β-amyloid protein (Aβ), the major component of plaque in the AD patient brain, play a fundamental role in the pathology of AD.[1–3] Aβ is produced *via* serial proteolysis of amyloid precursor protein (APP) by aspartyl protease β-site APP-cleaving enzyme (BACE), or β-secretase and γ-secretase. BACE cleaves APP to generate a 99-residue membrane-associated C-terminus fragment. The 99-residue membrane-associated C-terminus fragment is further cleaved by γ-secretase to release 4-kilodalton (kDa) Aβ and APP intracellular domain.[4–6] APP can also undergo caspase-mediated cleavage to generate a 90 kDa N-terminus APP caspase fragment (APP-N-caspase fragment).[7–9] Increasing evidence suggests a role for caspase activation and apoptosis in AD neuropathogenesis.[8–26]

An estimated 200 million patients worldwide undergo anesthesia and surgery each year. Even though anesthesia and surgery may not increase the incidence of AD,[27] it has been reported that age of onset of AD is inversely related to cumulative exposure to anesthesia and surgery before age 50.[28] A recent study also reported that patients having coronary artery bypass graft surgery under general anesthesia are at increased risk for AD, as compared with those having percutaneous transluminal coronary angioplasty under local anesthesia.[29] However, other studies have suggested that there is little or no relationship between anesthesia and AD.[30,31] More population studies, defining the role of anesthesia in AD, are necessary.[32]

Nevertheless, perioperative factors, including hypoxia,[33–37] hypocapnia,[38] and anesthetics,[7,24,39–46] have been reported to potentially contribute to AD neuropathogenesis in cultured cells and in animals. Nitrous oxide and isoflurane are common anesthetics for patients; however, the effects of nitrous oxide plus isoflurane on neurotoxicity such as AD neuropathogenesis, including caspase activation, apoptosis, and Aβ levels, have not been assessed.

In the present study, we set out to determine the effects of nitrous oxide plus isoflurane on caspase-3 activation, apoptosis, APP processing, and Aβ levels in H4 human neuroglioma cells (H4 naïve cells), H4 naïve cells stably transfected to express full-length (FL) APP (H4-APP cells) and primary neurons from naïve mice. We further studied the effects of the caspase inhibitor Z-VAD, γ-secretase inhibitor L-685,458, and Aβ on the nitrous oxide plus isoflurane–induced caspase-3 activation and Aβ accumulation.

Materials and Methods

Cell Lines

We employed H4 human neuroglioma cells (H4 naïve cells) and H4 naïve cells stably transfected to express FL APP (H4-APP cells) in the experiments. All cell lines were 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. Stably transfected H4 cells were in addition supplemented with 200 μg/ml G418.

Primary Neurons

This protocol was approved by the Massachusetts General Hospital Standing Committee on Animals (Boston, Massachusetts) on the Use of Animals in Research and Teaching. Naïve (C57BL/6J) mice with a gestation stage of Day 15 were killed 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 the 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 six well plates with a confluent rate of 50%. Ten days after the harvest, the neurons were exposed to nitrous oxide plus isoflurane.

Cell Treatment

The inhalation anesthetics nitrous oxide and/or isoflurane were delivered from an anesthesia machine to a sealed plastic box in a 37°C incubator containing six-well plates seeded with 1 million cells or 0.25 million neurons in 1.5

ml cell or neuron culture media. A Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) was used to continuously monitor the delivered concentrations of carbon dioxide, oxygen, nitrous oxide, and/or isoflurane. The cells were treated for 6 h with one of the following three conditions: Nitrous oxide alone (21% O_2, 5% CO_2, and 70% nitrous oxide), isoflurane alone (21% O_2, 5% CO_2, and 1% isoflurane), or nitrous oxide plus isoflurane (21% O_2, 5% CO_2, 70% nitrous oxide, and 1% isoflurane). The primary neurons were treated with nitrous oxide plus isoflurane for 6 h. In the interaction studies, the H4-APP cells were treated with Z-VAD (65 μM), L-685,458 (0.65 μM), and Aβ42 (7.5 μM) 1 h before nitrous oxide plus isoflurane treatment. The control for Z-VAD and L-685,458 was dimethyl sulfoxide, and the control for Aβ42 was saline. The control condition for nitrous oxide, isoflurane, and nitrous oxide plus isoflurane was 21% O_2 plus 5% CO_2, which did not affect caspase-3 activation, cell viability, APP processing, and Aβ generation.[40]

Cell or Neuron Lysis and Protein Amount Quantification

Cell or neuron pellets were detergent-extracted on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 13,000 revolutions per minute for 15 min, and quantified for total proteins with a bicinchoninic acid protein assay kit (Pierce, Iselin, NJ).

Western Blot Analysis

The cells or neurons were harvested at the end of the experiments and were subjected to Western blot analyses as described by Xie *et al.*[47] A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Inc., Beverly, MA) was used to recognize FL-caspase-3 (35–40 kDa) and caspase-3 fragment (17–20 kDa) resulting from cleavage at asparate position 175. Rabbit polyclonal anti-BACE-1 antibody (1:1,000; Abcam, Cambridge, MA) was used to detect protein levels of BACE (65 kDa). Antibody C66 (1:2,000; a generous gift of Dora Kovacs, Ph.D., Associate Professor of Neurology at Harvard Medical School, Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts) was used to visualize FL-APP (110 kDa) and APP-N-caspase (90 kDa) fragments. Antibody anti-β-actin (1:10,000; Sigma, St. Louis, MO) was used to detect β-actin (42 kDa). Each band in the Western blot represents an independent experiment. We have averaged results from 3–10 independent experiments. The quantification of Western blots was performed as described by Xie *et al.*[40] Briefly, the intensity of signals was analyzed by using the National Institutes of Health Image 1.62 program (National Institutes of Health, Bethesda,

MD). We quantified Western blots using two steps. First, we used levels of β-actin to normalize (*e.g.*, determine the ratio of FL-APP amount to β-actin amount) levels of FL-APP, APP-N-caspase fragment, FL-caspase-3, caspase-3 fragment, and BACE to control for any loading differences in total protein amounts. Second, we presented changes in levels of FL-APP, APP-N-caspase fragment, FL-caspase-3, caspase-3 fragment, and BACE in treated cells as percentages of those in cells treated with controls.

Quantification of Aβ Using a Sandwich Enzyme-linked Immunosorbent Assay

Secreted Aβ was measured with a Sandwich enzyme-linked immunosorbent assay by using an Aβ measurement kit (Wako, Richmond, VA) as described by Xie *et al.*[48] The monoclonal antibody BAN50, the epitope of which is human Aβ,[1–16] is coated on 96 well surfaces of a separable microplate and acts as a capture antibody for the N-terminal portion of human Aβ40. Captured human Aβ40 is recognized by antibody BA27, which specifically detects the C-terminal portion of Aβ40. The wells were incubated overnight at 4°C with test samples of conditioned cell culture media, and then BA27 was added. The plates were then developed with tetramethylbenzidine reagent, terminated by stop solution, and well absorbance was measured at 450 nm. Aβ levels in the test samples were determined by comparing results with signals from unconditioned media spiked with known quantities of Aβ40.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Staining

A TMR red kit (Roche Diagnostics, Mannheim, Germany) was used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Specifically, cells were grown on coverslips overnight in the cell culture medium. The cells or neurons were fixed in 4% paraformaldehyde for 60 min after treatment of nitrous oxide plus isoflurane. The coverslips were rinsed with phosphate-buffered saline three times for 5 min each time and then incubated in a permeabilization solution (0.1% TritonX-100 in 0.1% sodium citrate) at 4°C for 5 min. Finally, the coverslips were incubated with a TUNEL reaction mixture for 1 h at 37°C in a humidified dark chamber. The negative control was prepared by incubating the coverslips in label solution only. The coverslips for the positive control were incubated with deoxyribonuclease I recombinant. Finally, the coverslips were incubated with 10 μg/ml Hoechst 33342 in a humidified dark chamber for 10 min. The samples were then analyzed in mounting medium under a fluorescence microscope. The TUNEL-positive cells were counted manually in five randomly selected areas under a 20 × objective microscope lens by an investigator who was blinded to the experiments.

Cell Apoptosis Assay

Cell apoptosis was assessed by a cell death detection enzyme-linked immunosorbent assay kit (Roche, Palo Alto, CA), which assays cytoplasmic histone-associated deoxyribonucleic acid fragmentation associated with cellular apoptosis.

Statistics

Given the presence of background caspase-3 activation and apoptosis in the cells and neurons, we did not use absolute values to describe changes in caspase-3 activation and apoptosis. Instead, cell apoptosis and caspase-3 activation were presented as a percentage of those of the control group. One hundred percent caspase-3 activation or apoptosis refers to control levels for purposes of comparison with experimental conditions. Then, we presented changes in levels of caspase-3 activation, apoptosis, FL-APP, APP-N-caspase fragment, BACE, and Aβ in treated cells as percentages of those in cells treated with controls. Data were expressed as mean ± SD. The number of samples varied from 3–10, and the samples were normally distributed. ANOVA with repeated measurements or a *t* test was used to compare the difference from the control group. *P* values less than 0.05 (* or #) and 0.01 (** or ##) were considered statistically significant. The significance testing was two-tailed, and we used SAS software (SAS Institute, Inc., Cary, NC) to analyze the data.

Results

Nitrous Oxide Plus Isoflurane Induced Caspase-3 Activation and Apoptosis, and Increased Levels of BACE and Secreted Aβ in H4-APP Cells

Nitrous oxide plus isoflurane are common anesthetics for patients. We therefore assessed the effects of nitrous oxide plus isoflurane on apoptosis and secreted Aβ levels in H4-APP cells. Since caspase-3 activation is one of the final steps of cellular apoptosis,[49] we assessed the effects of nitrous oxide plus isoflurane on caspase-3 activation by quantitative Western blot analyses. Caspase-3 immunoblotting showed visible increases in protein levels of caspase-3 fragment after treatment with 70% nitrous oxide plus l% isoflurane for 6 h, as compared with the control condition (fig. 1A). We used staurosporine[50,51] in the experiments as a positive control to validate the caspase-3 antibody and to confirm that the visible band after the nitrous oxide plus isoflurane treatment is indeed the band representing cleaved caspase-3. Quantification of the Western blot by determining ratio of cleaved (activated) caspase-3 fragment (17–20 kDa) to FL-caspase-3 (35–40 kDa) revealed that treatment of nitrous oxide plus isoflurane led to a 264% increase in caspase-3 cleavage (activation), as compared with the control condition (fig. 1B) (*P* = 0.001). Given that caspase-3 activation alone may not represent apoptotic
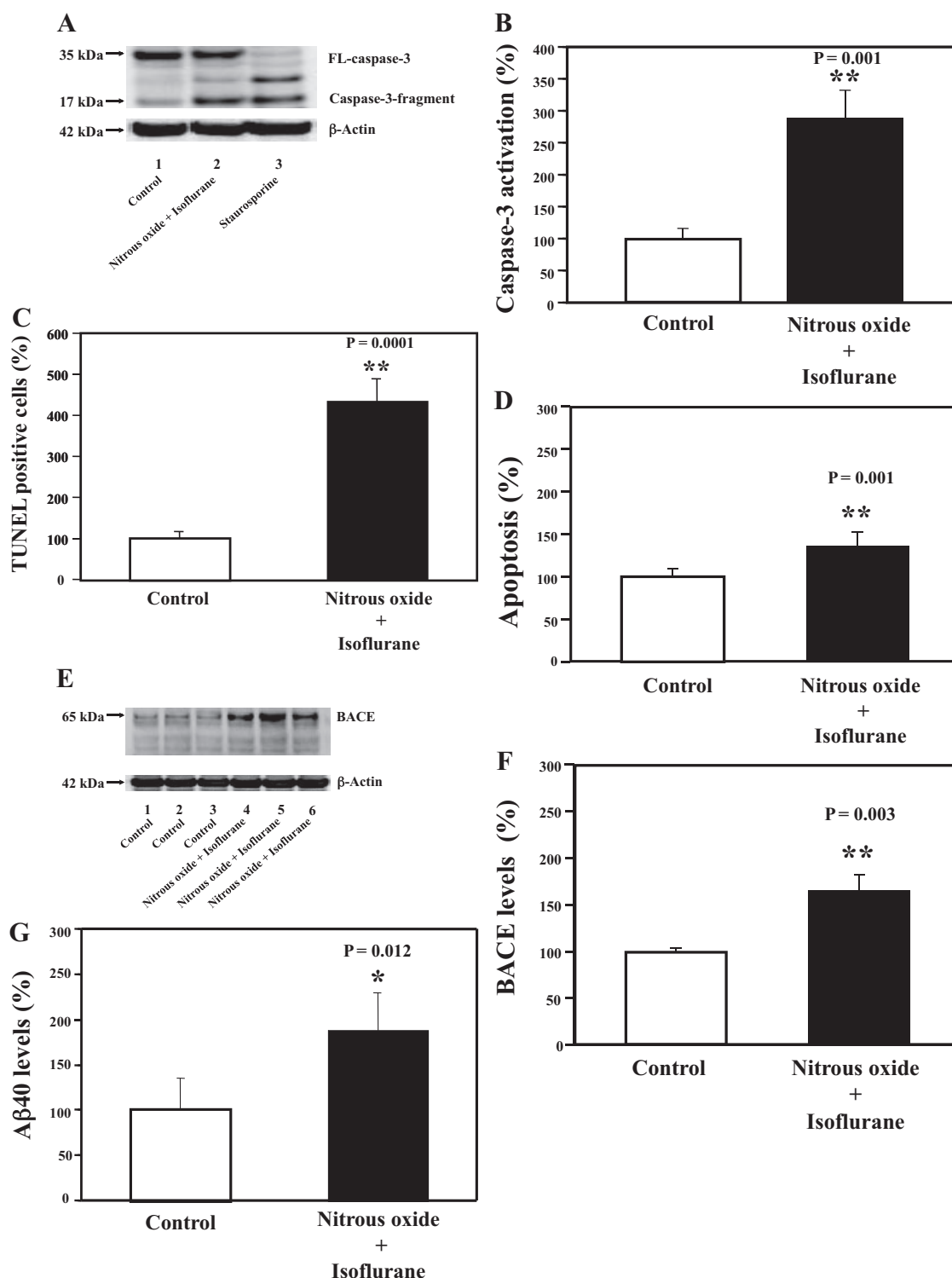
Fig. 1. Treatment of nitrous oxide plus isoflurane induces caspase-3 activation and apoptosis, and increases levels of BACE and Aβ in H4-APP cells. (A) Effects of 70% nitrous oxide plus 1% isoflurane for 6 h and staurosporine on caspase-3 activation. Each band in the Western blot represents an independent experiment. There is no significant difference in amounts of β-actin in the control condition-, staurosporine-, or nitrous oxide plus isoflurane–treated H4-APP cells. (B) Caspase-3 activation assessed by quantifying the ratio of caspase-3 fragment to FL caspase-3 in the Western blots, normalized to β-actin levels. We have averaged results from three independent experiments. (C) Effects of the nitrous oxide plus isoflurane treatment on TUNEL-positive cells. (D) Effects of the nitrous oxide plus isoflurane treatment on apoptosis. (E) Effects of the nitrous oxide plus isoflurane treatment on BACE levels. (F) Quantification of the Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (G) Effects of nitrous oxide plus isoflurane treatment on Aβ40 levels. We have averaged results from ten independent experiments. Aβ = β-amyloid protein; APP = amyloid precursor protein; BACE = β-site amyloid precursor protein-cleaving enzyme; FL = full-length; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling. $* P < 0.05$; $** P < 0.01$.
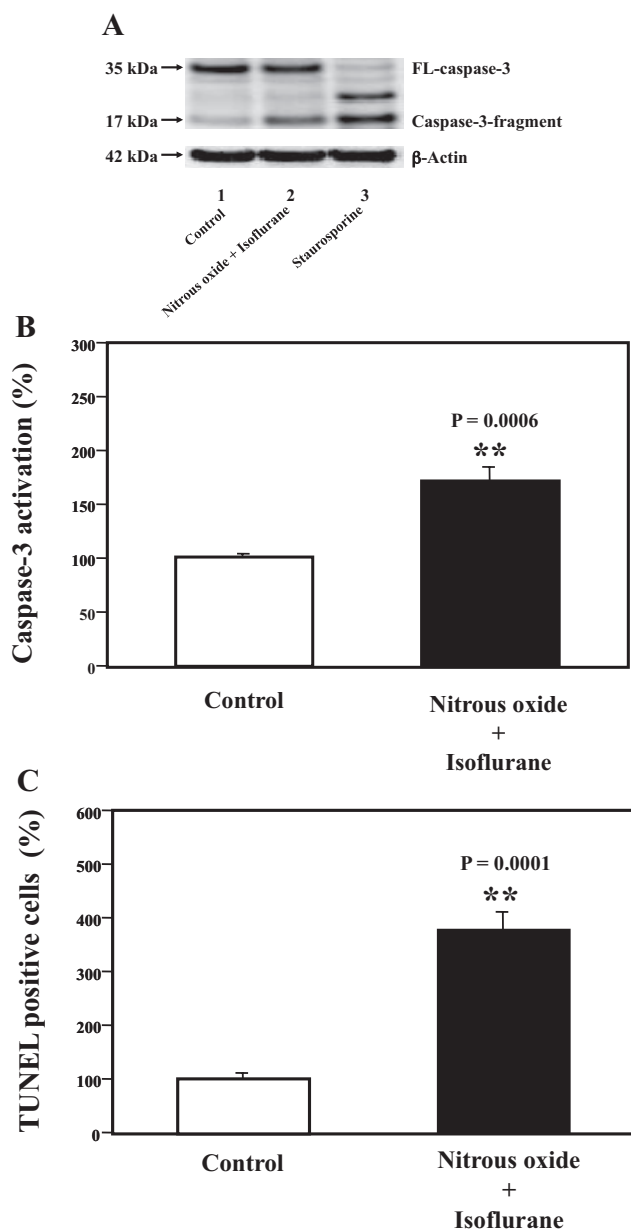
Fig. 2. Treatment of nitrous oxide plus isoflurane induces caspase-3 activation and apoptosis in H4 naïve cells. (A) Effects of 70% nitrous oxide plus 1% isoflurane for 6 h and staurosporine on caspase-3 activation. Each band in the Western blot represents an independent experiment. (B) Quantification of the Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (C) Effects of the nitrous oxide plus isoflurane treatment on TUNEL-positive cells. We have averaged results from four independent experiments. FL = full length; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling. ** P < 0.01.

cell damage,[52] we also assessed the effects of 70% nitrous oxide plus 1% isoflurane on cellular apoptosis by TUNEL study and by cell death detection enzyme-linked immunosorbent assay kit (detecting cytoplasmic histone-associated DNA fragmentation). We found that the treatment of nitrous oxide plus isoflurane increased TUNEL-positive cells (apoptosis), as compared with the control condition (see fig. 1, Supplemental Digital Content 1,

which is a histologic image showing the effects of nitrous oxide plus isoflurane on number of TUNEL-positive H4-APP cells, http://links.lww.com/ALN/A538; and fig. 1C): 100% *versus* 431%, ($P = 0.0001$). Moreover, we were able to show that the treatment of nitrous oxide plus isoflurane (fig. 1D, black bar) increased cytoplasmic histone-associated deoxyribonucleic acid fragmentation (apoptosis), as compared with the control condition (fig. 1D, white bar): 100% *versus* 136%, ($P = 0.001$). BACE is one of the enzymes for Aβ generation, so we asked whether the treatment of nitrous oxide plus isoflurane can increase levels of BACE. We were able to show that treatment with 70% nitrous oxide plus 1% isoflurane for 6 h caused visible increases in BACE levels, as compared with the control condition (fig. 1E). Quantification of the Western blot showed that the nitrous oxide plus isoflurane treatment led to a 171% increase in BACE levels, as compared with the control condition ($P = 0.003$, fig. 1F). Finally, the treatment of nitrous oxide plus isoflurane led to increases in secreted Aβ40 levels, as compared with the control condition in H4-APP cells (fig. 1G): 100% *versus* 188%, $P = 0.012$. These findings suggest that the treatment of 70% nitrous oxide plus 1% isoflurane may induce apoptosis in H4-APP cells, which enhances BACE levels to facilitate APP processing, leading to increases in Aβ levels.

Nitrous Oxide Plus Isoflurane Induced Caspase-3 Activation and Apoptosis in H4 Naïve Cells and Primary Neurons from Naïve Mice

Next, we asked whether the nitrous oxide plus isoflurane treatment can induce apoptosis in cells without overexpressing human APP and in healthy neurons. We were able to show that the treatment with 70% nitrous oxide plus 1% isoflurane for 6 h or staurosporine (the positive control in the experiments) induced caspase-3 activation, as compared with the control condition in H4 naïve cells (fig. 2A and B): 100% *versus* 172%, $P = 0.0006$, and primary neurons from naïve mice (fig. 3A and B): 100% *versus* 154%, $P = 0.0357$. Moreover, the treatment of nitrous oxide plus isoflurane increased TUNEL-positive cells (apoptosis), as compared with the control condition in H4 naïve cells (see fig. 2, Supplemental Digital Content 2, which is a histologic image showing the effects of nitrous oxide plus isoflurane on number of TUNEL-positive H4 naïve cells, http://links.lww.com/ALN/A539; and fig. 2C): 100% *versus* 376%, $P = 0.0001$, and in primary neurons from naïve mice (see fig. 3, Supplemental Digital Content 3, which is a histologic image showing the effects of nitrous oxide plus isoflurane on number of TUNEL-positive primary neurons, http://links.lww.com/ALN/A540; fig. 3C): 100% *versus* 298%, $P = 0.0005$. We were not able to detect alterations in secreted Aβ levels in H4 naïve cells or primary neurons from naïve mice, because the endogenous levels
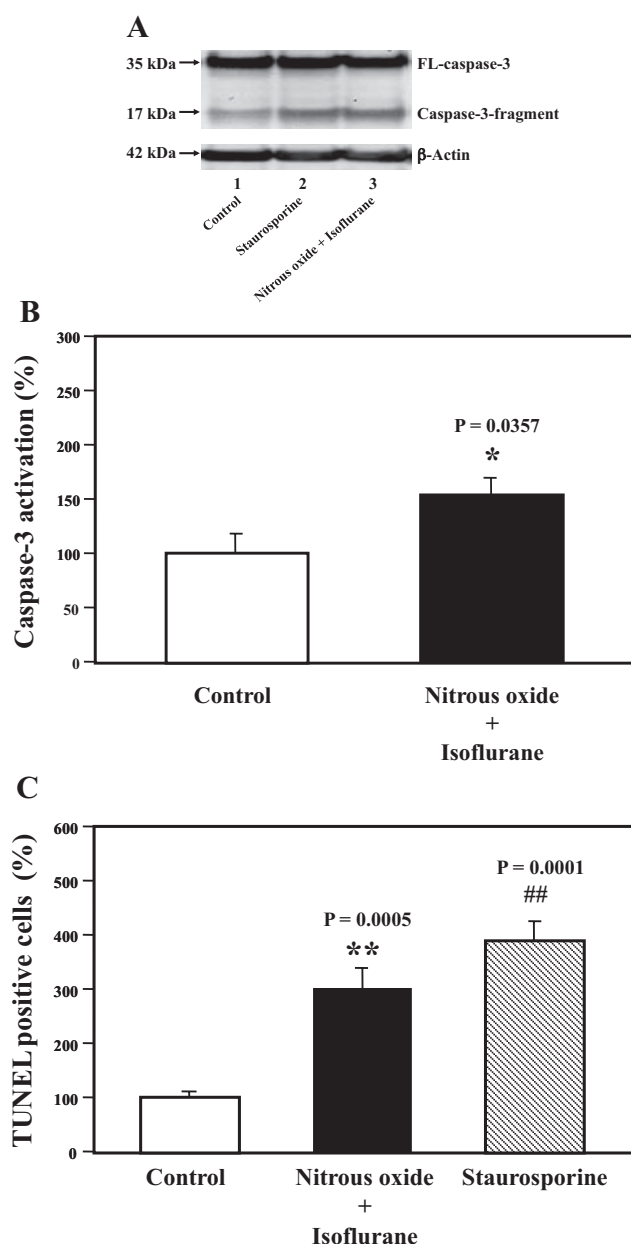
Fig. 3. Treatment of nitrous oxide plus isoflurane induces caspase-3 activation and apoptosis in primary neurons from naïve mice. (*A*) Effects of 70% nitrous oxide plus 1% isoflurane for 6 h and staurosporine on caspase-3 activation. Each band in the Western blot represents an independent experiment. (*B*) Quantification of the Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (*C*) Effects of the nitrous oxide plus isoflurane treatment and staurosporine treatment on TUNEL-positive cells. We have averaged results from four independent experiments. FL = full length; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling. * *P* < 0.05; ** *P* < 0.01; ## *P* < 0.01.

of Aβ in the cells and neurons are low. Collectively, these results illustrate that treatment with 70% nitrous oxide plus l% isoflurane for 6 h can induce caspase-3 activation and increase TUNEL-positive cells (apoptosis) in H4 naïve cells (without APP overload) and in primary neurons from naïve mice (healthy neurons), which suggests that the nitrous

oxide plus isoflurane treatment can induce apoptosis in healthy neurons and independent of APP overexpression.

Nitrous Oxide or Isoflurane Alone Induced Neither Apoptosis Nor Increases in Aβ Levels in H4-APP Cells

Given that the treatment of 70% nitrous oxide plus 1% isoflurane can induce caspase-3 activation and apoptosis, and can increase levels of BACE and secreted Aβ in H4-APP cells, we next asked whether treatment with 70% nitrous oxide or 1% isoflurane alone can cause the same effects. In the present experiment, treatment with 70% nitrous oxide for 6 h induced caspase-3 cleavage (activation) (fig. 4A and B); 100% *versus* 279% (*P* = 0.001). However, the nitrous oxide treatment induced neither apoptosis, assessed by detecting cytoplasmic histone-associated deoxyribonucleic acid fragmentation (fig. 4C; 100% *vs.* 109%, *P* = 0.06, not significant), nor increases in BACE levels (fig. 4D and E; 100% *vs.* 105%, *P* = 0.7978, N.S.), as compared with the control condition. Finally, the nitrous oxide treatment did not increase secreted Aβ40 levels, as compared with the control condition (fig. 4F); 100% *versus* 101% (*P* = 0.914, not significant). These results indicate that even 70% nitrous oxide induces caspase-3 activation; the nitrous oxide treatment alone induces neither apoptosis nor increases in BACE and Aβ levels. Next, we assessed whether 1% isoflurane alone can increase Aβ levels in H4-APP cells. Treatment with 1% isoflurane for 6 h did not induce caspase-3 activation (fig. 5A and B), and did not increase Aβ levels (fig. 5C). Taken together, these findings suggest that the apoptosis and Aβ level increase induced by 70% nitrous oxide plus 1% isoflurane likely resulted from the synergistic effects of nitrous oxide plus isoflurane.

Caspase Inhibitor Z-VAD Attenuated the Nitrous Oxide Plus Isoflurane–induced Caspase-3 Activation

Given the findings that nitrous oxide plus isoflurane can induce apoptosis and increase levels of BACE and Aβ, we next asked whether the nitrous oxide plus isoflurane–induced alteration in APP processing and Aβ levels is dependent on the ability of nitrous oxide plus isoflurane to induce caspase-3 activation and apoptosis. For this purpose, we set out to assess the effects of the caspase inhibitor Z-VAD on nitrous oxide plus isoflurane–induced alterations in caspase-3 activation, APP processing, and Aβ levels in H4-APP cells. Whereas Z-VAD alone did not affect caspase-3 activation, the Z-VAD treatment attenuated the nitrous oxide plus isoflurane–induced caspase-3 activation, as compared with the control condition (fig. 6A). The observed 20 kDa bands that only appeared in the Z-VAD-treated cells lysates in the Western blot were most likely caspase-dependent, nonspecific, cross-reacting bands. However, the identity of the
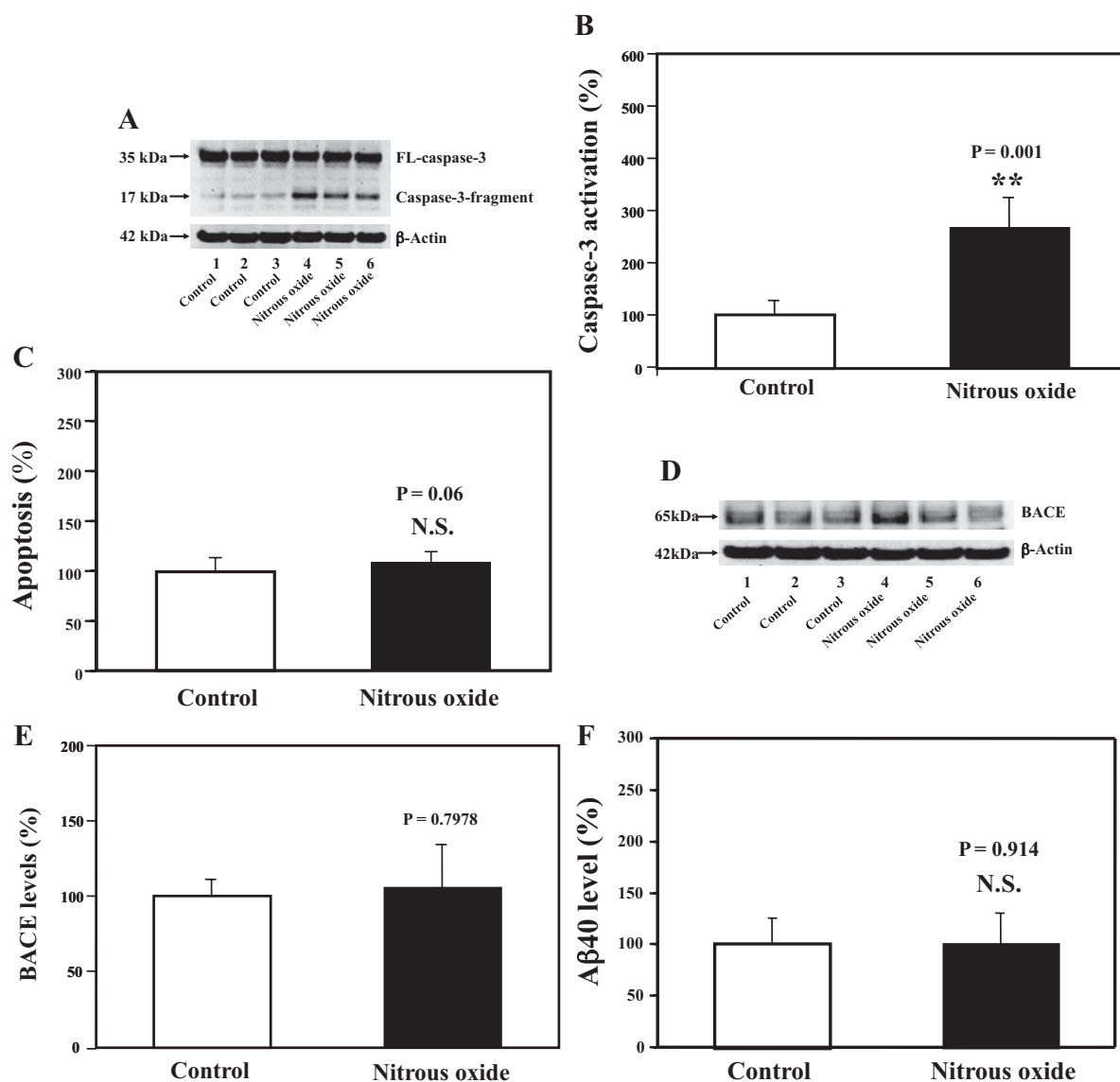
Fig. 4. Nitrous oxide treatment alone induces neither apoptosis nor increases in levels of BACE and Aβ in H4-APP cells. (*A*) Effects of 70% nitrous oxide on caspase-3 activation. Each band in the Western blot represents an independent experiment. (*B*) Quantification of Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (*C*) Effects of the nitrous oxide treatment on apoptosis. We have averaged results from six independent experiments. (*D*) Effects of the nitrous oxide treatment on BACE levels. Each band in the Western blot represents an independent experiment. (*E*) Quantification of the Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (*F*) Effects of the nitrous oxide treatment on Aβ levels. We have averaged results from six independent experiments. APP = amyloid precursor protein; Aβ = β-amyloid protein; BACE = β-site amyloid precursor protein-cleaving enzyme; FL = full length; N.S. = not significant. ** $P < 0.01$.

bands is unknown. Quantification of the Western blots revealed that Z-VAD attenuated nitrous oxide plus isoflurane–induced caspase-3 activation: 263% *versus* 131%, $P = 0.0001$ (fig. 6B). Furthermore, Z-VAD attenuated the nitrous oxide plus isoflurane–induced increases in levels of caspase-cleaved APP N-terminus fragment (fig. 6C and D), $P = 0.001$. Finally, Z-VAD reduced nitrous oxide plus isoflurane–induced increases in secreted Aβ40 levels in H4-APP cells, $P = 0.0004$ (fig. 6E). Collectively, these findings suggest that nitrous oxide plus isoflurane–induced APP processing and Aβ generation is dependent on the nitrous oxide plus isoflurane–induced caspase activation and apoptosis.

Nitrous Oxide Plus Isoflurane–induced Caspase-3 Activation Was Attenuated by the γ-Secretase Inhibitor L-685,458 but Was Potentiated by Aβ

Next, we asked whether pharmacologically based reductions in Aβ levels can attenuate nitrous oxide plus isoflurane–induced caspase-3 activation. For this purpose, we assessed the effects of the γ-secretase inhibitor L-685,458 on nitrous oxide plus isoflurane–induced caspase-3 activation in H4-APP cells. L-685,458 reduced the nitrous oxide plus isoflurane–induced caspase-3 activation; 287% *versus* 232%, $P = 0.0001$ (fig. 7A and B). Next, we asked whether a proapoptotic stimulus, 7.5 uM Aβ42, could potentiate nitrous oxide plus isoflurane–induced caspase-3 activation. We were
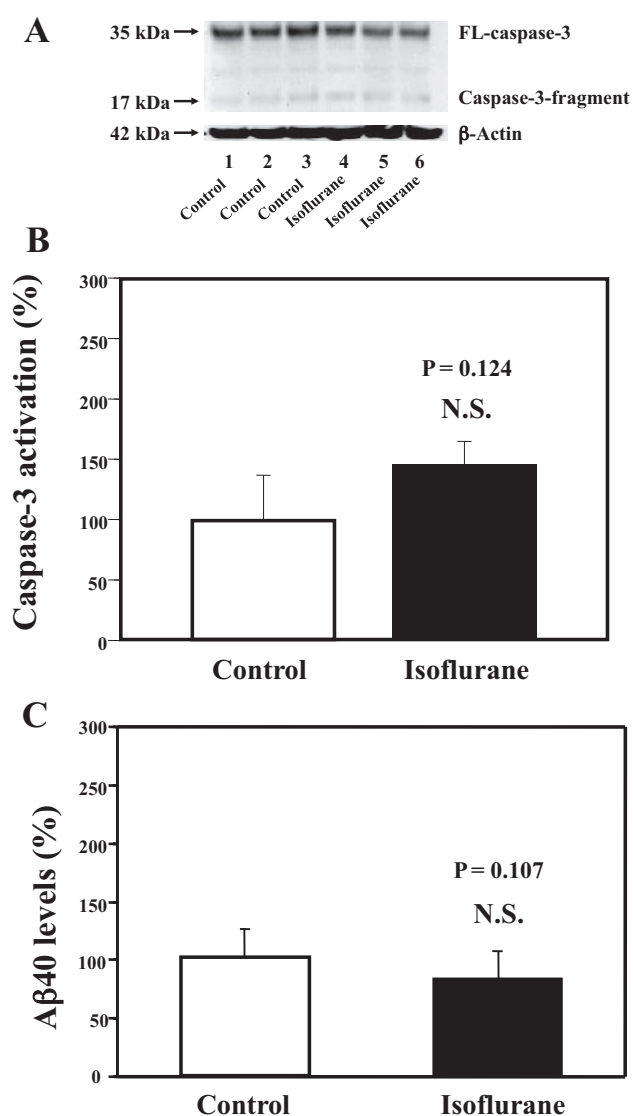
Fig. 5. Isoflurane treatment alone induces neither caspase-3 activation nor Aβ accumulation in H4-APP cells. (*A*) Effects of 1% isoflurane for 6 h on caspase-3 activation. Each band in the Western blot represents an independent experiment. (*B*) Quantification of Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (*C*) Effects of the isoflurane treatment on Aβ levels. We have averaged results from three independent experiments. APP = amyloid precursor protein; Aβ = β-amyloid protein; FL = full length; N.S. = not significant.

able to show that the Aβ treatment enhanced the nitrous oxide plus isoflurane–induced caspase-3 activation; 447% *versus* 772%, $P = 0.0004$ (fig. 7C and D). Collectively, these results suggest that Aβ can potentiate the nitrous oxide plus isoflurane–induced caspase-3 activation.

Discussion

The commonly used inhalation anesthetic isoflurane has previously been shown to promote Aβ aggregation and to enhance toxicity of Aβ.[39] We have shown that isoflurane,[7,40-42] sevoflurane,[53] and desflurane plus hyp-

oxia[54] can induce cellular apoptosis and increase Aβ generation. Nitrous oxide plus isoflurane are common anesthetics for patients. Moreover, animal studies have shown that anesthesia with 70% nitrous oxide plus 1.2% isoflurane can cause a long-term impairment of learning and memory in aged rats.[55] We therefore set out to assess the effects of nitrous oxide plus isoflurane on apoptosis and Aβ generation. We were able to show that treatment with 70% nitrous oxide plus 1% isoflurane for 6 h induced caspase activation and apoptosis, and increased levels of BACE and secreted Aβ in H4-APP cells. Moreover, we have found that the treatment with 70% nitrous oxide plus 1% isoflurane for 6 h can induce caspase activation and apoptosis without alteration in secreted Aβ levels in H4 naïve cells and primary neurons from naïve mice. However, it is possible that the treatment of isoflurane plus nitrous oxide may lead to undetectable changes in secreted Aβ levels in the H4 naïve cells and the primary neurons, because the endogenous levels of Aβ in the H4 naïve cells and the primary neurons from naïve mice are low. The cultured neurons in the experiments were Postnatal Day 10 neurons; therefore, the findings in the primary neurons that nitrous oxide plus isoflurane can induce caspase activation and apoptosis suggest that the clinically relevant treatment of nitrous oxide plus isoflurane may cause neurotoxicity in developing neurons. The findings in H4-APP cells that isoflurane plus nitrous oxide can induce caspase activation and apoptosis, and can increase levels of BACE and secreted Aβ, suggest that the clinically relevant treatment of nitrous oxide plus isoflurane may cause neurotoxicity, including apoptosis and Aβ generation, which are features of AD neuropathogenesis.

Consistent with our previous findings,[41] we were able to show that treatment with 1% isoflurane alone for 6 h did not induce caspase-3 activation. Furthermore, we found that the 1% isoflurane treatment did not increase Aβ levels in H4-APP cells. We found that treatment with 70% nitrous oxide alone for 6 h induced neither apoptosis nor increases in Aβ levels in current experiments. It is interesting that treatment with 70% nitrous oxide alone for 6 h induced casapse-3 activation in H4-APP cells. McLaughlin *et al.*[52] have shown that caspase-3 activation alone may not represent apoptotic cell damage; therefore, it is possible that the treatment of 70% nitrous oxide induces caspase-3 activation without causing apoptosis and increasing Aβ levels. In future studies we will employ cellular imaging methods to determine the location of the activated caspase-3, nuclear or cytoplasm, because activated caspase-3 in the nucleus may be strongly associated with apoptosis.[52] These results would further support our conclusion that apoptosis, but not caspase activation alone, can enhance BACE levels to facilitate APP processing, leading to Aβ generation.

It has been reported that N-methyl-D-aspartic acid (NMDA) receptor antagonists and γ-aminobutyric acid (GABA) receptor agonists can induce apoptosis in the de-
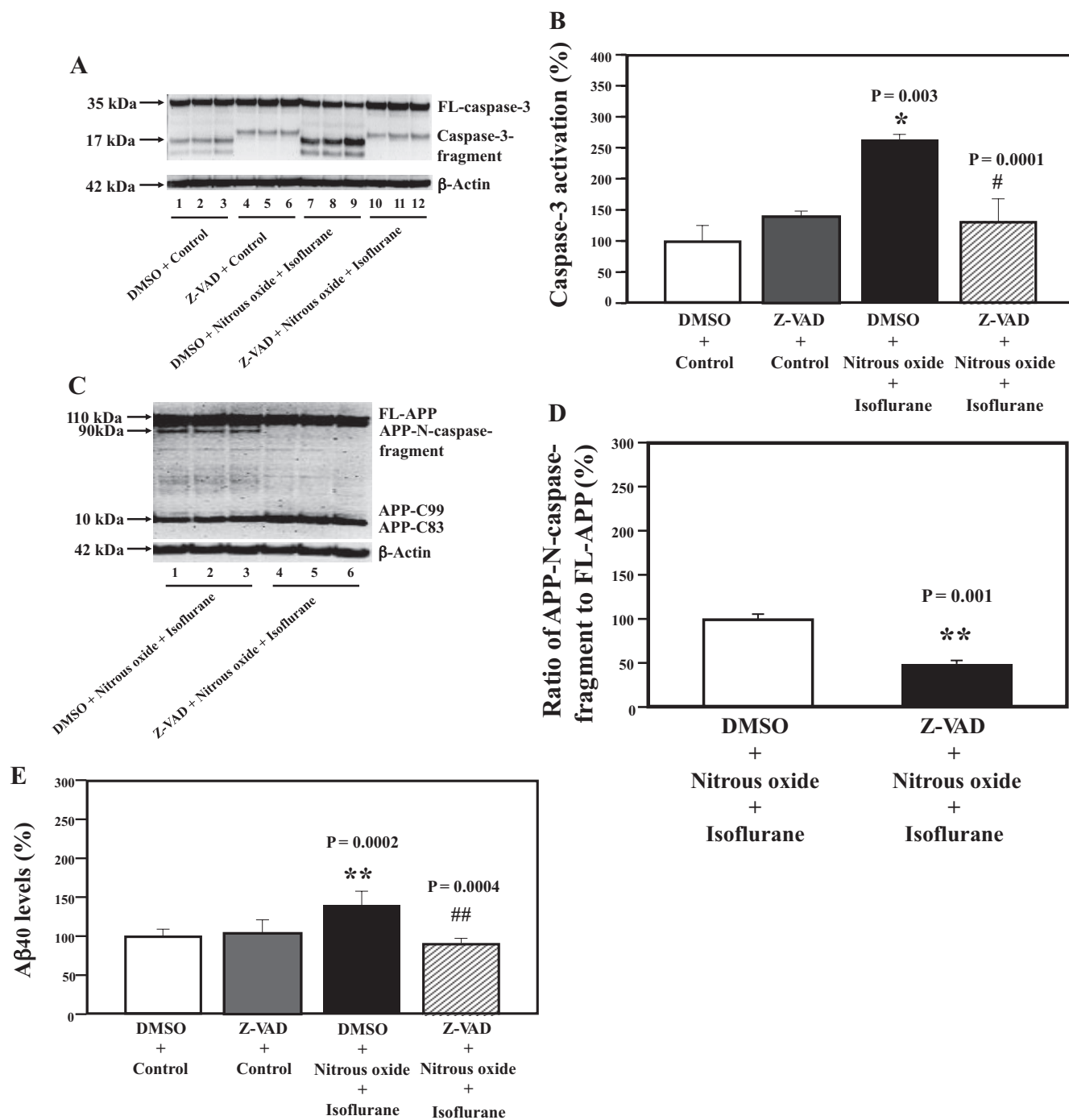
Fig. 6. Caspase inhibitor Z-VAD attenuates the nitrous oxide plus isoflurane–induced caspase-3 activation and Aβ generation in H4-APP cells. (*A*) Effects of Z-VAD on the nitrous oxide plus isoflurane–induced caspase-3 activation in H4-APP cells. Each band in the Western blot represents an independent experiment. (*B*) Quantification of the Western blot, normalized to β-actin levels. We have averaged results from three independent experiments. (*C*) Nitrous oxide plus isoflurane (*lanes 1–3*) increases levels of APP-N-caspase fragment, which is attenuated by Z-VAD treatment (*lanes 4–6*) in H4-APP cells. Each band in the Western blot represents an independent experiment. (*D*) Quantification of the Western blot shows that Z-VAD (*black bar*) decreases nitrous oxide plus isoflurane–induced increases in the ratio of APP-N-caspase-fragment to FL-APP, as compared with nitrous oxide plus isoflurane treatment (*white bar*), normalized to β-actin levels. We have averaged results from three independent experiments. (*E*) Z-VAD (*net bar*) reduces the nitrous oxide plus isoflurane–induced increases in Aβ40 levels in H4-APP cells. We have averaged results from six independent experiments. APP = amyloid precursor protein; DMSO = dimethyl sulfoxide; FL = full length. * $P < 0.05$; ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$.

veloping rodent brain. Moreover, simultaneous use of NMDA receptor antagonists and GABA receptor agonists can induce a greater degree of apoptosis.[56–58] Nitrous oxide is a NMDA receptor antagonist,[59] and isoflurane is a GABA receptor agonist.[60–62] Therefore, we have postulated that the NMDA receptor antagonist nitrous oxide (70%) and the GABA receptor agonist isoflurane (1%) can have a synergistic effect, leading to apoptosis, whereas 70%
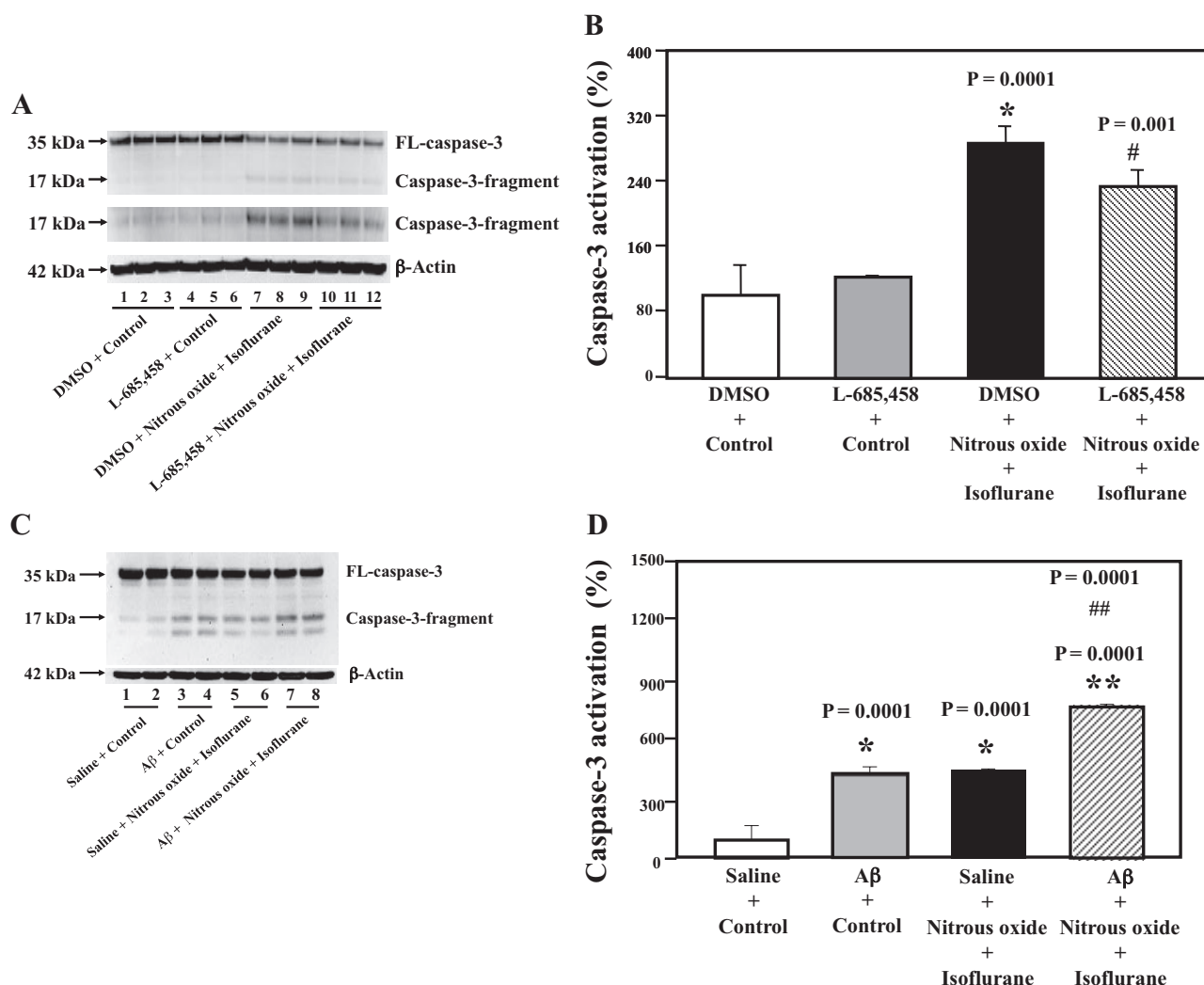
Fig. 7. γ-Secretase inhibitor L-685,458 attenuates, but Aβ potentiates, the nitrous oxide plus isoflurane–induced caspase-3 activation. (*A*) Effects of L-685,458 on the nitrous oxide plus isoflurane–induced caspase-3 activation. Each band in the Western blot represents an independent experiment. (*B*) Quantification of the Western blot shows that L-685,458 treatment (*net bar*) attenuates the nitrous oxide plus isoflurane–induced caspase-3 activation, normalized to β-actin levels. We have averaged results from three independent experiments. (*C*) Effects of Aβ on the nitrous oxide plus isoflurane–induced caspase-3 activation. Each band in the Western blot represents an independent experiment. (*D*) Quantification of the Western blot shows that Aβ treatment potentiates the nitrous oxide plus isoflurane–induced caspase-3 activation. We have averaged results from three independent experiments. Aβ = β-amyloid protein; DMSO = dimethyl sulfoxide; FL = full length. * $P < 0.05$; ** $P < 0.01$; # $P < 0.05$.

nitrous oxide or 1% isoflurane alone are not potent enough to induce the apoptosis. Previous studies have suggested that a treatment with 2% isoflurane for 6 h may induce apoptosis by elevating cytosolic calcium levels.[24] Collectively, we have hypothesized that low-concentration isoflurane (*e.g.*, 1%) may act as GABA receptor agonist, together with an NMDA receptor antagonist (*e.g.*, 70% nitrous oxide), to induce apoptosis, whereas high-concentration isoflurane (*e.g.*, 2%) alone will be able to induce apoptosis *via* enhancing cytosolic calcium levels. Future studies are needed to test this hypothesis by systemically assessing the effects of nitrous oxide and/or isoflurane, as well as other NMDA receptor antagonists and GABA agonists, alone or in combination, on caspase activation and apoptosis.

To further explore the mechanisms by which nitrous oxide plus isoflurane induces apoptosis, affects APP pro-

cessing, and increases Aβ levels, we assessed the effects of nitrous oxide plus isoflurane on levels of BACE, the enzyme that cleaves APP to generate APP-C99, leading to Aβ generation. We have found that treatment of nitrous oxide plus isoflurane can increase levels of BACE. Early studies have shown that caspase activation and apoptosis, induced by isoflurane *in vitro*[42] and *in vivo*,[7] sevoflurane,[53] desflurane plus hypoxia,[54] and ischemia[9] can increase BACE levels, leading to Aβ generation. Nitrous oxide plus isoflurane has been shown to induce caspase activation and apoptosis to increase levels of BACE and Aβ in the present experiments. Thus it is likely that treatment with nitrous oxide plus isoflurane can cause apoptosis, which then increases levels and activity of BACE to facilitate APP processing, finally leading to an increase in Aβ levels.

The mechanism by which nitrous oxide plus isoflurane increases BACE levels is still unknown. Recent studies have suggested that isoflurane[7] and ischemia[9] can induce caspase activation and apoptosis, which subsequently cause reductions in protein levels of Golgi-localized γ-ear–containing adenonisine disphosphate-ribosylation factor binding protein-3, a protein that can facilitate trafficking and metabolism of BACE, ultimately leading to increases in levels of BACE and activity of β-secretase. Future studies should include assessing whether the treatment with nitrous oxide plus isoflurane might likewise reduce levels of Golgi-localized γ-ear–containing adenonisine disphosphate-ribosylation factor binding protein-3 to enhance BACE levels and β-secretase activity subsequent to caspase activation and apoptosis *in vitro* and *in vivo*.

Z-VAD, a broad caspase activation inhibitor, has been shown to attenuate not only the nitrous oxide plus isoflurane–induced caspase-3 activation, but also the nitrous oxide plus isoflurane–induced APP processing and Aβ generation. Moreover, the same treatment of nitrous oxide plus isoflurane can induce caspase-3 activation and apoptosis without detectable changes in APP processing and Aβ levels in H4 naïve cells and primary neurons from naïve mice. Collectively, these results suggest that the nitrous oxide plus isoflurane–induced alterations in APP processing and Aβ generation are largely dependent on the ability of nitrous oxide plus isoflurane to induce caspase activation and apoptosis.

Finally, we assessed whether increases and reductions in Aβ levels can potentiate and attenuate, respectively, nitrous oxide plus isoflurane–induced caspase-3 activation. We have found that L-685,458, a γ-secretase inhibitor that reduces Aβ generation, can inhibit caspase-3 activation. Conversely, treatment with Aβ can potentiate nitrous oxide plus isoflurane-induced caspase-3 activation. Taken together, these results further suggest that nitrous oxide plus isoflurane can induce apoptosis, which then facilitates APP processing, leading to increases in Aβ generation. The increased Aβ levels can potentiate nitrous oxide plus isoflurane–induced apoptosis to form another round of apoptosis and Aβ generation.

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

In conclusion, we have found that treatment with 70% nitrous oxide plus 1% isoflurane, but neither alone, can induce apoptosis, facilitate APP processing, and increase Aβ generation. Figure 8 shows a scheme in which the treatment of nitrous oxide plus isoflurane induces apoptosis, which leads to increases in BACE and Aβ levels. In-
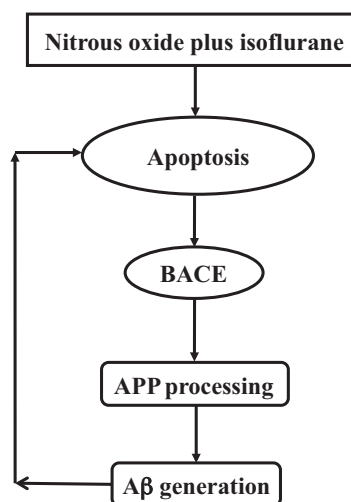

Fig. 8. Hypothetical pathway by which nitrous oxide plus isoflurane induces apoptosis and Aβ generation. Nitrous oxide plus isoflurane induces apoptosis. Apoptosis, in turn, increases BACE levels, which serves to facilitate APP processing and increase Aβ generation. Elevated Aβ generation then further induces apoptosis, leading to another round of nitrous oxide plus isoflurane–induced apoptosis and Aβ generation. Aβ = β-amyloid protein; APP = amyloid precursor protein; BACE = β-site amyloid precursor protein-cleaving enzyme.

creased Aβ levels would then induce further apoptosis, resulting in another round of apoptosis and Aβ generation. Further investigation, especially *in vivo* studies, will be necessary to assess the potential role of nitrous oxide and/or isoflurane in triggering or driving neurotoxicity, including AD neuropathogenesis. These efforts should promote more attempts to assess the effects of anesthetics on AD neuropathogenesis *in vitro* and *in vivo*. The findings from all of these studies will ultimately lead to safer and better anesthesia care for patients, especially elderly patients and AD patients.

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