but not the IP3 receptor total knock-out cells. Thapsigargin, a time-dependently, and sequentially elevated cytosolic and then rane-evoked changes of calcium concentration in cytosol and mitochondria in both chicken B-lymphocyte wild-type and IP3 receptor total knock-out cells. Isoflurane induced significantly more neurotoxicity and greater calcium release from the ER in L286V PC12 and Q111 Huntingtin striatal cells than in their corresponding wild-type controls, both of which were significantly inhibited by the IP3 receptor antagonist xestospongion C.

Background: Isoflurane induces cell apoptosis by an unknown mechanism. The authors hypothesized that isoflurane activates inositol 1,4,5-trisphosphate (IP3) receptors on the endoplasmic reticulum (ER) membrane, causing excessive calcium release, triggering apoptosis.

Methods: The authors determined isoflurane-induced cytotoxicity by measuring caspase-3 activity, lactate dehydrogenase release, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reduction, and imaging analysis of cell damage markers (annexin V and propidium iodide staining) in different cell types. The authors used the chicken B lymphocyte with a total knock-out of IP3 receptors or on the L286V PC12 cells with elevated IP3 receptor activity (transfected with L286V presenilin 1), striatal cells with a knock-in of Q111 Huntingtin, and each cell line’s corresponding wild-type controls. The authors also measured the isoflurane-evoked changes of calcium concentration in cytosol and/or mitochondria in these cells.

Results: Isoflurane induced apoptosis concentration- and time-dependently, and sequentially elevated cytosolic and then mitochondrial calcium in the chicken B-lymphocyte wild-type but not the IP3 receptor total knock-out cells. Thapsigargin, a calcium adenosine triphosphatase inhibitor on ER membranes, induced apoptosis and elevations of calcium in cytosol and mitochondria in both chicken B-lymphocyte wild-type and IP3 receptor total knock-out cells. Isoflurane induced significantly more neurotoxicity and greater calcium release from the ER in L286V PC12 and Q111 Huntingtin striatal cells than in their corresponding wild-type controls, both of which were significantly inhibited by the IP3 receptor antagonist xestospongion C.

Conclusion: These findings suggest that isoflurane activates the ER membrane IP3 receptor, producing excessive calcium release and triggering apoptosis. Neurons with enhanced IP3 receptor activity, as in certain cases of familial Alzheimer or Huntington disease, may be especially vulnerable to isoflurane cytotoxicity.
current study that isoflurane induces apoptosis by excessive calcium release from ER via overactivation of IP₃ receptor. To test this hypothesis, we studied apoptosis and calcium release from the ER in a variety of cell types with varying IP₃ receptor expression or activity.

Materials and Methods

Cell Cultures

Cells of wild-type (WT) chicken B lymphocyte (DT40) cell line, and its total IP₃ receptor knock-out (DT40 IP₃R TKO) type, were cultured in RPMI 1640 with 10% fetal calf serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 4 mm L-glutamine, and antibiotics in a 95% air, 5% CO₂ humidified atmosphere at 38°C as previously described.²⁶ Rat pheochromocytoma (PC12) cells transfected with WT presenilin 1 (PS1) or point mutated PS1 (L286V) were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, 200 μg/ml G418, and antibiotics in a 95% air, 5% CO₂ humidified atmosphere at 37°C as described.¹,²⁷ The transfection of the WT and mutant PS1 has been described and confirmed in detail previously.²⁸,²⁹

Mouse HD knock-in striatal cells (STHdbQ111/Q111) and their WT control cells (STHdbS77/Q77) were generated and cultured as described previously.³⁰,³¹ Briefly, cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, 400 μg/ml G418, and antibiotics in a 95% air, 5% CO₂ humidified atmosphere at 33°C.

Anesthetic Exposure

DT40 cells (WT and IP₃R TKO), rat PC12 cells (WT and L286V), and mouse striatal cells (WT and HD) were exposed to isoflurane at different concentrations for various durations in a gastight chamber inside the culture incubator (Bellco Glass, Inc., Vineland, NJ), with humidified 5% CO₂–21% O₂–balanced N₂ (AirGas East, Bellmawr, NJ) going through a calibrated agent-specific vaporizer as described previously.¹ Gas phase concentrations in the gas chamber were verified and maintained at the desired concentration throughout the experiments using an infrared Ohmeda 5330 agent monitor (Coast to Coast Medical, Fall River, MA). In a pilot study, the cell medium was aspirated and extracted into hexane for high-performance liquid chromatography measurement (System Gold; Beckman Coulter, Fullerton, CA) to verify that the various anesthetic concentrations in the medium in millimolars are equivalent to the minimal alveolar concentration (MAC) in the gas phase inside the gas chamber using the concentration correlation previously described.³²

Imaging Analysis of Annexin V and Propidium Iodide

Translocation of membrane phospholipid phosphatidylinerine from the inner to the outer leaflet of the plasma membrane is an early indication of cell damage. Annexin V, a phospholipid binding protein with a high affinity for phospholipid phosphatidylserine, can bind to phospholipid phosphatidylserine once it is exposed to the extracellular environment. Propidium iodide (PI) can bind to nucleic acid after penetrating a breached plasma membrane, as occurs in the later stages of cell damage. We treated DT40 cells, grown floating in the medium, with different concentrations of isoflurane (0.6, 1.2, and 2.4%) for 24 h, as well as with 2.4% isoflurane for different times (6, 12, and 24 h). Immediately after treatment, we determined annexin V- or PI–positive cells by the methods described previously.²⁶ Cells were dropped onto 25-mm cover slips and stained with annexin V or PI. The stained cells were visualized and counted by two persons blinded to the treatments. The percentage of annexin V- or PI–positive cells averaged from four areas on each cover slip was then calculated and compared.

Detection of Caspase-3 Activity

Increased caspase-3 activity is a typical marker for apoptosis. The assay is based on the ability of the active enzymes to cleave the fluorogenic substrates Ac-DEVD-AFC (caspase 3; Calbiochem, San Diego, CA) and was performed as per instructions and as described previously.²⁶ DT40 cells grown on six well plates were treated with 2.4% isoflurane for 24 h and then were harvested via trypsinization and washed with phosphate-buffered saline. The cell pellet was gently resuspended in CeLytic M lysis buffer and protease inhibitor cocktail (Sigma, St. Louis, MO), lysed, and centrifuged; the supernatant was used for the assay. Caspase substrates were added to a final concentration of 50 μM, and the samples were incubated at 37°C for 45 min in caspase assay buffer. Incubated samples were measured at an excitation of 400 nm and an emission of 505 nm in a multiwavelength-excitation dual wavelength-emission fluorometer (Delta RAM; photon Technology International, Birmingham, NJ). We determined the caspase-3 activity immediately after treating DT40 cells with 2.4% isoflurane for 24 h.

Cytotoxicity Assays

The inhibition of isoflurane-induced cytotoxicity by xestospongin C, a potent antagonist of IP₃ receptor, was assessed by the lactate dehydrogenase (LDH) release assay in PC12 cells and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reduction assay in mouse striatal cells. LDH release reflects plasma membrane integrity, is a relatively late event in cytotoxicity, and is shared by both apoptosis and necrosis pathways, and its measurement is well described.¹ The MTS reduction assay reflects mito-
chondrial function and is considered a middle event in both apoptosis and necrosis. The tetrazolium compound is bioreduced by normal mitochondria into a colored formazan product measured by absorbance. We followed the standard experimental protocol for MTS reduction assay from Promega (Madison, WI). We treated PC12 and striatal neurons, grown on 24 well plates, with 2.4% isoflurane for 24 h. Immediately (PC12 cells) or 24 h (striatal neurons) after treatment, LDH or MTS assay was performed, respectively. Xestospongin C at 100 nm was used to inhibit IP₃ receptor activity. The results of LDH release and MTS reduction assays were expressed as a percentage of the control without anesthetic treatment.

**Simultaneous Confocal Imaging of Cytosolic and Mitochondrial Ca²⁺**

The method used was the same as described previously.²⁶ DT40 cells (WT and IP₃R TKO) grown on 25-mm cover slips were loaded with 2 μM rhod-2/AM in cell medium containing 2.0% bovine serum albumin in the presence of 0.003% Pluronic acid at 37°C for 50 min. Cells loaded with rhod-2 dye were washed and then reloaded with fluo-4/AM for an additional 30 min at room temperature. Cells were placed on a stage and exposed to 2 MAC (0.7 mM) isoflurane dissolved in the perfusion buffer. The fluorescent measurements were calibrated by bathing cells in the HEPES buffer containing ionomycin 20 nm for maximum or 20 nm EGTA for minimum calcium values. The intracellular calcium concentration ([Ca²⁺]ᵢ) was calculated by the ratio method of Grynkiewicz et al.³⁴ using 224 nm as the Kd of fura-2. The final result of [Ca²⁺]ᵢ was averaged from the cells of at least three separate experiments. We used 0.7 mM (2 MAC) isoflurane to elevate [Ca²⁺]ᵢ in all cells. Xestospongin C at 1 μM was used to inhibit isoflurane-mediated calcium release from the ER.

**Measurement of Cytosolic Calcium Concentration**

Cytosolic calcium concentration was measured using fura-2 fluorescence (Molecular Probes, Eugene, OR) with a photometer coupled to an Olympus IX70 inverted microscope (Olympus America Inc., Center Valley, PA) and IPLab version 3.7 imaging processing and analysis software (Biovision Technologies, Exton, PA). The protocol to determine [Ca²⁺]ᵢ was similar to that previously described, with some modifications.³⁵ Briefly, cells grown on 25-mm round glass cover slips were washed three times with Krebs-Ringer’s buffer without addition of calcium and then loaded with 2.5 μM fura-2/AM (Molecular Probes) for 30 min at room temperature. The cells were then placed in a sealed chamber (Warner Instrument Inc., Hamden, CT) connected with multiple inflow infusion tubes and one outflow tube, which provided constant flow to the chamber. The cells were first washed with Krebs-Ringer’s buffer through one inflow tube for the baseline measurement of [Ca²⁺]ᵢ, and then were exposed to isoflurane via a separate inflow infusion tubes driven by a syringe pump (Braintree Scientific Inc., Braintree, MA). The fluorescence signals were measured with excitation at 340 and 380 alternatively and emission at 510 nm for a period up to 18 min for each treatment. A pilot study confirmed that the cells were still viable at the end of experiments for calcium measurement. The fluorescence measurements were calibrated by bathing cells in the HEPES buffer containing ionomycin 20 nm for maximum or 20 nm EGTA for minimum calcium values. The intracellular calcium concentration ([Ca²⁺]ᵢ) was calculated by the ratio method of Grynkiewicz et al.³⁴ using 224 nm as the Kd of fura-2. The final result of [Ca²⁺]ᵢ was averaged from the cells of at least three separate experiments. We used 0.7 mM (2 MAC) isoflurane to elevate [Ca²⁺]ᵢ in all cells. Xestospongin C at 1 μM was used to inhibit isoflurane-mediated calcium release from the ER.

**Statistics**

We used GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA) and STATA version 7 Software (StataCorp LP, College Station, TX) for all statistical analysis. Annexin V and PI staining, LDH release, and MTS reduction were all expressed as percentage of those in the control. Peak [Ca²⁺]ᵢ in PC12 and HD striatum cells was expressed as a percentage of its own baseline. We analyzed the data with one-way analysis of variance followed by Newman-Keuls multiple comparison tests using GraphPad Prism. We also confirm our analysis of dose response and time response of image analysis of cell damage marker annexin V and PI in DT40 cells with a mixed mode of regression using STATA version 7. A significantly increased odds ratio risk was determined if the estimate for the greater time point or dose exceeded the upper limit of the 95% confidence interval for the lower time point or dose. P < 0.05 was considered statistically significant.

**Results**

**DT40 IP₃R TKO Cells Were Resistant to Isoflurane-induced Apoptosis**

To investigate whether isoflurane induces apoptosis in the absence of IP₃ receptors, we first studied its cytotoxic effects in the DT40 IP₃R TKO cells and compared it with the corresponding WT cells. Isoflurane induced cell damage determined by both annexin V and PI staining were all expressed as percentage of those in the control. Peak [Ca²⁺]ᵢ in PC12 and HD striatum cells was expressed as a percentage of its own baseline. We analyzed the data with one-way analysis of variance followed by Newman-Keuls multiple comparison tests using GraphPad Prism. We also confirm our analysis of dose response and time response of image analysis of cell damage marker annexin V and PI in DT40 cells with a mixed mode of regression using STATA version 7. A significantly increased odds ratio risk was determined if the estimate for the greater time point or dose exceeded the upper limit of the 95% confidence interval for the lower time point or dose. P < 0.05 was considered statistically significant.
Isoflurane-induced Apoptosis Was Associated with Elevation of $[\text{Ca}^{2+}]_c$ and Then $[\text{Ca}^{2+}]_m$ in DT40 WT but Not IP$_3$R TKO Cells

To further examine the hypothesis that isoflurane induced apoptosis by disruption of intracellular calcium homeostasis, we first assayed the cytotoxic effects of thapsigargin, a selective inhibitor of the ER calcium adenosine triphosphatase, which can cause ER calcium to passively leak even without any contribution from IP$_3$ receptors. Thus, in this positive control experiment, thapsigargin (100 nM for 2 h) induced similar apoptosis in both WT and TKO cells (fig. 2B), whereas 2.4% isoflurane for 24 h only induced apoptosis in DT40 WT cells. Isoflurane induced a sequential elevation of $[\text{Ca}^{2+}]_c$ and then mitochondria calcium concentration ($[\text{Ca}^{2+}]_m$) only in DT40 WT but not TKO cells (figs. 2C and D), whereas thapsigargin produced this sequential calcium elevation in both WT and IP$_3$R TKO cells (fig. 2E). These results show that the IP$_3$ receptor knock-out did not diminish the cells response to stress or calcium transients in general, and thereby strengthen the hypothesis that isoflurane induced apoptosis by causing calcium release from the ER via overactivation of IP$_3$ receptors.

Elevated Activity of IP$_3$ Receptors Enhances Isoflurane-induced Apoptosis

The mutation of PS1, a protein located primarily on ER membranes, appears in most cases of familial Alzheimer disease, and is associated with increased expression of ryanodine receptors$^{27}$ and increased activity of IP$_3$ receptors.$^{36,37}$ If isoflurane induces apoptosis by overactivation of IP$_3$ receptor, cells with enhanced expression or activity of this receptor should be more vulnerable to
isoflurane-induced cytotoxicity and exhibit greater calcium release from the ER. Consistent with this hypothesis, isoflurane caused significantly more PI-positive cells in PC12 cells transfected with L286V PS1 than in its WT vector control (figs. 3A and B). To establish the linkage of these cytotoxic effects of isoflurane to IP$_3$ receptors, we co-cultivated cells with xestospongin C, a potent inhibitor of the IP$_3$ receptor.\textsuperscript{40,59} Xestospongin C abolished the cell damage represented by elevated LDH (fig. 3C) and also nearly abolished the calcium release from the ER caused by isoflurane (figs. 3D and E) in L286V PC12 cells. Although xestospongin C may also inhibit calcium adenosine triphosphatase in some cell cultures,\textsuperscript{40} it did not affect adenosine triphosphatase activity in this system because xestospongin C alone did not increase [Ca$^{2+}$]$_i$ in L286V PC12 cells (data not shown).

We also examined isoflurane effects in another model of elevated IP$_3$ receptor activity. Abnormal calcium release from ER via elevated activity of IP$_3$ receptors is thought to play an important role in the neurodegeneration of HD.\textsuperscript{33,34,42} Mutated Huntingtin protein with an enlarged polyglutamine repeat section causes excessive calcium release from the ER upon the activation of IP$_3$ receptors by an agonist.\textsuperscript{23} Therefore, if isoflurane causes apoptosis via activation of IP$_3$ receptors, the elevated activity of IP$_3$ receptors in mouse striatal cells with an overexpression of Q111 Huntingtin should not only render these cells more vulnerable to isoflurane-induced neurotoxicity and calcium release from the ER, but also xestospongin C should inhibit these effects. In accordance with this hypothesis, 2.4% isoflurane for 24 h induced more cell damage (PI-positive cells) in Q111 Huntingtin knock-in than in the corresponding WT control striatal cells (figs. 4A and B), and xestospongin C (100 nM) abolished this cytotoxic effect (fig. 4C). In the absence of extracellular calcium, 2.4% isoflurane (0.7 mM) induced significantly greater elevation of [Ca$^{2+}$]$_c$ (representing calcium release from the ER) in Q111 Huntingtin knock-in than the WT control striatal cells, an effect that was also nearly abolished by pretreatment of xestospongin C (figs. 4D and E).

**Discussion**

Our results are most consistent with the hypothesis that isoflurane, a commonly used inhalational anesthetic, induces cell apoptosis by excessive calcium release from the ER via overactivation of IP$_3$ receptors. First, cells depleted of IP$_3$ receptors were resistant to isoflurane-induced apoptosis and calcium elevation, whereas cells with enhanced activity of the IP$_3$ receptor were more vulnerable to isoflurane-induced apoptosis. Finally, the IP$_3$ receptor antagonist xestospongin C significantly inhibited isoflurane-induced cell damage and calcium elevations in the vulnerable cell models.

Our results suggest a direct interaction between isoflurane and the receptor protein, but cannot prove it. Alternative possibilities are that isoflurane interacts with an associated protein or signaling system, or the ER lipid...
membrane itself. But it has been demonstrated that anesthetics similar to isoflurane can bind specifically to many membrane proteins, so it is plausible that the effect on IP₃ receptors may be the result of a direct interaction.

Consistent with our previous study and others, isoflurane induced apoptosis in DT40 WT cells concentration- and time-dependently. It is important to note that there exists considerable variation in cell sensitivity to isoflurane-induced apoptosis. For example, the minimal time to induce apoptosis in DT40 cells with 2.4% isoflurane was only 6 h, with a minimal concentration of 1.2%. In rat cerebral cortical neurons or PC12 cells, the minimal concentration and time needed to induce apoptosis was 2.4% isoflurane for 24 h. Immortalized striatal neurons also needed a minimal exposure of 2.4% isoflurane for 24 h to induce modest cell damage in this study. In normal human peripheral lymphocytes, only 0.85% isoflurane was needed to induce apoptosis, and it is likely that normal human neurons are similarly sensitive. Although this study suggests that the IP₃ receptor is an important target underlying isoflurane-induced apoptosis, this variability in sensitivity suggests that there may be other targets that contribute, or that downstream effects are considerably different. In addition, the shortest times for 2.4% isoflurane to induce apoptosis in chicken lymphocytes are 6 h (fig. 1E) and 12 h (fig. 1F), respectively, when techniques to detect early-phase (annexin V) apoptosis or late-phase (PI staining, primarily necrosis) cell damage were used. This demonstrates that the detection of vulnerability to isoflurane cytotoxicity will vary depending on the assays used to measure cell damage. Isoflurane may produce only modestly enhanced LDH release in PS1 mutated PC12 cells (fig. 3C) because the LDH method detects severe late cell damage, whereas the cell injury induced by these concentrations of isoflurane must be more subtle, given the lack of overt clinical sequelae. Further studies are needed to

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Fig. 3. The inositol 1,4,5-trisphosphate (IP₃) receptor antagonist xestospongin C (Xc) inhibited isoflurane-induced cytotoxicity and calcium elevations in PC12 cells transfected with L286V presenilin 1. (A) Representative images of phase contrast (PC) or propidium iodide (PI) staining of L286V or wild-type (WT) PC12 cells immediately after treatment of 2.4% isoflurane for 24 h. Scale bar = 100 μm. (B) Quantification of percentage of PI-positive cells (late cell damage) after treating L286V or WT PC12 cells with 2.4% isoflurane for 24 h. L286V PC12 cells were more vulnerable than WT PC12 cells to isoflurane-induced cytotoxicity. Data represent mean ± SE from 25 repeats of three separate experiments. ** or *** P < 0.01 compared with control in L286V or isoflurane treatment in WT, respectively. (C) Pretreatment of 100 nM Xc for 30 min abolished the lactate dehydrogenase (LDH) release (late cell damage) induced by 2.4% isoflurane for 24 h in L286V cells. Data represent mean ± SE from 12 repeats of three separate experiments. * P < 0.05 compared with control. ** P < 0.01 compared with isoflurane treatment alone. (D) Representative tracing reveals averaged isoflurane-evoked elevation of cytosolic calcium concentration ([Ca²⁺]ₗ) with or without pretreatment of Xc in the absence of extracellular calcium in both WT and L286V PC12 cells. (E) Isoflurane at 2.4% induced significantly higher peak elevation of [Ca²⁺]ₗ in the absence of extracellular calcium in L286V than in WT PC12 cells. Xc (1 μM) significantly inhibited isoflurane-induced peak elevation of [Ca²⁺]ₗ in the absence of extracellular calcium in both WT and L286V cells. Data represent mean ± SE from three separate experiments. * or ** P < 0.05 or P < 0.01 compared with isoflurane treatment alone without Xc pretreatment. ## P < 0.01 compared with WT PC12 cells treated with isoflurane alone. ISO = isoflurane; L286V = PC12 cells with presenilin-1 mutation.
**Fig. 4.** Xestospongin C (Xc) inhibited isoflurane-induced cytotoxicity and calcium elevations in mutated Huntington disease (HD) knocked-in striatal cells. (A) Representative images of phase contrast (PC) or propidium iodide (PI) staining of HD or wild-type (WT) striatal cells at 24 h after completion of treating cells with 2.4% isoflurane for 24 h. Scale bar = 100 μm. (B) Isoflurane at 2.4% for 24 h significantly increased percentage of cell damage determined by PI staining in HD knocked-in but not in WT striatal cells. Data represent mean ± SE of 18 repeats from three separate experiments. *P < 0.05 compared with control in HD striatal cells. (C) Pretreatment of 100 nM Xc for 30 min abolished the MTS reduction induced by 2.4% isoflurane for 24 h in HD striatal cells. Data represent mean ± SE from 12 repeats of three separate experiments. *** or ### P < 0.001 compared with control or isoflurane treatment alone. (D) Representative tracing reveals averaged isoflurane-evoked elevation of cytosolic calcium concentration ([Ca^{2+}]_c) with or without pretreatment of Xc in the absence of extracellular calcium in both WT and HD cells. (E) Isoflurane at 2.4% induced significantly higher peak elevation of [Ca^{2+}]_c in the absence of extracellular calcium in HD knocked-in than in WT striatal cells. Xc (1 μM) nearly abolished isoflurane-induced peak elevation of [Ca^{2+}]_c in the absence of extracellular calcium in both WT and HD striatal cells. Data represent mean ± SE from three separate experiments. ** P < 0.01 compared with WT striatal cells treated with isoflurane alone. ISO = isoflurane; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
determine the features that underlie this differing vulnerability to isoflurane-induced toxicity.

Increased calcium release from the ER via the IP$_3$ receptors may contribute to neurodegeneration in other conditions, such as Alzheimer disease and HD. Various mutations of PS1, a protein located primarily in ER membranes, appear in most cases of familial Alzheimer disease. This PS1 mutation has been associated with increased activity of the IP$_3$ receptors, which may render neurons more vulnerable to apoptosis induced by a variety of factors that trigger calcium release (e.g., excitotoxicity). Our results are consistent with this hypothesis in that PC12 with L286V PS1 were more vulnerable to isoflurane cytotoxicity than its WT control, in a xestospongin C-dependent manner. It should be noted that many other PS1 mutations, such as PS1 delta 9, M146V, or just enhanced expression of full-length a xestospongin C–dependent manner. It should be noted that another anesthetic, sevoflurane, did not induce similar neuronal apoptosis as isoflurane at equipotent concentrations, so there is hope that other anesthetic drugs might prove to be less neurotoxic, at least in in vitro studies. Clinical studies are ultimately required to demonstrate whether these cell culture studies have any relevance to humans.

It should be noted that isoflurane has been long considered an agent for cardioprotection and neuroprotection. It is likely that isoflurane is both neurotoxic and neuroprotective, depending on the concentration and duration of exposure, and the degree of patient vulnerability. Isoflurane may be inherently cytotoxic, but it provides cardioprotection or neuroprotection via a preconditioning mechanism, much like hypoxia. Mild calcium release from the ER and moderate elevation of [Ca$^{2+}$], by isoflurane at low concentrations and short duration may trigger the ER stress response, marked by the expression of genes characterizing the well-known “preconditioning” effect. Longer exposures to isoflurane, producing extensive and prolonged calcium release from ER, may deplete ER calcium and shut down protein synthesis, leading to “cytotoxicity” effects. Therefore, like ischemic preconditioning, isoflurane for short durations might provide cytoprotection via preconditioning, whereas prolonged exposures produce cytotoxicity directly. This hypothesis was supported by our recent experiments, which demonstrated that 2.4% isoflurane preconditioning for 1 h abolished neurotoxicity induced by isoflurane itself for 24 h in cerebral cortical neurons. These potential dual features of isoflurane should be considered in future studies, and perhaps in clinical application in vulnerable populations.

This study has several limitations that should influence in vivo interpretations: (1) The cells with total IP$_3$R knock-out are chicken B lymphocytes, not neurons. Although the ER calcium handling mechanisms are thought to be the same, downstream effects of the calcium transients might be considerably different. To our knowledge, there are no immortal neuronal cell lines with IP$_3$R knock-out, but transgenic mice with type I IP$_3$R$^{−/−}$ or type II and III$^{−/−}$ knock-out have been reported, from which cultures of primary neurons could be studied. Expression of IP$_3$ receptors could also be reduced via small interfering RNA in normal neurons. (2) All of the results from this study are from cell lines, which are immortal transformed cells. Although clearly different from normal cells, in general, such cells are more resistant to stressors, making the results of our study somewhat more relevant to the in vivo situation. Future in vivo studies are needed to investigate whether IP$_3$R also plays a role in isoflurane-mediated neurotoxicity in animals or patients.

Taken together, our findings suggest that the commonly used inhalational anesthetic isoflurane may induce cell apoptosis by triggering abnormal calcium release from the ER via activation of IP$_3$ receptors. Preexisting genetic features may render some populations at increased risk from this effect.

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