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The Potential Dual Effects of Anesthetic Isoflurane on Hypoxia-Induced Caspase-3 Activation and Increases in β -Site Amyloid Precursor Protein-Cleaving Enzyme Levels

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Abstract

Background— β -amyloid protein ($A\beta$) accumulation, caspase activation, apoptosis, and hypoxia-induced neurotoxicity have been suggested to be involved in Alzheimer disease neuropathogenesis. $A\beta$ is produced from amyloid precursor protein (APP) through proteolytic processing by the aspartyl protease β -site APP-cleaving enzyme (BACE) and γ -secretase. Inhaled anesthetics have long been considered to protect against neurotoxicity. However, recent studies have suggested that the inhaled anesthetic isoflurane may promote neurotoxicity by inducing caspase activation and apoptosis, and by increasing levels of BACE and $A\beta$. We therefore sought to determine whether isoflurane can induce concentration-dependent dual effects on hypoxia-induced caspase-3 activation and increases in BACE levels: protection versus promotion.

Methods—H4 human neuroglioma cells were treated with hypoxia (3% O_2) alone, different concentrations of isoflurane (0.5% and 2%), and the combination of hypoxia and 0.5% or 2% isoflurane. The levels of caspase-3 cleavage (activation), BACE, and Bcl-2 were determined by Western blot analysis.

Results—Here we show for the first time that treatment with 0.5% isoflurane for eight hours attenuated, whereas treatment with 2% isoflurane for eight hours enhanced, hypoxia-induced caspase-3 activation and increases in BACE levels. The 2% isoflurane treatment also enhanced a hypoxia-induced decrease in Bcl-2 levels.

Conclusions—These results suggest a potential concept that isoflurane has dual effects (protection versus promotion) on hypoxia-induced toxicity, which may act through Bcl-2 family proteins. These findings could lead to more systematic studies to determine the potential dual effects of anesthetics on Alzheimer disease-associated neurotoxicity.

Introduction

Alzheimer disease (AD), an insidious and progressive neurodegenerative disorder, is characterized by global cognitive decline, and robust accumulation of amyloid deposits and neurofibrillary tangles in the brain.(1) Genetic evidence, confirmed by neuropathological and biochemical findings, indicates that excessive production and/or accumulation of β -amyloid protein ($A\beta$) play a fundamental role in the pathology of AD.(2,3) $A\beta$ is produced from amyloid precursor protein (APP) through proteolytic processing by the aspartyl protease β -site APP-cleaving enzyme (BACE) and γ -secretase.(4) Moreover, increasing evidence suggests a role for caspase activation and apoptosis in AD neuropathogenesis.(5,6) Finally, hypoxia and cerebral ischemia have been reported to play roles in neurodegenerative diseases, including AD.(7–13) Therefore, the overarching goal is to determine whether anesthetics can affect hypoxia-induced AD neuropathogenesis, e.g., caspase activation. We started these studies with H4 human neuroglioma cells (H4 naïve cells) because these cells are more physiologically relevant without the artificial over-expression of human APP.

Recent studies have suggested that isoflurane, a commonly used inhaled anesthetic, can induce caspase activation and apoptosis, affect APP processing, increase $A\beta$ levels, and enhance $A\beta$ aggregation.(14–21) However, other reports have suggested that isoflurane protects against apoptosis (22–32). Specifically, isoflurane has been shown to attenuate hypoxia-induced neurotoxicity *in vitro* and *in vivo* (33–35). The reason for these different effects of isoflurane is currently unknown.

Bcl-2 protein family members, including antiapoptotic protein Bcl-2 and proapoptotic protein Bax, can regulate apoptosis by modulating outer mitochondrial membrane permeability (36,37). Other studies showed that a short duration of isoflurane treatment increases Bcl-2 levels in rats (23), whereas a long duration of isoflurane treatment can decrease Bcl-2 levels in cultured cells (15).

Given these observations, we sought to determine if different concentrations of isoflurane may have differential effects on hypoxia-induced caspase-3 activation, changes in the levels of BACE, Bax, and Bcl-2 in H4 naïve cells. We observed the dual effects of isoflurane (promotion versus protection) on hypoxia-induced caspase-3 activation and increases in BACE levels.

Materials and Methods

Cell lines

We used H4 human neuroglioma cells (H4 naïve cells) in the current experiments. The cells were cultured in Dulbecco's modified Eagle medium (high glucose) containing 9% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine.

Cell treatments

The cell treatments were similar to those in our previous studies (16,17,38). We chose 0.5% isoflurane (air component: 0.5% isoflurane, 5% CO₂, 21% O₂, and balanced nitrogen) as the low, and 2% isoflurane (air component: 2% isoflurane, 5% CO₂, 21% O₂ and balanced nitrogen) as the high concentration isoflurane treatments, respectively. The control condition included 5% CO₂ plus 21% O₂ (air component: 5% CO₂, 21% O₂ and balanced nitrogen), which did not affect caspase-3 activation or BACE levels (data not shown). The cells were treated with hypoxia (3% O₂, air component: 5% CO₂, 3% O₂, and balanced nitrogen) plus the control condition, 0.5%, or 2% isoflurane for eight hours. The delivery of gases was similar to that described in our previous studies (16,17). Briefly, 3 or 21% O₂, 5% CO₂, and 0.5 or 2% isoflurane were delivered from an anesthesia machine to a sealed plastic box (airtight chamber) in a 37 degree C incubator containing six-well plates seeded with one million cells in 1.5 ml cell culture media. The Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) was used to continuously monitor the delivered CO₂, O₂, and isoflurane concentrations.

Lysis of cells and protein amount quantification

The pellets of the cells 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 pepstatin A). The lysates were collected, centrifuged at 12,000 × g for 10 min, and quantified for total proteins using the bicinchoninic acid protein assay kit (Pierce, Iselin, NJ).

Western blot analysis

The cells were harvested at the end of the experiments and were subjected to Western blot analyses as described by Xie et al. (17). A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Inc. Beverly, MA) was used to recognize the caspase-3 fragment (17–20 kDa), which results from cleavage at the aspartate position 175, and caspase-3 FL (35–40 kDa). BACE antibody (1:1,000 dilution; Abcam, Cambridge, MA) was used to recognize BACE (65 kDa). Bcl-2 antibody (1:1,000 dilution; Cell Signaling Technology, Inc.) was used to recognize Bcl-2 (28 kDa). Antibody to the nontargeted protein β-Actin (42 kDa, 1:5,000, Sigma, St. Louis, MO) was used to control for loading differences in total protein amounts. Each band in Western blot represents an independent experiment. We averaged the results from 3–6 independent experiments. The intensity of signals in each Western blot was analyzed using the National Institute of Health image program (National Institute of Health Image 1.62, Bethesda, MD). We quantified Western blots using two steps. First, we used levels of β-Actin to normalize (e.g., determine the ratio of the amount of full length (FL)-caspase-3 to the amount of β-Actin) the levels of BACE, Bcl-2, and caspase-3 to control for any loading differences in total protein amounts. Second, we presented changes in the levels of BACE, Bcl-2, and caspase-3 in the treated cells as percentages of those in cells from the control condition.

Statistics

Given the presence of background caspase-3 activation, BACE and Bcl-2 levels in the cells cultured in serum-free media, we did not use absolute values to describe changes in caspase-3 activation, BACE, and Bcl-2 levels. Instead, these changes were presented as a percentage of those of the control group. One hundred percent levels of caspase-3 activation, BACE, or Bcl-2 refer to control levels for the purpose of comparison to experimental conditions. Data were expressed as mean + S.D. The number of samples varied from 3–6, and the samples were normally distributed. We used a two-tailed t-test to determine the effects of 0.5% isoflurane, 2% isoflurane or 3% O₂ alone on caspase-3 activation, and levels

of BACE and Bcl-2, as well as to compare the effects of 0.5% isoflurane or 2% isoflurane on hypoxia (3% O₂)-induced caspase-3 activation, increases in BACE levels, and decreases in Bcl-2 levels. P-values less than 0.05 (* or #) and 0.01 (** or ##) were considered statistically significant.

Results

Low concentration isoflurane treatment attenuates hypoxia-induced caspase-3 activation

We previously reported that the common inhaled anesthetic isoflurane can induce caspase activation and apoptosis *in vitro* (16,17,38) and *in vivo* (18). However, many other studies have shown that isoflurane may protect against apoptosis (22–31). One of the reasons for this discrepancy could be that treatments with different concentrations of isoflurane may have different effects on caspase activation. Thus, we sought to determine the effects of low (0.5%) and high (2%) concentrations of isoflurane on hypoxia-induced caspase-3 activation in the same cell line, i.e., H4 naïve cells.

H4 naïve cells were treated with 3% O₂ plus 0.5% isoflurane for eight hours. The cells were harvested at the end of the experiment and were subjected to Western blot analysis. Caspase-3 immunoblotting revealed that hypoxia (3% O₂) induced caspase-3 activation (Figure 1A) as evidenced by increased ratios of cleaved (activated) caspase-3 fragment (17 kDa) to FL (35–40 kDa) caspase-3. Treatment with 0.5% isoflurane for eight hours alone induced caspase-3 activation as well, but the same 0.5% isoflurane treatment attenuated hypoxia-induced caspase-3 activation (Figure 1A). Quantification of Western blots, based on the ratio of caspase-3 fragment to FL caspase-3, revealed that hypoxia led to caspase-3 activation as compared to the control condition: 100% versus 286% (P = 0.00004). Treatment with 0.5% isoflurane for eight hours also induced caspase-3 activation: 100% versus 212%, P = 0.0008. However, the combination of hypoxia and treatment of 0.5% isoflurane for eight hours led to an attenuation in caspase-3 activation as compared to hypoxia alone: 166% versus 286%, P = 0.007. These findings suggest that low concentration isoflurane may mitigate hypoxia-induced caspase-3 activation. The combination of hypoxia and treatment of 0.5% isoflurane for eight hours also led to an attenuation in caspase-3 activation as compared to 0.5% isoflurane treatment alone: 166% versus 212%. Thus, it is possible that the hypoxia condition can attenuate isoflurane-induced caspase-3 activation. Thus, we next asked whether a high concentration of isoflurane can, like low concentration isoflurane, attenuate hypoxia-induced caspase-3 activation.

High concentration isoflurane treatment enhances hypoxia-induced caspase-3 activation

We treated H4 naïve cells with 3% O₂ plus 2% isoflurane for eight hours. The cells were harvested at the end of the experiment and were subjected to Western blot analysis. We were able to show that both hypoxia (3% O₂) and treatment with 2% isoflurane for eight hours induced caspase-3 activation (Figure 2A). However, treatment with 2% isoflurane for eight hours, different from treatment with low (0.5%) concentration isoflurane, enhanced hypoxia-induced caspase-3 activation (Figure 2A). Quantification of Western blots, based on the ratio of caspase-3 fragment to FL caspase-3, revealed that the hypoxia condition led to caspase-3 activation as compared to the control condition: 100% versus 288% (P = 0.003). Treatment with 2% isoflurane for eight hours also induced caspase-3 activation: 100% versus 385%, P = 0.0008. However, the combination of hypoxia and treatment with 2% isoflurane for eight hours led to an enhancement of caspase-3 activation as compared to hypoxia alone: 477% versus 288%, P = 0.015, or 2% isoflurane treatment alone: 477% versus 385%. These findings suggest that hypoxia does not always attenuate isoflurane-induced caspase-3 activation. Furthermore, these findings suggest that high concentrations of

isoflurane may promote hypoxia-induced caspase-3 activation, whereas low concentrations of isoflurane may attenuate it.

Low concentration isoflurane treatment attenuates hypoxia-induced increases in BACE levels

Our early studies have suggested that isoflurane-induced caspase-3 activation may lead to increases in BACE levels (17). Other studies have shown that hypoxia and ischemia can increase BACE levels (39). Therefore, we asked whether isoflurane could also have dual effects on hypoxia-induced increases in BACE levels.

H4 naïve cells were treated with 3% O₂ plus 0.5% for eight hours. The cells were harvested at the end of the experiment and were subjected to Western blot analysis. As can be seen in Figure 3A, both the hypoxia condition (3% O₂) and treatment with 0.5% isoflurane for eight hours increased BACE levels as compared to the control condition: 100% versus 313%, $P = 0.0026$, 100% versus 207%, $P = 0.011$. The 0.5% isoflurane treatment attenuated hypoxia-induced increases in BACE levels: 313% versus 119%, $P = 0.003$. These findings suggest that the low concentration isoflurane treatment is able to mitigate hypoxia-induced increases in BACE levels.

High concentration isoflurane treatment enhances the hypoxia-induced increases in BACE levels

Finally, we found that both hypoxia (100% versus 327%) and treatment with 2% isoflurane for eight hours (100% versus 262%) enhanced BACE levels (Figure 4A and 4B). The 2% isoflurane treatment slightly enhanced hypoxia-induced increases in BACE levels: 378% versus 327%. Taken together, these findings suggest that isoflurane may have dual effects on hypoxia-induced caspase activation as well as hypoxia-induced increases in BACE levels.

High concentration isoflurane treatment enhances hypoxia-induced decrease in Bcl-2 levels

Given that isoflurane may induce dual effects, either promoting or protecting the hypoxia-induced caspase-3 activation and elevation of BACE levels, we next investigated the potential underlying molecular mechanisms. Bcl-2 protein family members, including Bcl-2, the antiapoptotic protein, and Bax, the proapoptotic protein, can regulate apoptosis by modulating outer mitochondrial membrane permeability (36,37). Therefore, we sought to determine the effects of hypoxia or hypoxia plus isoflurane on the levels of Bax and Bcl-2.

Bcl-2 immunoblotting revealed that hypoxia (3% O₂) reduced levels of Bcl-2 as compared to the control condition (Figure 6A). Treatment of 2% isoflurane for eight hours enhances hypoxia-induced reduction of Bcl-2 levels (Figure 6A). Quantification of Western blot (Figure 6B) showed that hypoxia decreased Bcl-2 levels: 100% versus 67%, $P = 0.037$, and 2% isoflurane treatment plus hypoxia further decreased Bcl-2 levels: 67% versus 33%, $P = 0.002$. These results have illustrated that high concentration isoflurane may enhance hypoxia-induced reduction of Bcl-2 levels. Interestingly, hypoxia alone did not change the Bax levels (data not shown), and 0.5% isoflurane did not alter the hypoxia-induced reduction of Bcl-2 levels (Figure 5). Taken together, these findings suggest that high concentrations of isoflurane may enhance hypoxia-induced caspase-3 activation and increases in BACE levels through reducing the levels of Bcl-2 protein. At this time, the underlying mechanisms of low concentrations of isoflurane attenuate hypoxia-induced caspase-3 activation and increases in BACE levels remain to be determined.

Discussion

There have been different reports regarding the effects of the common inhaled anesthetic isoflurane on neurotoxicity. Many studies have suggested that isoflurane can protect against apoptosis (22–32). Specifically, isoflurane has been shown to mitigate hypoxia-induced neurotoxicity *in vitro* and *in vivo* (33–35). However, recent studies have suggested that isoflurane can induce caspase activation and apoptosis, affect APP processing, increase A β levels, and enhance A β aggregation.(14–21) This difference could be due to different isoflurane treatments, e.g., different concentrations. We therefore compared the effects of high versus low concentration isoflurane treatment on hypoxia-induced caspase-3 activation, increases in BACE levels, and reduction in Bcl-2 levels.

We have found that treatment with 0.5% isoflurane or hypoxia (3% O₂) for eight hours can induce caspase-3 activation and increase BACE levels (Figure 1 and 3). The combination of 0.5% isoflurane treatment and hypoxia leads to an attenuation of caspase-3 activation (Figure 1) and of increases in BACE levels (Figure 3). These findings suggest that low concentrations of isoflurane may mitigate hypoxia-induced caspase-3 activation or the hypoxia condition may attenuate isoflurane-induced caspase-3 activation. However, the findings that the combination of 2% isoflurane treatment and hypoxia for eight hours leads to a greater degree of caspase-3 activation and increases in BACE levels suggest that hypoxia may not attenuate isoflurane's effects, rather, isoflurane can induce a dual effect on hypoxia-induced neurotoxicity. Specifically, low concentrations of isoflurane mitigate but high concentrations of isoflurane promote hypoxia-induced caspase-3 activation and increases in BACE levels.

Our findings are supported by other studies. Wei et al. (40) reported that treatment with isoflurane for one hour inhibited cell death induced by treatment with isoflurane for 24 hours in a dose-dependent manner. These findings suggest that short duration isoflurane treatment may attenuate long duration isoflurane treatment-induced neurotoxicity (protection effects) (40). In another study, Lee et al. (33) showed that isoflurane provided postconditioning effects on ischemia *in vitro* and *in vivo*. Specifically, treatments with 2% isoflurane for 20 or 30 minutes mitigated oxygen-glucose deprivation-induced cell injury. However, the postconditioning effects of isoflurane disappeared when the isoflurane treatment time was increased to 60 minutes [Figure 3A in (33)]. The protective effects of isoflurane are also concentration-dependent. Treatments with 1.5% and 2.0% isoflurane for 30 minutes provided postconditioning effects, but these postconditioning effects disappeared when the isoflurane concentration was increased to 2.5% and 3.0% [Figure 3B in (33)]. These results suggest that isoflurane may have concentration- and duration-dependent dual effects (promotion and protection) on anesthesia- or oxygen-glucose deprivation-induced cell toxicity, which supports our findings that low concentrations of isoflurane attenuate (protection) but high concentrations of isoflurane enhance (promotion) hypoxia-induced caspase-3 activation and increases in BACE levels. Moreover, our current results are consistent with our recent findings that isoflurane has a dual effect (promotion and protection) on A β -induced caspase-3 activation and apoptosis (41).

It has been reported that Bcl-2 protein family members, including Bcl-2 (antiapoptotic protein) and Bax (proapoptotic protein), can regulate apoptosis by modulating outer mitochondrial membrane permeability (36,37). In our current study, we first found that hypoxia can decrease levels of antiapoptotic protein Bcl-2, and then treatment with 2% isoflurane for eight hours enhanced hypoxia-induced reduction of Bcl-2 levels (Figure 6). These findings suggest that isoflurane may affect the hypoxia effects on cell injury via the Bcl-2 protein family members. Interestingly, the hypoxia condition in the current experiments did not change Bax levels, and low concentration isoflurane did not affect the

hypoxia-induced reduction in Bcl-2 levels (Figure 5). These could be due to the specific type of cells used in the experiments. Our previous studies (41,42) illustrated that it could be easier to detect the effects of isoflurane on Bax and Bcl-2 levels in the primary neurons than in H4 cells. Thus, future studies could include assessing the potential dual effects of isoflurane in primary neurons.

The mechanisms by which hypoxia and isoflurane affect the levels of Bcl-2 are largely unknown. Recent studies by Zhang et al. (42) have shown that isoflurane can change mRNA levels of Bcl-2 and Bax. Therefore, it is possible that hypoxia and isoflurane may regulate generation of Bcl-2 by affecting gene expression of Bcl-2. Future studies could include assessment of the effects of hypoxia and isoflurane on gene expression of Bcl-2, as well as other Bcl-2 protein family members in vitro and in vivo. Our previous studies have shown that treatments with 2% isoflurane for six hours (17), 4.1% sevoflurane for six hours (43), 12% desflurane plus hypoxia for six hours (44), and 70% nitrous oxide plus 1% isoflurane for six hours (45) all can induce caspase-3 activation, which then increases the levels of BACE. The enhanced BACE levels will promote APP processing to increase A β generation. Collectively, findings from our current study suggested that hypoxia may decrease levels of antiapoptotic factor Bcl-2 to induce caspase-3 activation, which increases BACE levels, leading to the promotion of APP processing and A β generation. Moreover, treatment with a high concentration of isoflurane (e.g., 2%) can augment the hypoxic effects on Bcl-2 levels, leading to the enhancement of hypoxic effects on caspase-3 activation and BACE levels. It has been postulated that low concentrations of isoflurane (e.g., 0.5%) may mitigate hypoxic effects on Bcl-2 levels, consequently attenuate hypoxic effects on caspase-3 activation and the increase of BACE levels. More studies are needed to further test this hypothesis.

We chose to use H4 naïve cells because these cells are more physiologically relevant without the artificial over-expression of human APP. However, it is technically difficult to detect APP processing and A β levels in these nonhuman APP over-expression cells, as described in our previous studies (17). Therefore, one limitation in our current study is that we were unable to directly assess the effects of hypoxia with and without isoflurane on APP processing and A β generation. However, increases of BACE levels strongly suggest the promotion of APP processing and A β generation (17,46). Many studies have shown that hypoxia promotes APP processing and A β generation by enhancing BACE levels (39,47–51). Taken together, our findings that a high concentration of isoflurane enhances, but a low concentration of isoflurane mitigates, the hypoxic effects in enhancing BACE levels suggest that high concentrations of isoflurane enhance, but low concentrations of isoflurane mitigate, hypoxic effects in promoting APP processing and A β generation. Our future studies will include in vivo relevance studies in primary (cortical/hippocampal) cultured neurons, wild type mice, and AD transgenic mice (which have higher A β levels for a relatively easy measurement of A β) to further assess the potential dual effects of isoflurane, as well as other anesthetics (e.g., sevoflurane), on hypoxia-induced caspase activation, and increases in BACE levels, APP processing, and A β accumulation.

Another limitation in our current study is that we only investigated the potential dual effects of isoflurane on caspase-3 activation and apoptosis in H4 naïve cells. It is possible that isoflurane may not show such dual effects in other cell lines or with other insults. Finally, we did not investigate the effects of isoflurane treatment with different durations (e.g., one versus eight hours) on hypoxia-induced caspase-3 activation and increases in BACE levels. The changes in caspase-3 activation, levels of BACE, and Bcl-2 were limited to prolonged exposure (8 hours) with a high concentration (2%) of isoflurane. It is conceivable that short exposure of hypoxia and anesthetics may not lead to caspase-3 activation, and increases in BACE levels. The results from our current study only demonstrated that isoflurane may have dual effects on hypoxia-induced neurotoxicity. Future studies should include an

assessment of time-dependent effects of anesthetics and hypoxia on AD neuropathogenesis. Indeed, the findings from our previous studies showed that treatment with 0.5% isoflurane for 30 minutes did not induce caspase-3 activation (41). Whereas the results from the current experiments suggest that treatment with 0.5% isoflurane for eight hours induced caspase-3 activation. Collectively, these findings illustrate that isoflurane's effects on cellular toxicity could be time dependent.

We performed the current experiments to embark on a series of studies to demonstrate the potential dual effects of anesthetics and their underlying mechanisms. Our future studies will include measurement of caspase-3 activation, cell viability, cellular apoptosis, cytosol calcium levels, and mitochondrial activity (e.g., mitochondrial permeability transition pore) after different treatments with anesthetics. Future studies should also determine whether these effects can be mitigated by inhibitors of caspase-3 (e.g., Z-VAD) and other inhibitors of mitochondrial pathways of apoptosis.

In conclusion, we found that different concentrations of isoflurane treatments could have different effects on hypoxia-induced neurotoxicity. Specifically, a low concentration isoflurane treatment may protect against hypoxia-induced caspase-3 activation and increases in BACE levels, whereas high concentration isoflurane treatment may promote hypoxia-induced caspase-3 activation and increases in BACE levels. Furthermore, isoflurane may enhance hypoxia-induced caspase-3 activation and increases in BACE levels (AD neuropathogenesis) via reduction in Bcl-2 levels. These findings should facilitate future studies to determine whether inhaled anesthetics may induce neurotoxic and neuroprotective effects depending on different concentrations, which may eventually lead to safer anesthesia care for patients, especially senior and AD patients.

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Figure 1. Low concentration isoflurane treatment attenuates hypoxia-induced caspase-3 activation in H4 naïve cells

A. Hypoxia treatment (lanes 7 to 9) induces caspase-3 cleavage (activation) by increasing caspase-3 fragment levels and decreasing full length (FL)-caspase-3 levels as compared to the control condition (lanes 1 to 3) in Western blots. Treatment with 0.5% isoflurane for 8 hours (lanes 4 to 6) induces caspase-3 activation as compared to the control condition (lanes 1 to 3), but 0.5% isoflurane treatment attenuates hypoxia-induced caspase-3 activation (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blots shows that both isoflurane treatment (gray bar, ** P = 0.008) and hypoxia (black bar, ** P = 0.0004) induce caspase-3 activation, assessed by quantifying the ratio of caspase-3 fragment to FL-caspase-3, as compared to that of the control condition (white bar). Isoflurane 0.5% (striped bar, ## P = 0.007) attenuates hypoxia-induced caspase-3 activation as compared to hypoxia alone (black bar).

A **B**

Figure 2. High concentration isoflurane treatment enhances hypoxia-induced caspase-3 activation in H4 naïve cells

A. Hypoxia (lanes 7 to 9) induces caspase-3 activation as compared to control condition (lanes 1 to 3) in Western blots analysis. Treatment with 2% isoflurane for eight hours (lanes 4 to 6) induces caspase-3 activation as compared to control condition (lanes 1 to 3), and enhances hypoxia-induced caspase-3 activation (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blots shows that both 2% isoflurane (gray bar, ** P = 0.0008) and hypoxia (black bar, ** P = 0.003) induce caspase-3 activation as compared to control condition (white bar). Isoflurane 2% (striped bar, # P = 0.015) enhances hypoxia-induced caspase-3 activation as compared to hypoxia alone (black bar).

Figure 3. Low concentration isoflurane treatment attenuates hypoxia-induced elevation of BACE levels in H4 naïve cells

A. Hypoxia treatment (lanes 7 to 9) increases levels of BACE as compared to the control condition (lanes 1 to 3) in Western blots. Treatment with 0.5% isoflurane for 8 hours (lanes 4 to 6) increases levels of BACE as compared to the control condition (lanes 1 to 3), but 0.5% isoflurane treatment attenuates hypoxia-induced elevation of BACE levels (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blots shows that both 0.5% isoflurane treatment (gray bar, * $P = 0.011$) and hypoxia (black bar, ** $P = 0.0026$) increase BACE levels as compared to the control condition (white bar). Isoflurane 0.5% (striped bar, ### $P = 0.003$) attenuates hypoxia-induced elevation of BACE levels as compared to hypoxia alone (black bar).

A **B**

Figure 4. High concentration isoflurane treatment enhances hypoxia-induced elevation of BACE levels in H4 naïve cells

A. Hypoxia (lanes 7 to 9) increases BACE levels as compared to control condition (lanes 1 to 3) in Western blot analysis. Treatment with 2% isoflurane for eight hours (lanes 4 to 6) increases BACE levels as compared to control condition (lanes 1 to 3), and enhances hypoxia-induced elevation of BACE levels (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blots shows that both 2% isoflurane (gray bar, * $P = 0.02$) and hypoxia (black bar, * $P = 0.04$) increase BACE levels as compared to control condition (white bar). Two percent isoflurane (striped bar, * $P = 0.011$) enhances hypoxia-induced elevation of BACE levels as compared to hypoxia alone (black bar).

A **B**

Figure 5. Low concentration isoflurane treatment does not enhance hypoxia-induced decreases in the Bcl-2 levels in H4 naïve cells

A. Hypoxia (lanes 7 to 9) decreases Bcl-2 levels as compared to the control condition (lanes 1 to 3) in Western blots. Treatment with 0.5% isoflurane for eight does not enhance hypoxia-induced reduction of Bcl-2 levels (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blot shows that hypoxia (black bar, ** P = 0.007) decreases levels of Bcl-2 as compared to control condition (white bar). Isoflurane 0.5% does not enhance hypoxia-induced reduction of Bcl-2 levels (striped bar, P = 0.135, N.S.).

A **B**

Figure 6. High concentration isoflurane treatment potentiates hypoxia-induced decreases in the Bcl-2 levels in H4 naïve cells

A. Hypoxia (lanes 7 to 9) decreases Bcl-2 levels as compared to the control condition (lanes 1 to 3) in Western blots. Treatment with 2% isoflurane for eight hours enhances hypoxia-induced reduction of Bcl-2 levels (lanes 10 to 12) as compared to hypoxia alone (lanes 7 to 9). **B.** Quantification of Western blot shows that hypoxia (black bar, * P = 0.037) decreases levels of Bcl-2 as compared to control condition (white bar). Two % isoflurane treatment enhances hypoxia-induced reduction of Bcl-2 levels (striped bar, ## P = 0.002) as compared to hypoxia alone (black bar).