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# The Effect of Endoplasmic Reticulum Stress on Neurotoxicity Caused by Inhaled Anesthetics

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**BACKGROUND:** The mechanisms by which inhaled anesthetics cause neurotoxicity are not well clarified. Exposure to inhaled anesthetics induces a release of $Ca^{2+}$ from the endoplasmic reticulum (ER) into the cytosol. Aberrant $Ca^{2+}$ mobilization may alter the protein-folding environment in the ER, causing ER stress. Binding immunoglobulin protein (BiP) is an ER chaperone that is critical to ER functions. Because ER stress leads to cellular dysfunction and apoptotic cell death, leading to diverse human disorders such as neurodegenerative diseases, we hypothesized ER stress may play a role in neurotoxicity caused by inhaled anesthetics.

**METHODS:** We investigated the relationship between ER stress and neurodegeneration caused by inhaled anesthetics by using knock-in mice expressing a mutant BiP and neuronal culture cells. Neuronal culture cells and mutant BiP pregnant mice were exposed to 3% sevoflurane. The levels of BiP and C/EBP homologous protein (CHOP), a transcription factor related to cell death during ER stress, were evaluated by Western blot in neuronal cells and fetal brains delivered by cesarean delivery. Cell death in the fetal brains was evaluated with TUNEL staining. Statistical significance was assessed using unpaired *t* test and analysis of variance followed by multiple comparison tests.

**RESULTS:** Sevoflurane exposure enhanced the expression of BiP and CHOP significantly in neuronal culture cells. A chemical chaperone that assisted ER functions reduced the expression of CHOP induced by sevoflurane exposure. In an in vivo study, we observed that an enhanced expression of CHOP and significantly more apoptotic cells in the brains of homozygous mutant BiP fetuses compared with the wild type. Mouse embryonic fibroblasts derived from the mutant BiP mice also exhibited enhanced levels of CHOP and cleaved caspase-3 after sevoflurane exposure.

**CONCLUSIONS:** Sevoflurane exposure may cause ER stress, which is tolerated to some extent in wild-type cells. When this tolerance is limited, like in cells with mutant BiP, the exposure leads to cell death in the brain, suggesting that ER stress may partially mediate neurotoxicity caused by inhaled anesthetics. This study suggests that patients with certain conditions sensitive to ER stress such as ischemia, hypoxia, developing brain, or neurodegenerative diseases may be vulnerable to inhaled anesthetics. (Anesth Analg 2013;117:1197–204)

General anesthetics have been widely used in patient care, assuming that the effects of anesthetics are transient and reversible. However, growing findings suggest that general anesthetics may induce permanent damage in several types of cells including neurons, especially in the developing brain.[1–4] Neuronal injury or neuronal cell death in the developing brain may lead to cognitive dysfunctions later in life.[5] The mechanisms by which inhaled anesthetics injure neurons are not well known, though recent studies have suggested that it is mediated in part by the dysregulation of intracellular $Ca^{2+}$ homeostasis.[6]

Cytosolic $Ca^{2+}$ plays an important role as an intracellular messenger. Excessive cytosolic $Ca^{2+}$ activates calpain and caspases, resulting in cell death.[7] The endoplasmic reticulum (ER) is a major $Ca^{2+}$ pool in most cells. $Ca^{2+}$ is released from the ER into the cytosol through the inositol 1, 4, 5-trisphosphate receptor (IP3R) and the ryanodine receptor on the ER membrane, while the extracellular $Ca^{2+}$ enters the cytosol through the voltage-dependent calcium channel and the *N*-methyl-D-aspartatic acid glutamate receptor on the plasma membrane.[8,9] Inhaled anesthetics like isoflurane have been reported to enhance the cytosolic $Ca^{2+}$ oscillations by activating γ-aminobutyric acid A receptors.[10] Inhaled anesthetics also activate the IP3Rs and ryanodine receptors, inducing $Ca^{2+}$ release from the ER.[11] Aberrant $Ca^{2+}$ mobilization may alter the protein-folding environment in the ER, leading to ER stress.

The ER provides a folding environment for newly synthesized secretory and membrane proteins.[12] Those proteins are synthesized on the ER membrane and translocated into the ER, where they are subjected to quality control through interactions with molecular chaperones such as binding immunoglobulin protein (BiP), which function as intermediaries for protein folding or degradation.[12] Insults such as ischemia, hypoxia, and toxic substances cause aberrant protein folding and an accumulation of misfolded proteins in the ER. This condition initiates the unfolded protein response (UPR), which enhances the capacity for ER quality control by reducing general protein synthesis, up-regulating ER chaperones, and promoting ER-associated protein degradation.[13,14] Further insults beyond these adaptive

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mechanisms can cause cellular dysfunction and death, resulting in diverse human disorders[15–17] such as neurodegenerative diseases, diabetes, cardiomyopathy,[18] morphine tolerance,[19] and renal diseases.[20]

BiP, one of the most abundant ER chaperones, plays a critical role in ER functions, assisting in protein translocation, folding, degradation, and regulation of the UPR.[21] Since complete depletion of BiP is lethal in early embryonic cells,[22] we used a previously generated mutant BiP knock-in mouse where the retrieval of BiP from post-ER compartments is impaired.[23] BiP is usually retained within the ER by association with other ER proteins, though it is sometimes secreted to post-ER compartments. When wild-type BiP is secreted from the ER, the carboxyl-terminal Lys-Asp-Glu-Leu (KDEL) sequence of BiP is recognized by the KDEL receptor on the Golgi, thereby facilitating the retrieval of BiP to the ER.[24,25] This process is disrupted in our knock-in mice, priming these mice for ER stress. Using culture cells and tissues from these mice, we examined the relationship between ER stress and neurotoxicity caused by inhaled anesthetics.

## METHODS

### Animals

All animal experimental procedures were performed in accordance with a protocol approved by the Institutional Animal Care Committee of Chiba University, Chiba, Japan. The study was conducted in a manner that does not inflict unnecessary pain or discomfort on the animal, as outlined by the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (1996), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

In a previous study, we generated knock-in mice expressing a mutant BiP lacking the carboxyl-terminal KDEL sequence.[23] The missing KDEL sequence was replaced by a hemagglutinin (HA) tag. The heterozygous mutant BiP mice were maintained over 10 generations while backcrossing to C57BL/6 mice. Mutant BiP mice (20–25 g body weight, 12- to 14-week-old) were used in this study. All mice were provided with food and water ad libitum before the experiments.

### Cells and Reagents

Mouse embryonic fibroblasts (MEFs) were prepared from 13.5-day-old embryos.[23] MEFs and the murine neuroblastoma cell line, neuro2a, were grown in a complete medium that consisted of Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., Irvine, United Kingdom) with 10% fetal bovine serum, 2 mM glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin G at 37°C in a 5% $CO_2$ incubator.

The following antibodies were used: mouse mAb 15E6 against the hemagglutinin epitope (a kind gift from VW Hsu, Harvard Medical School, Boston, MA), mouse mAb against γ-tubulin (Sigma Chemical), mouse mAb SPA-827 against BiP (KDEL sequence) (Stressgen, Victoria, Canada), rabbit antiserum against CHOP/GADD153 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antiserum against cleaved caspase-3 (Cell signaling technology, Danvers, MA), Cy-3-conjugated donkey antibody against mouse immunoglobulin G, and Cy-2-conjugated donkey antibody against rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA). The following reagents were used: Hoechst 33258 (Life Technologies, Grand Island, NY) and tauroursodeoxycholic acid (TUDCA, Calbiochem, San Diego, CA).

### Immunofluorescence Microscopy

Cells on coverslips were fixed in cold methanol for 10 minutes at −20°C and then processed as previously described.[26] The stained cells were examined by fluorescence microscopy (Axiovert 200M, Carl Zeiss, Oberkochen, Germany). Immunolocalization was observed by a fluorescence microscope using FITC/rhodamine filters with a Plan-Neofluar 20x NA 0.50 and a Plan-Neofluar 40x NA 0.75 objectives. Immunofluorescence images were captured with a digital camera (AxioCam MRm, Carl Zeiss, Oberkochen, Germany). The brightness and contrast were optimized by AxioVision 4.4 software (Carl Zeiss), and the mean grey values of the cells with the background subtraction were used for densitometry.

### Sevoflurane Exposure

#### *Cell Cultures*

Neuro2a cells and MEFs were placed in an acrylic resin chamber and exposed to 3% sevoflurane, delivered from a calibrated vaporizer for 2.5 to 7.5 hours, in a gas mixture of 5% $CO_2$, 40% $O_2$ balanced with air at a flow rate of 1 L/min. The concentration of sevoflurane was monitored continuously by an anesthetic agent monitor (Atom 303, Atom, Tokyo, Japan). The temperature was kept at 37°C with a heater (T/Pump, Gaymar, NY). Control cells were exposed to a gas mixture of 5% $CO_2$, 40% $O_2$ balanced with air in the same way.

#### *Mice Treatment*

Heterozygous mutant BiP pregnant mice with embryos at embryonic day 17.5 (E17.5) were randomly assigned to an anesthesia or control group. We chose 3% sevoflurane treatment in this study, since previous studies suggested that 3.0% sevoflurane was almost equivalent to 0.6 minimum alveolar concentration (MAC) for neonatal mice and caused significant neuronal damage.[5,27] Mice assigned to the anesthesia group ($n = 6$) received 3% sevoflurane in 40% oxygen, balanced with air for 3 hours in an anesthetizing chamber at a flow rate of 1 L/min. The control mice ($n = 7$) received 40% oxygen in the same way. The mice breathed spontaneously, and anesthetic and oxygen concentrations were measured continuously (Datex-Ohmeda RGM 5250, Louisville, CO). Skin temperature was measured intermittently (CODA Monitor; Kent Scientific Corporation, Torrington, CT), and the temperature of the anesthetizing chamber was controlled to maintain skin temperature of the animals at 37°C. Twenty-four hours after sevoflurane exposure, the pregnant mice were deeply anesthetized by peritoneal injection of pentobarbital (Nembutal, Dainippon Sumitomo Pharma, Osaka, Japan), and the E18.5 embryos were removed by cesarean delivery. The fetuses were anesthetized with pentobarbital through the maternal mice.

### TUNEL Staining

The fetal brains (total; $n = 30$, $n = 5$ in each group) were isolated, and apoptotic cells were visualized by terminal

deoxynucleotidetransferase-mediated dUTP nick end-labeling (TUNEL) assay based on the manufacturer's protocol (Roche, Basel, Switzerland) as previously described.[18] TUNEL staining was observed with a microscope using a N-Achroplan 40x NA 0.65 objective (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). The brightness and contrast were optimized by AxioVision Rel.4.7 software (Carl Zeiss), and images were captured with a digital camera (AxioCam MRc, Carl Zeiss).

### Western Blotting

The fetal brains were homogenized by sonication (UR-20P, TOMY, Tokyo, Japan) in a buffer containing 0.4% (w/v) Nonidet P-40, 0.2% N-lauroylsarcosine, 30 mM Tris/HCl pH 8.0, 1 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 30 μg/mL N-acetyl-L-leucinal-L-lecinal-L-norleucinal (ALLN, Sigma Chemical). Cultured cells were washed twice with ice-cold phosphate buffered saline and then homogenized in the same buffer. The lysates were centrifuged, the supernatants were resuspended in SDS-PAGE sample buffer, and then separated by SDS-PAGE under reducing conditions. Western blotting was done as previously described.[18] Imaging was obtained by LAS-1000 and Image Gauge software (Fuji Photo Film Co. Ltd., Tokyo, Japan). Densitometry was performed using LAS-1000 and ImageJ software (Wayne Rasband, NIH, Bethesda, MD).

### siRNA

siRNA oligonucleotides for BiP (sc-45064) and control (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For siRNA uptake, neuro2a cells were transfected with siRNA oligonucleotide according to manufacturer's instructions. At 24 hours after transfection, the medium was changed to a complete medium, and cells were exposed to sevoflurane.

### Statistical Analysis

The statistical analyses (KaleidaGraph Version 3.6, Reading, PA) are specified in the figure legends. Statistical significance was accepted at $P < 0.01$.

## RESULTS

### Exposure to Sevoflurane Leads to Enhanced Levels of ER Chaperones

To evaluate whether inhaled anesthetics affect ER functions, we first tested the effects of sevoflurane on neuronal cultured cells. Neuro2a cells were exposed to 3% sevoflurane with 40% $O_2$ and 5% $CO_2$ at 37°C for 5 hours, followed by incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours. Control cells were exposed to a gas mixture of 5% $CO_2$, 40% $O_2$ balanced with air in the same way. Those cells were fixed and double stained with a monoclonal anti-KDEL antibody to detect ER chaperones and with Hoechst 33342 for nuclear staining. The anti-KDEL antibody recognizes BiP as well as other KDEL-containing ER chaperone proteins, such as GRP94. Immunofluorescence microscopy with densitometry revealed that the expression of ER chaperones was significantly enhanced after exposure to sevoflurane, suggesting that sevoflurane exposure causes ER stress (Fig. 1).

We then examined whether persistent sevoflurane exposure might cause more ER stress on neuronal cultured cells.

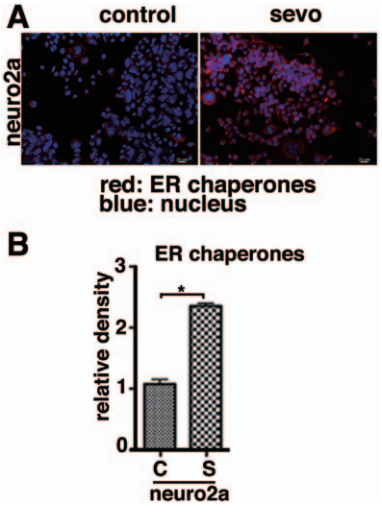


**Figure 1.** The levels of endoplasmic reticulum chaperones were enhanced in neuronal cells after sevoflurane exposure. After the exposure, neuro2a cells were fixed and double-stained with a monoclonal anti-KDEL antibody to detect endoplasmic reticulum (ER) chaperones and with Hoechst 33342 for nuclear staining. Scale bars represent 20 μm (A). Immunofluorescence microscopy with densitometry revealed that the levels of ER chaperones were significantly enhanced after exposure. The densitometry was done with 10 cells in each group. C = control, S = sevoflurane. Data are shown as mean + SEM (B). To compare values between the 2 groups, unpaired *t* test was used. Corrected **P* < 0.0001.

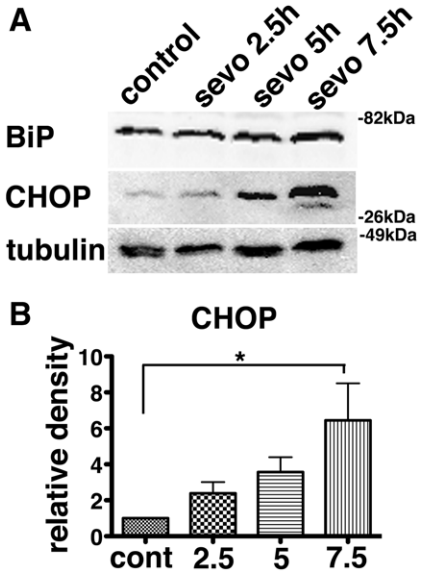


**Figure 2.** Sevoflurane exposure may enhance the levels of CHOP in neuro2a cells. Neuro2a cells were exposed to 3% sevoflurane with 40% $O_2$ and 5% $CO_2$ at 37°C for 2.5, 5 or 7.5 hours, followed by incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours. The cells were collected and subjected to Western blot analysis (A). Densitometry revealed that sevoflurane exposure for 7.5 hours enhanced the expression of CHOP in neuro2a cells. The expression of CHOP was normalized to that of γ-tubulin. The results of densitometry are shown as the average of 3 separate identically treated groups + SEM (B). To compare values between groups, 1-way ANOVA and the Dunnett's multiple comparison tests were used. Corrected **P* =0.0316. CHOP = C/EBP homologous protein; BiP = binding immunoglobulin protein.

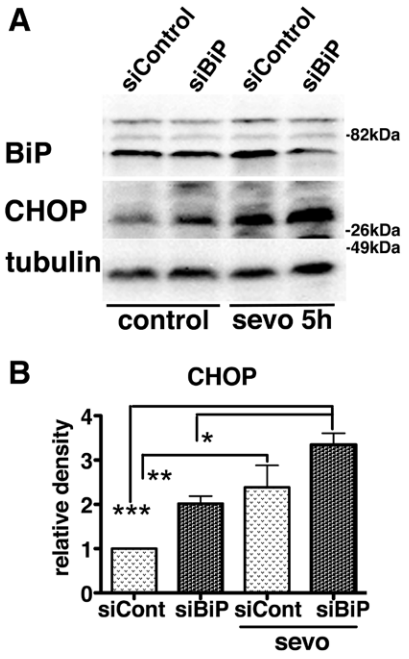


**Figure 3.** Knockdown of binding immunoglobulin protein enhanced the expression of CHOP in neuro2a cells after sevoflurane exposure. Neuro2a cells with siRNA for binding immunoglobulin protein (BiP) or control were exposed to 3% sevoflurane (sevo 5 hours) or not (control) with 40% $O_2$ and 5% $CO_2$ at 37°C for 5 hours, followed by incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours. The cells were collected and subjected to Western blot analysis with an anti-KDEL mouse mAb and an anti-CHOP rabbit antiserum (A). The results of densitometry are shown as the average of 3 separate identically treated groups + SEM (B). To compare values between groups, 1-way ANOVA followed by the Tukey's multiple comparison tests was used. Corrected $*P$ =0.05, $**P$ =0.0414, $***P$ =0.0021. CHOP = C/EBP homologous protein.

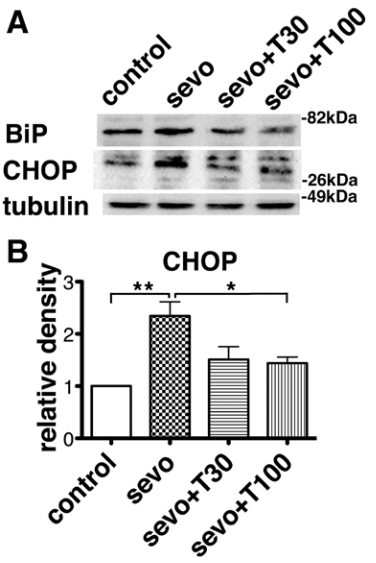


**Figure 4.** A chemical chaperone reduced the levels of CHOP in neuro2a cells after sevoflurane exposure. Neuro2a cells were exposed to 3% sevoflurane with 40% $O_2$ and 5% $CO_2$ at 37°C with or without TUDCA (T30; 30, T100; 100 μg/mL) for 5 hours, followed by incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours. Cells were collected and subjected to Western blot analysis (A). Densitometry revealed that sevoflurane exposure significantly induced expression of CHOP and TUDCA may have reduced the expression of CHOP in neuro2a cells. The expression of CHOP was normalized to that of γ-tubulin. The results of densitometry are shown as the average of 3 separate identically treated groups + SEM (B). To compare values between groups, 1-way ANOVA followed by the Tukey's multiple comparison tests was used. Corrected $*P$ =0.0416, $**P$ =0.0049. CHOP = C/EBP homologous protein; BiP = binding immunoglobulin protein.

Neuro2a cells were exposed to 3% sevoflurane for 2.5, 5, or 7.5 hours. The cells were collected and subjected to Western blot analysis (Fig. 2). Sevoflurane exposure for 7.5 hours may enhance expression of C/EBP homologous protein (CHOP), a transcription factor related to apoptotic cell death during ER stress, on neuro2a cells. Thus, the effects of sevoflurane exposure would be time dependent.

To examine the relationship between ER stress and the toxicity of sevoflurane exposure, we used siRNA for BiP to reduce the chaperone expression and evaluated the effect of ER stress on neurotoxicity by sevoflurane exposure in neuro2a cells (Fig. 3). siRNA for BiP may reduce the expression of BiP in neuro2a cells, and sevoflurane exposure may cause ER stress. Indeed, the expression of CHOP was enhanced in cells with siRNA for BiP and sevoflurane exposure. BiP expression may play a role in protective effects against sevoflurane exposure.

We then used a chemical chaperone. TUDCA, a derivative of an endogenous bile acid, is thought to increase ER folding capacity.[28,29] Neuro2a cells were exposed to 3% sevoflurane with or without TUDCA (30, 100 μg/mL, Fig. 4). Sevoflurane exposure enhanced expression of CHOP on neuro2a cells, while co-incubation with TUDCA may have reduced expression. Thus, the chemical chaperone TUDCA may suppress the toxicity of sevoflurane exposure, suggesting a mechanistic relationship between ER chaperones and neuronal damage due to inhaled anesthetics.

## Sevoflurane Exposure Causes Cell Death in the Mutant Bip Fetal Brains

We next examined the effect of sevoflurane exposure on neuronal cells in vivo. The homozygous mutant BiP neonates have defects in alveolar type II epithelial cells and die from respiratory failure.[23] Thus, we exposed mutant BiP pregnant mice to sevoflurane. Heterozygous female mutant BiP mice were mated with heterozygous male mutant BiP mice. Pregnant mice with embryos at day E17.5 were exposed to 3% sevoflurane with 40% $O_2$ for 3 hours. Twenty-four hours later, the pregnant mice were anesthetized by pentobarbital, and E18.5 embryos were removed by cesarean delivery to assay the effects of sevoflurane exposure on developing neurons harvested from the littermates with different genotypes under the same physiological conditions. We found significantly more apoptotic cells with TUNEL staining in the homozygous mutant BiP brains after sevoflurane exposure compared with the wild-type brains (Fig. 5). Western blotting revealed that sevoflurane exposure enhanced the levels of CHOP as well as mutant BiP in the homozygous fetal mutant brain (Fig. 6). In the heterozygous mutant brains, sevoflurane exposure enhanced the expression of BiP but resulted in less induction of CHOP compared with homozygous mutants.

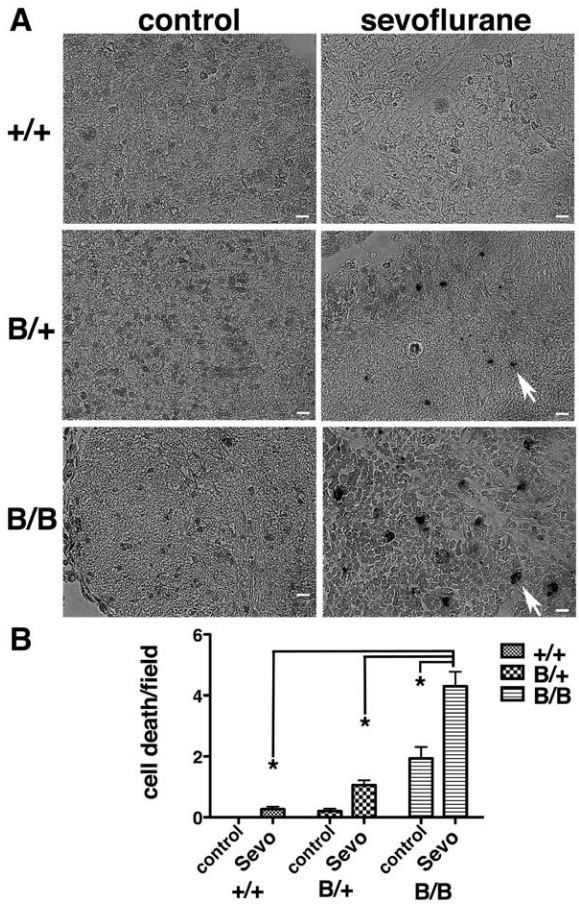


**Figure 5.** Sevoflurane exposure caused cell death in the homozygous mutant brain. Pregnant mutant mice at embryonic day 17.5 (E17.5) were exposed to 3% sevoflurane with 40% $O_2$ for 3 hours (Sevo, $n = 6$). Control pregnant mutant mice received 40% oxygen in the same way (control, $n = 7$). Twenty-four hours later, the pregnant mice were anesthetized by pentobarbital, and the E18.5 embryos were removed by cesarean delivery (+/+, wild-type; B/+, heterozygous; B/B, homozygous). The brains were removed from the fetuses and subjected to histochemistry with TUNEL staining for detection of neuronal cell death in the CA1 region of hippocampus (white arrow heads). Scale bars represent 10 μm (A). Sevoflurane (Sevo) exposure significantly induced apoptotic cell death in the homozygous mutant binding immunoglobulin protein (BiP) brain. We analyzed 30 fetal brains (5 fetuses in each group; wild-type control, wild-type Sevo, heterozygous control, heterozygous Sevo, homozygous control, and homozygous Sevo group). Twenty-five fields were examined in each group (5 fields in each fetus, 5 fetuses in each group). We took 1 homozygous, 1 heterozygous mutant BiP, and 1 wild-type fetus from each pregnant mouse for TUNEL analysis. Data are shown as mean + SEM (B). To compare values between groups, 1-way ANOVA followed by the Tukey's multiple comparison tests was used. Corrected $*P < 0.0001$.

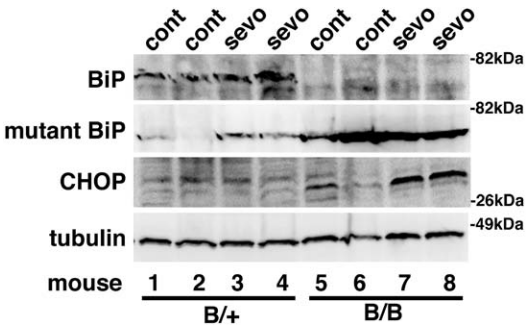


**Figure 6.** Sevoflurane exposure enhanced the levels of CHOP in the homozygous mutant brain. Pregnant binding immunoglobulin protein (BiP) mutant mice with embryos at embryonic day 17.5 (E17.5) were exposed to 3% sevoflurane with 40% $O_2$ for 3 hours (sevo) or exposed to 40% $O_2$ for 3 hours (cont). Twenty-four hours later, the pregnant mice were anesthetized by pentobarbital, and the E18.5 embryos (B/+; heterozygous, B/B; homozygous) were delivered by cesarean delivery. The brains were removed from the fetuses and subjected to Western blot analysis with an anti-KDEL mouse mAb for BiP, an anti-HA mouse mAb for mutant BiP and an anti-CHOP rabbit antiserum. Each lane represents a distinct mouse. Mice 1, 2, 5, 6 were littermates born from pregnant mice exposed to 40% $O_2$. Mice 3, 4, 7, 8 were littermates born from pregnant mice exposed to 3% sevoflurane. CHOP = C/EBP homologous protein.

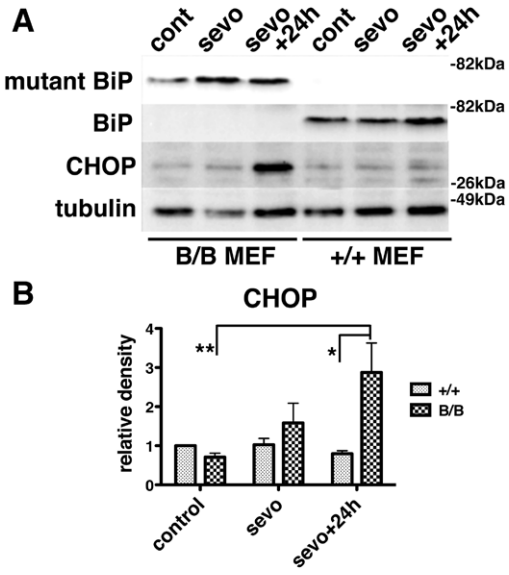


**Figure 7.** Sevoflurane exposure enhanced the levels of CHOP in mutant binding immunoglobulin protein (BiP) mouse embryonic fibroblasts (MEFs). Wild-type MEFs and BiP MEFs were exposed to 3% sevoflurane with 40% $O_2$ and 5% $CO_2$ at 37°C for 5 hours (sevo), followed by an incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours (sevo + 24 hours). Control groups were exposed to 40% $O_2$ and 5% $CO_2$ at 37°C for 5 hours, followed by incubation with 40% $O_2$ and 5% $CO_2$ at 37°C for 24 hours (cont). Cells were subjected to Western blot analysis with an anti-KDEL mouse mAb for BiP, an anti-HA mouse mAb for mutant BiP and an anti-CHOP rabbit antiserum. The results of densitometry are shown as the average of 4 separate identically treated groups + SEM (B). To compare values among groups, 1-way ANOVA followed by the Dunnett's multiple comparison tests was used. Corrected $*P = 0.0048$, $**P = 0.0033$. CHOP = C/EBP homologous protein.

These results suggest that sevoflurane exposure causes ER stress, leading to cell death in the homozygous mutant BiP fetal brains shown by TUNEL staining, where the compensation by the UPR is limited by the absence of wild-type BiP.

The MEFs derived from mice homozygous for a mutant BiP grew as well as wild-type MEFs.[23] To evaluate the effect of the mutant BiP, the MEFs were exposed to sevoflurane and subjected to Western blot analysis. We found that sevoflurane exposure induced the expression of wild-type BiP or mutant BiP in the wild-type or mutant MEFs, respectively, but only in the mutant MEFs were elevated levels of CHOP detected (Fig. 7). The MEFs were also stained with a monoclonal anti-KDEL antibody and a polyclonal anti-activated caspase-3 antiserum for the detection of cell death. While

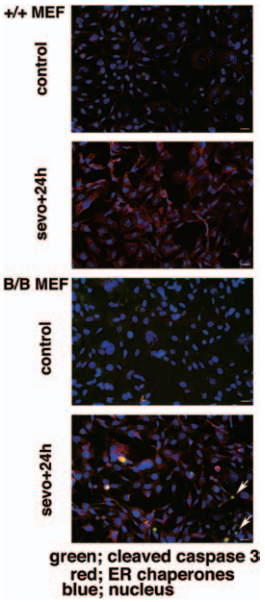


**Figure 8.** The activation of caspase-3 was enhanced in mutant binding immunoglobulin protein (BiP) mouse embryonic fibroblasts (MEFs) after sevoflurane exposure. Twenty-four hours after sevoflurane exposure as described in Figure 7, wild-type MEFs (+/+) and BiP MEFs (B/B) were stained with an anti-KDEL mAb to detect endoplasmic reticulum (ER) chaperones (red) and an anti-activated caspase-3 antiserum for the detection of apoptotic cell death (green). Nucleus was stained with Hoechst 33342 (blue). Scale bars represent 20 μm. Arrows point out cells with positive cleaved caspase-3.

we observed an enhanced expression of ER chaperones in both cells after sevoflurane exposure, activated caspase-3 was seen only in the mutant BiP MEFs, not in the wild-type MEFs (Fig. 8).

These results indicate that sevoflurane exposure causes ER stress. The effect of sevoflurane exposure was tolerated in the wild-type MEFs, while it might have led to cell death in the mutant BiP MEFs, possibly because the mutant BiP MEFs were sensitive to ER stress due to the absence of wild-type BiP.

## DISCUSSION

We found that sevoflurane exposure induced expression of ER chaperones in neuronal culture cells, indicating that sevoflurane exposure causes ER stress. Furthermore, we observed more cell death in the mutant BiP fetal brains than in the wild type after sevoflurane exposure.

ER chaperones are localized to the ER by 2 mechanisms: retention and retrieval.[30] BiP is retained in the ER through interaction with other ER proteins and the ER matrix. When misfolded proteins accumulate in the ER, BiP dissociates from some ER membrane kinases. BiP dissociation activates these kinases and transcription factors and initiates the UPR.[31] When BiP is secreted from the ER along with misfolded proteins,[26,32] the carboxyl-terminal KDEL sequence of BiP is recognized by the KDEL receptor, thereby facilitating the retrieval of BiP from post-ER compartments to the ER.[24,25] We used knock-in mice expressing a mutant BiP lacking the retrieval sequence.[23] This mutant allows us to examine the effects of a defect in ER function without completely eliminating BiP function. Deletion of the retrieval sequence from BiP, and the consequent loss of BiP from the ER, could result in impairment of the folding environment in the ER. Mutant BiP is functional as long as it remains in the ER. Therefore, constitutive activation of the UPR could compensate for the altered folding environment by producing mutant BiP in quantities sufficient for cell survival. As a result, mutant BiP cells and mutant BiP mice would be sensitive to ER stress.

An increase in misfolded proteins in the ER evokes the UPR to cope with the protein overload in the ER. A further accumulation of misfolded proteins despite these responses leads to protein aggregation in the cell, frequently accompanied by cellular dysfunction and death.[15,16] In fact, we observed more apoptotic cells in the brain of the homozygous mutant BiP fetuses compared with the wild type. Thus, sevoflurane exposure causes ER stress, which is tolerated by the UPR in the wild-type cells to some extent; however, it has deleterious effects on the mutant cells where the compensation is limited due to the absence of the wild-type BiP.

Sevoflurane is a frequently used inhaled anesthetic, especially for slow induction procedures in pediatric anesthesia. We used pregnant mice exposed to 3% sevoflurane in this study to evaluate the effects of the anesthetic on fetuses with different genotypes in the BiP allele under the same physiological conditions. Three hours of exposure to sevoflurane might have had a minimal impact on blood flow of the pregnant mice, since wild-type fetuses delivered from the pregnant mice with sevoflurane exposure had few apoptotic cells in this study (Fig. 5), suggesting that the negative effects of sevoflurane on mutant fetuses may act through ER stress. Istaphanous et al.[27] showed that 6-hour exposure to 2.9% sevoflurane in neonatal mice caused significant amounts of neuronal cell death. Kodama et al.[5] also reported that 6-hour exposure to 3% sevoflurane caused neuroapoptosis in neonatal mice, while they showed that 3-hour exposure had fewer effects. Therefore, the effects of sevoflurane exposure should be time- and dose-dependent.[33] Our experiment with neuronal cells showed that sevoflurane exposure for 7.5 hours might enhance the expression of CHOP (Fig. 2).

Our experimental conditions may have some limitations. Exposure times were different for most of the in vitro (5 hours or longer) and in vivo experiments (3 hours). Culture cells like neuro2a cells and embryonic fibroblasts seem to be more resistant to environmental stress including sevoflurane exposure. Fibroblasts are nonneural cells, and neuro2a cells are different in many ways from normal neurons, with respect to signaling characteristics. Since our preliminary experiments showed that pregnant mice did not tolerate exposure to sevoflurane for >3 hours, we exposed pregnant mice for 3 hours in the in vivo experiments. We cannot exclude maternal effects on fetuses during sevoflurane exposure. Physiological conditions during the exposure such as hypotension and hypoxia other than from sevoflurane exposure may also have caused ER stress (Fig. 6).

Our in vivo experiments were performed late in gestation, not during the early postnatal period. This is also an important limitation because we know that sensitivity to anesthetic neurotoxicity is highly developmental age sensitive. Our experimental conditions with sevoflurane exposure seemed to have a significant effect on homozygous

mutant BiP fetuses but a lesser effect on the wild types. Thus, mice with defects in ER stress responses like the mutant BiP mice may be more sensitive to sevoflurane exposure, when mice with a normal ER stress response could tolerate the insult. Some human mental disorders such as neurodegenerative diseases, bipolar disorders, and ischemic neuronal injury involve ER stress. Missense mutations in the human presenilin-1 gene may increase vulnerability to ER stress by altering the UPR signaling pathway, leading to early-onset familial Alzheimer's disease.[34] Kakiuchi et al.[35] identified XBP1, a pivotal gene in the ER stress response, as contributing to the genetic risk factor for bipolar disorder. Tajiri et al.[36] showed that ischemia-induced neuronal cell death was mediated by the ER stress pathway involving CHOP induction. People with these diseases may be especially sensitive to inhaled anesthetics. In fact, sevoflurane anesthesia caused a greater degree of neurotoxicity in the brain tissue of the transgenic mouse model for Alzheimer's disease than in that of the wild-type mouse.[37]

Inhaled anesthetics have been assumed to be safe drugs, even though in certain situations they may be neurotoxic. Neurons in the developing brain and certain neurons with ischemia, hypoxia, or degenerative diseases may be vulnerable to the neurotoxicity of inhaled anesthetics. Liang et al.[38] reported that isoflurane causes greater neurodegeneration than an equivalent exposure to sevoflurane in the developing brain of neonatal mice. Yang et al.[11] revealed that inhaled anesthetics may induce cell damage by causing abnormal calcium release from the ER via excessive activation of IP3 receptors and that isoflurane has greater potency than sevoflurane or desflurane to cause calcium release from the ER and to induce cell damage. However, neonatal desflurane exposure has been reported to induce more robust neuroapoptosis than isoflurane and sevoflurane,[5] while another group reported similar neurotoxicity from isoflurane, sevoflurane, and desflurane.[27] Zhang et al.[39] also showed that propofol may mitigate the caspase-3 activation by attenuating isoflurane-induced Abeta42 oligomerization.

While the mechanisms of inhaled anesthetic-mediated neuronal injury are not fully understood, calcium dysregulation leading to ER stress is one of the possible mechanisms. In this regard, chemical chaperones like TUDCA which increase ER folding capacity[28,29] may be useful to reduce neurotoxicity. A preconditioning procedure that causes a minor insult may induce the UPR, promoting the expression of ER chaperones. This enhanced expression of ER chaperones including BiP would be useful to cease the next insult.

In conclusion, sevoflurane exposure causes ER stress, which is tolerated in wild-type cells. When tolerance is limited as in the BiP mutant, the exposure leads to cell death in the brain, suggesting that ER stress may play a role in neurodegeneration caused by exposure to inhaled anesthetics. This study suggests additional precautions would be taken for patients with certain conditions sensitive to ER stress.

## DISCLOSURES

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**Attestation:** Mari Komita approved the final manuscript. Mari Koimita attests to the integrity of the original data and the analysis reported in this manuscript.
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