

Inhalational Anesthetics Induce Cell Damage by Disruption of Intracellular Calcium Homeostasis with Different Potencies

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Background: **The authors hypothesized that inhalational anesthetics induced cell damage by causing abnormal calcium release from the endoplasmic reticulum *via* excessive activation of inositol 1,4,5-trisphosphate (IP_3) receptors, with isoflurane having greater potency than sevoflurane or desflurane.**

Methods: **The authors treated DT40 chicken B lymphocytes with total IP_3 receptor knockout or their corresponding wild-type control cells with equipotent exposure to isoflurane, sevoflurane, and desflurane. The authors then determined the degree of cell damage by counting the percentage of annexin V– or propidium iodide–positively stained cells or measuring caspase-3 activity. They also studied the changes of calcium concentrations in the endoplasmic reticulum, cytosol, and mitochondria evoked by equipotent concentrations of isoflurane, sevoflurane, and desflurane in both types of DT40 cells.**

Results: **Prolonged use of 2 minimal alveolar concentration sevoflurane or desflurane (24 h) induced significant cell damage only in DT40 wild-type and not in IP_3 receptor total knockout cells, but with significantly less potency than isoflurane. In accord, all three inhalational anesthetics induced significant decrease of calcium concentrations in the endoplasmic reticulum, accompanied by a subsequent significant increase in the cytosol and mitochondrial calcium concentrations only in DT40 wild-type and not in IP_3 receptor total knockout cells. Isoflurane treatment showed significantly greater potency of effect than sevoflurane or desflurane.**

Conclusion: **Inhalational anesthetics may induce cell damage by causing abnormal calcium release from the endoplasmic reticulum *via* excessive activation of IP_3 receptors. Isoflurane has greater potency than sevoflurane or desflurane to cause calcium release from the endoplasmic reticulum and to induce cell damage.**

IT is important for a surgical patient to be immunocompetent to prevent perioperative infection and related complications. Various instances of immune system dysfunction have been reported after surgery[1,2] and were associated with lymphocytopenia.[3] Recent studies suggest that inhalational anesthetics commonly used for various kinds of surgery may affect the immune system by causing peripheral lymphocytopenia.[4,5] Inhalational anesthetics induced cell damage by apoptosis in lymphocytes in a dose- and time-dependent manner, which may be mediated by mitochondria.[4,5] Inhalational anesthetics may also cause DNA damage in human peripheral blood lymphocytes.[6] However, the exact mechanisms for inhalational anesthetic–mediated cell damage are still unclear.

Our recent study suggested that the commonly used inhalational anesthetic isoflurane may induce cell apoptosis by disruption of intracellular calcium homeostasis.[7–9] The inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors, two calcium channels located on the endoplasmic reticular (ER) membrane, play important physiologic roles in normal cells, including lymphocytes.[10,11] However, abnormal calcium release from the ER *via* excessive activation of either IP_3 or ryanodine receptors on the ER membrane may result in abnormal elevation of cytosolic calcium concentrations ($[Ca^{2+}]_c$), calcium overload in mitochondria, and depletion of ER calcium, all of which can contribute to cell death.[12,13] In addition, cytochrome *c* released from mitochondria due to calcium overload can remove the negative feedback inhibition of IP_3 receptors (IP_3Rs) by cytosolic calcium, leading to a vicious cycle of excessive calcium release from the ER *via* IP_3R.[13,14] Cytochrome *c* release also activates caspase 3, which in turn cleaves IP_3R, resulting in a permanent leak of calcium from the ER.[15,16] Furthermore, ryanodine and IP_3Rs are both calcium-activated calcium-release channels. Calcium release from the ER *via* activation of ryanodine receptors can activate IP_3R and *vice versa*.[17] Consistent with this theory, our previous studies have demonstrated that lymphocytes deficient of IP_3R were resistant to isoflurane-induced cell apoptosis, as well as the elevation of calcium concentrations in cytosol and mitochondria, but thapsigargin, which can release calcium from the ER independent of IP_3Rs, still induced apoptosis in the lymphocytes even with total knockout of IP_3Rs.[8] This suggests an important role of IP_3R activation on isoflurane-mediated cell damage. In addition, inhibition of excessive calcium release from the ER through either IP_3R or ryanodine calcium channel receptors by dantrolene or xestos-

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pongin C significantly inhibited isoflurane-induced cell damage.[7,8]

The three commonly used inhalational anesthetics seem to have quite different potencies to cause apoptosis in different kinds of cells, with an unclear mechanism.

Isoflurane was significantly more toxic than sevoflurane or desflurane in different lymphocytes.[4,5] We also have demonstrated that only isoflurane, not sevoflurane nor desflurane at equipotent exposures, induce cell damage in different kinds of neurons.[7,9] This unexpected phenomenon may provide an important basis for anesthesiologists to use inhalational anesthetics differently in their practice, especially for those patients vulnerable to anesthesia-mediated cell damage. We have hypothesized that inhalational anesthetics have different potencies to induce calcium release from the ER *via* activation of IP_3R and therefore have different toxic potencies. To confirm this hypothesis, we comparatively studied the effect of three commonly used inhalational anesthetics (isoflurane, sevoflurane, and desflurane) on cell apoptosis, calcium concentrations in the ER, cytosol, and mitochondria in chicken B lymphocytes with total knockout of IP_3R (DT40 IP_3R TKO) or its corresponding wild-type (WT) control.

Materials and Methods

Cell Cultures

DT40 WT and its IP_3R TKO cells 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% carbon dioxide humidified atmosphere at 38°C as we previously described.[8,18]

Anesthetic Exposure

DT40 cells (WT and IP_3R TKO) were exposed to the equivalent of 2 minimum alveolar concentration (MAC) isoflurane (2.4%), sevoflurane (4%), and desflurane (12%) for 24 h in a gastight chamber inside the culture incubator (Bellco Glass, Inc., Vineland, NJ), with humidified 5% CO_2–21% O_2–balanced N_2 (AirGas East, Bellmawr, NJ) going through a calibrated agent-specific vaporizer as we have previously described.[7–9] 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 media 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 MACs in the gas phase inside the gas chamber using the concentration correlation previously described.[19]

Imaging Analysis of Annexin V and Propidium Iodide

Translocation of membrane phospholipid phosphatidylserine 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 when 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 equivalent 2 MAC isoflurane (2.4%), sevoflurane (4%), and desflurane (12%) for 24 h. Our previous study demonstrated that the minimal exposure of 2 MAC for 24 h of isoflurane, but not its equivalent of sevoflurane, induced cell apoptosis in the rat primary cortical neurons.[7] However, it only required 1.2% isoflurane for 6 h to induce apoptosis in DT40 WT cells.[8] We hypothesized that sevoflurane or desflurane could still induce apoptosis but with much less potency than isoflurane in the vulnerable DT40 WT cells. As a result, we elected to use relatively high concentrations (2 MAC) for a prolonged time (24 h) of three inhalational anesthetics so that the possible toxic effects of sevoflurane or desflurane could be detected. Immediately after treatment, we determined annexin V– or PI–positive cells by the methods we have previously described.[8] Briefly, cells were dropped onto 25-mm coverslips and stained with annexin V or PI. Annexin V– and PI–positive cells and normal cells were counted by two persons blinded to the treatments. The percentage of annexin V– or PI–positive cells was calculated by dividing the positively stained cells by the total number of counted cells in at least four areas on each coverslip.

Detection of Caspase-3 Activity

Increased caspase-3 activity is a hall marker for cell damage by apoptosis. We measured the caspase-3 activity immediately after treatment using a method we have described previously.[8,18] 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). DT40 cells grown on six-well plates were treated with equipotent concentrations of isoflurane (2.4%), sevoflurane (4%), and desflurane (12%) for 24 h and then were harvested *via* trypsinization and washed with phosphate-buffered saline. The cell pellet was gently resuspended in CelLytic 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).

Measurement of Cytosolic Calcium Concentration

Cytosolic calcium concentration was measured using fura-2/AM fluorescence (Molecular Probes, Eugene, OR) with a photometer coupled to an Olympus 1X70 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 $[Ca^{2+}]_c$ was same as that which we previously described.[8,9] Briefly, cells attached to Cell-Tak (BD Biosciences, Bedford, MA)–coated glass coverslips 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. All bubbles in the chamber were flushed out at the beginning so that there was no gas phase in the sealed chamber during measurement of calcium concentration in the buffer. The cells were first washed with Krebs-Ringer's buffer through one inflow tube for the baseline measurement of $[Ca^{2+}]_c$ and then were exposed to the approximate equivalent of 2 MAC isoflurane (0.64 mM), sevoflurane (0.96 mM), and desflurane (1.2 mM)[19] *via* a separate inflow infusion tube driven by a syringe pump (Braintree Scientific Inc., Braintree, MA). The anesthetic concentrations in the buffer were determined with high-performance liquid chromatography (System Gold; Beckman Coulter) as we described previously.[9,20] The fluorescence signals were measured with excitation at 340 and 380 alternatively and emission at 510 nM for a period up to 15 min for each treatment. The ratios of F_{340}/F_{380} reflected the cytosolic calcium concentrations and were used for comparison among three inhalational anesthetics. The final result of F_{340}/F_{380} was averaged from the cells of at least three separate experiments. A pilot study confirmed that the cells were still viable at the end of experiments for calcium measurement.

Measurement of ER Calcium Concentration

We measured the calcium concentrations inside the ER ($[Ca^{2+}]_e$) using the dye mag-fura-2/AM (Molecular Probes) and a previously described protocol.[21] Briefly, DT40 cells were incubated with 5 μM mag-fura-2/AM in modified Hanks balanced salt solution buffered with HEPES (HBSS-H: 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 0.34 mM Na2HPO$_4$, 0.44 mM NaH2PO$_4$, 5.5 mM glucose, and 20 mM HEPES/NaOH [pH 7.4]) containing 1% bovine serum albumin for 45 min at 37°C in a dark room. The mag-fura-2/AM–loaded cells were then washed with bovine serum albumin–free HBSS-H and attached to Cell-Tak–coated glass coverslips placed at the bottom of the recording chambers. The cells were then exposed to intracellular-like medium containing 125 mM KCl, 19 mM NaCl, 10 mM HEPES (pH7.3 with KOH), 330 μM $CaCl_2$, and 100μg/ml (wt/vol) saponin (Sigma) for 1–2 min to permeabilize the cells. Thereafter, the permeabilized cells were washed with intracellular-like medium and then incubated in intracellular-like medium containing 3 mM ATP and 1.4 mM $MgCl_2$ for at least 5 min. The coverslips 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 washed with running buffer through one inflow tube for the baseline measurement of $[Ca^{2+}]_e$ at first and then were exposed to equivalent concentrations of isoflurane (0.64 mM), sevoflurane (0.96 mM), or desflurane (1.2 mM) *via* a separate inflow infusion tube driven by a syringe pump (Braintree Scientific Inc.). The fluorescence signals were measured with excitation at 340 and 380 alternatively and emission at 510 nM for a period up to 6 min for each treatment. The ratios of F_{340}/F_{380} reflected the ER calcium concentrations relatively and were used for comparison among three inhalational anesthetics. The final results of F_{340}/F_{380} were averaged from the cells of at least three separate experiments and compared among three inhalational anesthetics. All these experiments were performed at room temperature.

Confocal Imaging of Mitochondrial Ca^{2+}

The method used was the same as we described previously.[8,18] DT40 cells (WT and IP_3R TKO) 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, then attached to Cell-Tak–coated glass coverslips. The cells were washed and placed on a stage and exposed to equivalent concentrations of isoflurane (0.64 mM), sevoflurane (0.96 mM), or desflurane (1.2 mM) dissolved in the perfusion buffer. The images were recorded using the Radiance 200 imaging system (Bio-Rad Laboratories, Hercules, CA) with excitation at 568 nm. The relative changes of fluorescence signals were determined and compared among three inhalational anesthetics.

Statistical Analysis

We used GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA) for all statistical analysis. Annexin V and propidium iodide staining were expressed as percentage of total cells examined. Changes of calcium concentrations in ER ($[Ca^{2+}]_e$), cytosol ($[Ca^{2+}]_c$), and mitochondria ($[Ca^{2+}]_m$) were expressed as a percentage of their own baseline. All data were expressed as mean ± SD. We analyzed all the data with one-way analysis of variance followed by Tukey multiple

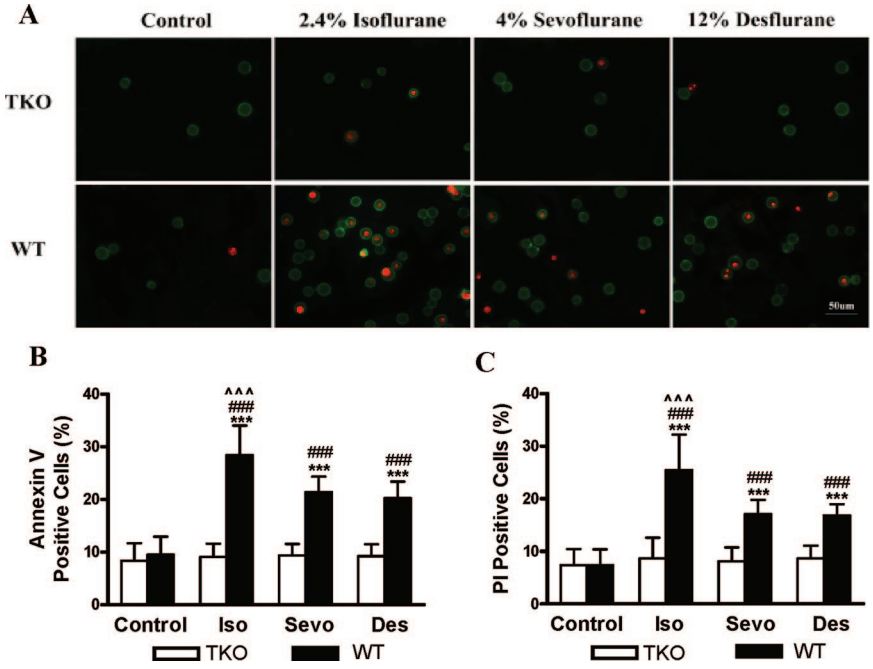

Fig. 1. Isoflurane induced significantly greater cell damage than sevoflurane or desflurane only in DT40 wild-type (WT) cells. The early stage of cell damage by apoptosis was determined by the externalization of anionic phospholipid, phosphatidyl serine–annexin V binding (annexin V). The late stage of cell damage was determined by propidium iodide (PI) staining, representing loss of plasma membrane integrity. All three inhalational anesthetics induced significant increase of annexin V–positive (*green circle*) and PI-positive (*red dot*) cells (*A*). *Scale bar* = 50 μM. Isoflurane (Iso) also induced significantly more early (annexin V; *B*) and late cell damage (PI; *C*) than either sevoflurane (Sevo) or desflurane (Des). (*B* and *C*) Data represent mean ± SD from at least three separate experiments. *, ### $P < 0.001$ compared with DT40 inositol 1,4,5-trisphosphate receptor total knockout (TKO) cells or the DT40 WT cell controls, respectively. ^^^ $P < 0.001$ compared with DT40 WT cells treated with either sevoflurane or desflurane (*B* and *C*).**

comparison tests; $P < 0.05$ was considered statistically significant.

Results

Isoflurane Induced Significantly Greater Cell Damage than Sevoflurane or Desflurane Only in DT40 WT Cells

We have previously demonstrated that isoflurane induced apoptosis only in DT40 WT and not in IP_3R TKO in a dose- and time-dependent manner.[8] Because sevoflurane and desflurane, at equipotent exposure to isoflurane, seemed not to induce cell damage in neuronal cultures in our previous studies,[7,9] we investigated whether the three inhalational anesthetics also have different potencies to induce apoptosis. All three inhalational anesthetics induced significant cell damage determined by both annexin V and PI staining only in DT40 WT and not in IP_3R TKO (fig. 1A). Isoflurane significantly increased the percentage of annexin V– and PI–positive cells from 9.1 ± 2.4% and 8.7 ± 3.9% in controls to 28.4 ± 5.6% and 25.4 ± 6.7%, respectively, after treatments only in DT40 WT cells ($P < 0.001$, n = 29 for all controls, n = 28 for all treatments; figs. 1B and C). Compared with control, sevoflurane and desflurane also significantly increased the percentage of annexin V–positive cells from 9.4 ± 2.2% and 9.2 ± 2.3% in controls to 21.4 ± 2.9% and 20.2 ± 3.1%, respectively, after treatment only in DT40 WT cells ($P < 0.001$ for all, n = 29 for all controls, n = 28 for all treatments; fig. 1B). Similarly, sevoflurane and desflurane significantly increased the percentage of PI–positive cells from 8.1 ± 2.6% and 8.6 ± 2.4% in controls to 17 ± 2.7% and 16.9 ± 2.1%, respectively, after treatments ($P < 0.001$ for all, n = 29 for all controls, n = 28 for all treatments; fig. 1C) only in WT DT40 cells. Consistent with our previous finding,[7,9] isoflurane, at equipotent exposure to sevoflurane and desflurane, induced significantly greater cell damage than either sevoflurane or desflurane in WT DT40 cells ($P < 0.001$ for both annexin V and PI; figs. 1B and C). We also compared the potency of three commonly used inhalational anesthetics on induction of apoptosis in chicken B lymphocytes by measuring caspase-3 activity, which is one of the hallmarks of cell death by apoptosis. Isoflurane dramatically increased caspase-3 activity as a percentage of control to 358 ± 60% ($P < 0.001$, n = 8 for control, n = 9 for isoflurane treatment; fig. 2) only in WT DT40 cells, whereas sevoflurane and desflurane did not

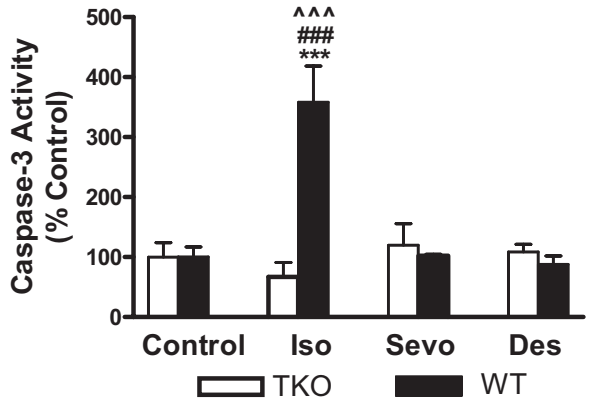

Fig. 2. Isoflurane, but not sevoflurane or desflurane, significantly elevated caspase-3 activity only in wild-type (WT) DT40 cells. Data represent mean ± SD from minimum four repeats. *, ### $P < 0.001$ compared with DT40 inositol 1,4,5-trisphosphate receptor total knockout (TKO) cells or the DT40 WT cell controls, respectively. ^^^ $P < 0.001$ compared with DT40 WT cells treated with either sevoflurane (Sevo) or desflurane (Des). Iso = isoflurane.**

increase caspase-3 activity significantly (102 ± 2.6% and 87 ± 14%, respectively, as a percentage of control, $P > 0.05$, n = 4 for both controls and treatments; fig. 2). Isoflurane at equipotent concentrations caused a markedly greater caspase-3 activity in comparison with sevoflurane and desflurane ($P < 0.001$; fig. 2) only in DT40 WT cells. All three inhalational anesthetics did not induce significant increases of caspase-3 activity in DT40 IP_3R TKO cells.

Isoflurane Induced a Significantly Greater Decrease of ER Calcium than Sevoflurane or Desflurane Only in DT40 WT Cells

To support our hypothesis that inhalational anesthetics can induce cell damage by causing calcium release from the ER *via* activation of IP_3R, we measured the change of ER calcium after exposure of DT40 cells to these anesthetics at equipotent concentrations. All three inhalational anesthetics significantly decreased ER calcium selectively only in DT40 WT and not in IP_3R TKO cells (fig. 3A), indicating that calcium is released from the ER *via* activation of IP_3R. Isoflurane, sevoflurane, and desflurane significantly reduced ER calcium concentration as percentage decrease below their initial baseline maximally by 17.6 ± 6.6% (n = 21), 11.6 ± 3.8% (n = 18), and 11 ± 4.2% (n = 22), respectively ($P < 0.001$ for all; figs. 3B and C), in DT40 WT cells. However, isoflurane, sevoflurane, and desflurane only reduced ER calcium level as percentage decrease below their baseline level by 1.4 ± 0.9% (n = 18), 1.9 ± 0.8% (n = 21), and 1.2 ± 0.3% (n = 35), respectively, in DT40 IP_3R TKO cells, significantly less than in their corresponding WT DT40 cells ($P < 0.001$ for all; fig. 3C). These results were consistent with the greater potency of isoflurane than sevoflurane or desflurane to induce cell damage by apoptosis (figs. 1 and 2).

Isoflurane Induced a Significantly Greater Increase of Cytosolic and Mitochondria Calcium than Sevoflurane or Desflurane Only in DT40 WT Cells

To confirm that the calcium released from the ER by inhalational anesthetic–mediated activation of IP_3R was transferred into mitochondria *via* the cytosolic space, we measured the changes of calcium concentrations in both the cytosolic space and the mitochondria. Isoflurane, sevoflurane, and desflurane significantly increased peak cytosolic calcium (F_{340}/F_{380} ratio) as percentages above their own baseline controls to 162.7 ± 64% (n = 39), 79.4 ± 19% (n = 15), and 67.5 ± 29% (n = 21) ($P < 0.001$ for all; figs. 4A and B) only in DT40 WT cells, which were also significantly higher than their effects in DT40 IP_3R TKO cells (6.3 ± 3.5%, n = 23, 3 ± 1.4%, n = 38, and 3 ± 1.4%, n = 39, respectively, $P < 0.001$ for all; figs. 4A and B). Similarly, all three inhalational anesthetics significantly increased mitochondrial calcium concentrations only in DT40 WT cells (fig. 5). Isoflurane, sevoflurane, and desflurane significantly increased mitochondrial calcium concentrations as percentages of increase above their own baseline control to 121.1 ± 80% (n = 26), 51.5 ± 22.5% (n = 25), and 64.8 ± 34% (n = 27) ($P < 0.001$ for all; figs. 5A and B) only in DT40 WT cells, which were also significantly higher than their effects in DT40 IP_3R TKO cells (2.7 ± 2.7%, n = 27, 1.4 ± 2.8%, n = 28, and 1.3 ± 2.3%, n = 28, respectively, $P <$

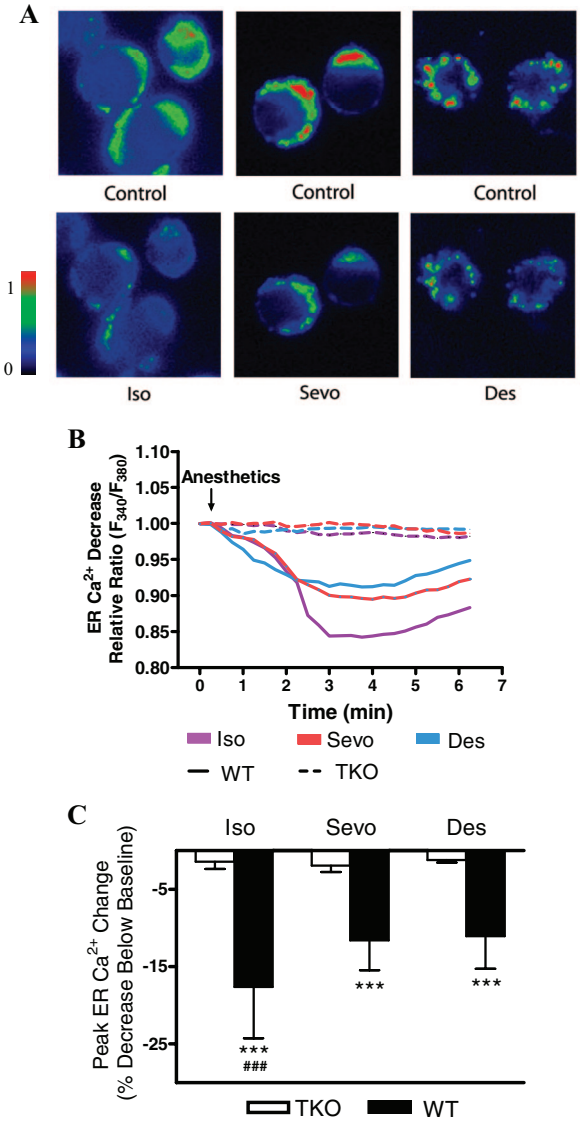

Fig. 3. Isoflurane induced significantly greater decrease of the endoplasmic reticulum (ER) calcium than sevoflurane or desflurane only in DT40 wild-type (WT) cells. DT40 WT cells were permeabilized with saponin to allow the dye mag-fura-2/AM loaded into ER and then exposed to three inhalational anesthetics of isoflurane (Iso), sevoflurane (Sevo), and desflurane (Des) to induce calcium release from the ER (*A*). (*B* and *C*) Isoflurane induced significantly more peak ER calcium decrease over its own baseline represented by the relative ratio (F_{340}/F_{380}) than sevoflurane or desflurane only in DT40 WT cells. Data represent mean ± SD from at least three separate experiments. *** $P < 0.001$ compared with inositol 1,4,5-trisphosphate receptor total knockout (TKO) DT40 cells. ### $P < 0.001$ compared with WT DT40 cells treated with either sevoflurane or desflurane.

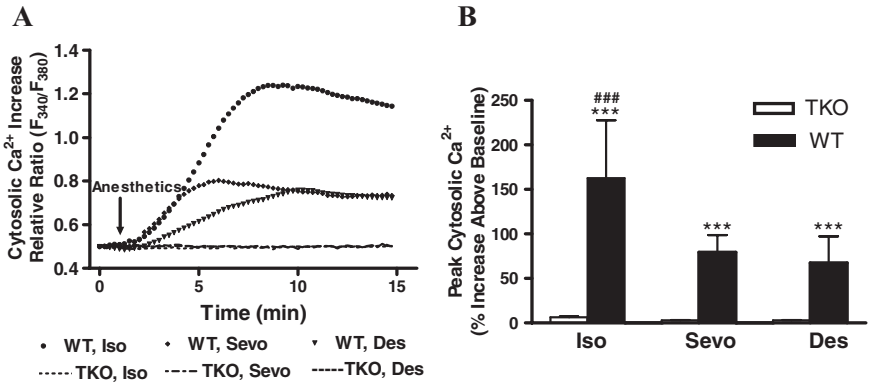

Fig. 4. Isoflurane induced significantly greater increase of cytosolic calcium than sevoflurane or desflurane only in wild-type (WT) DT40 cells. Isoflurane (Iso) induced significantly more elevation of peak cytosolic calcium represented by the relative ratio (F_{340}/F_{380}) than sevoflurane (Sevo) or desflurane (Des) only in DT40 WT cells (*A* and *B*). (*B*) Data represent mean ± SE from at least three separate experiments. *** $P < 0.001$ compared with inositol 1,4,5-trisphosphate receptor total knockout (TKO) DT40 cells. ### $P < 0.001$ compared with DT40 WT cells treated with either sevoflurane or desflurane.

0.001 for all; figs. 5A and B). These results were consistent with their effects on decreasing ER calcium (fig. 3), increasing cytosolic calcium (fig. 4), and inducing cell damage (fig. 1 and 2).

Discussion

Our results suggest that inhalational anesthetics induce apoptosis in lymphocytes *via* excessive activation of IP_3R on the ER membrane. The apoptosis induced by inhalational anesthetics was associated with a decrease of calcium concentrations in the ER, and the sequential increase of calcium concentrations in the cytosolic space and then the mitochondria. This most likely represented transfer of ER calcium into mitochondria *via* the cytosolic space, which is much more efficient than the calcium transfer originating from the calcium influx from the extracellular space.[22] It is generally believed that calcium ions in cytosolic space are transferred into mitochondria by a calcium ion uniporter.[22] The excessive cytosolic calcium originating from the ER can cause overloading of mitochondria with calcium, collapse of mitochondrial membrane potential, and subsequent release of cytochrome *c* from mitochondria, triggering cell death by apoptosis.[23] The current study provides a novel finding that all three inhalational anesthetics actually decrease ER calcium by directly measuring ER calcium concentrations. The significant decrease of ER calcium concentrations after exposing DT40 to isoflurane in WT but not IP_3R TKO cells further supports the finding from our previous reports[8,9] that isoflurane induced calcium release from the ER *via* activation of IP_3R. We did not test the effect of the IP_3R antagonist xestospongin C on cell damage induced by inhalational anesthetics in the current study because xestospongin C does not behave consistently as a selective IP_3R antagonist[24] and we have previously demonstrated the inhibitory effects of xestospongin C on isoflurane-mediated cell damage in other types of cells.[8] The major advantage of using the DT40 IP_3R TKO cell line is to selectively study the role of this receptor on excessive calcium release from the ER and the subsequent apoptosis mediated by inhalational anesthetics, which is a more powerful tool than the relatively less selective IP_3R antagonist xestospongin C. More studies are needed to investigate whether inhalational anesthetics can directly activate IP_3R on the ER membrane or simply increase the production of IP_3 or augment the opening probability of IP_3R upon activation by their agonists.

Isoflurane seems to behave consistently throughout our studies in having greater potency to induce apoptosis and calcium release from the ER than sevoflurane or desflurane.[7–9] This is especially obvious on comparison of activation of caspase 3 induced by three inhalational anesthetics, with isoflurane but not sevoflurane or desflurane inducing remarkable elevation of caspase-3 activ-

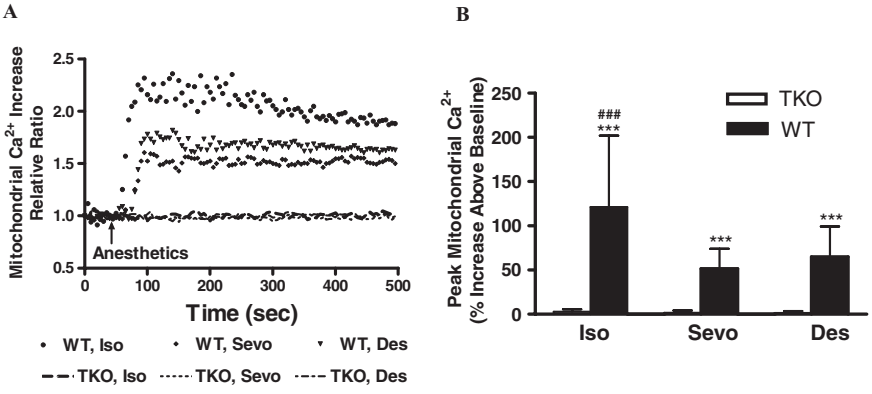

Fig. 5. Isoflurane induced significant greater increase of mitochondrial calcium than sevoflurane or desflurane only in wild-type (WT) DT40 cells. Isoflurane (Iso) induced significantly more increase of peak mitochondrial calcium represented by the relative ratio as percentage increase above their own baselines than sevoflurane (Sevo) or desflurane (Des) only in DT40 WT cells (*A*). (*B*) Data represent mean ± SD from at least three separate experiments. *** $P < 0.001$ compared with DT40 inositol 1,4,5-trisphosphate receptor total knockout (TKO) cells. ### $P < 0.001$ compared with DT40 WT cells treated with either sevoflurane or desflurane.

ity, a hallmark of cell death by apoptosis in WT DT40 cells. It is still not clear why sevoflurane and desflurane significantly increased annexin V and PI but not caspase 3–positive cells compared with their corresponding controls. Annexin V, a phospholipid-binding protein with a high affinity for phospholipid phosphatidylserine, can bind to phospholipid phosphatidylserine when it is exposed to the extracellular environment during early stage of cell damage. Propidium iodide (PI) can bind to nucleic acid after penetrating a breached plasma membrane, as occurs in the later stages of cell damage. Caspase-3 activation is the hallmark of apoptosis. We speculated that annexin V might also detect some damaged cells without apoptosis process, whereas caspase-3 activity assay detected only apoptotic cells. It is possible that sevoflurane or desflurane did not activate caspase 3 (fig. 2). DT40 IP_3R TKO chicken lymphocytes were resistant to the transfer of ER calcium into mitochondria *via* cytosolic space and apoptosis induced by all three inhalational anesthetics. Isoflurane has greater potency to induce calcium release from the ER, most likely derived from its greater activation of IP_3Rs. It seems that neurons are more resistant to inhalational anesthetic–induced apoptosis than lymphocytes because the minimum exposure of isoflurane required to induce neuronal damage was 2 MAC for 24 h.[7,8,25] It should be noted that sevoflurane or desflurane could also induce cell damage determined by annexin V and PI staining in the vulnerable lymphocytes, but with significantly less potency than isoflurane. Our previous studies suggested that isoflurane induced apoptosis in DT40 WT cells and primary cortical neurons in a concentration- and time-dependent manner.[7,8] The minimal exposure for isoflurane to induce apoptosis in DT40 cells was approximately 1.2% for 6 h.[8] In normal human peripheral lymphocytes, only 0.85% isoflurane was needed to induce apoptosis.[4] We have chosen a treatment regiment with relatively high concentrations (equivalent to 2 MAC) for a long duration (24 h) to test the hypothesis that sevoflurane and desflurane could still induce apoptosis as isoflurane, but with much lower potencies. An intervention with low concentration and duration would be less likely to cause any detectible sevoflurane or desflurane cytotoxicity, as we have demonstrated previously in other types of cells.[7,25] Our results endorse the hypothesis that sevoflurane and desflurane at equipotent exposure of 2 MAC for 24 h induces cell damage determined with annexin V and PI staining in DT40 lymphocytes, but with significantly less potency than isoflurane. In peripheral human lymphocytes, both isoflurane and sevoflurane induced apoptosis,[4,5] with isoflurane having greater potency than sevoflurane at the same concentrations.[4] Loop *et al.*[5] also demonstrated that isoflurane and sevoflurane, but not desflurane, induced apoptosis dose dependently in human T lymphocytes. Further animal and clinical studies are needed to determine whether desflurane may be a better choice than other inhalational anesthetics in patients with peripheral lymphocytopenia.

All inhalational anesthetics evidently affect intracellular calcium homeostasis. A transient and moderate elevation of cytosolic calcium by isoflurane may provide cytoprotection through up-regulation of host preconditioning responses,[26,27] but prolonged exposure to higher concentrations of isoflurane, such as this study, may maintain the IP_3R in an open state, in turn excessively elevating $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, depleting ER calcium, and ultimately leading to cell damage.[28,29] It seemed that the potency for inhalational anesthetics to induce cell apoptosis correlated with their potency to decrease ER calcium concentrations and subsequently their capacity to increase cytosolic and mitochondrial calcium. The greater likelihood of isoflurane to induce calcium release from ER than sevoflurane may also contribute to its greater ability to precondition neurons and provide neuroprotection against neurotoxicity induced by the prolonged use of isoflurane, as demonstrated in our previous study.[25] Future studies are needed to quantify the amount of ER calcium decrease required to convert the short-exposure neuroprotective effects into neurotoxic effects by a prolonged isoflurane exposure.

This study has several limitations that should influence *in vivo* interpretations: (1) DT40 chicken lymphocytes are from immortal transformed cell lines. 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. Although the mechanisms for this vulnerability variance among different types of cells are elusive, it seems that neurons in cell cultures are relatively less vulnerable to isoflurane-mediated neurotoxicity.[7–9,25] (2) Because the DT40 cells are chicken lymphocytes, not human lymphocytes, it is difficult to refer the finding in this study to clinical importance. Previous studies have demonstrated that inhalational anesthetics induced apoptosis in human lymphocytes with different potencies,[4,5] whereas the current study provided mechanisms of how the inhalational anesthetics induce apoptosis in lymphocytes. (3) We used inhalational anesthetics at relatively high concentrations for a prolonged time, which is less ideal to mimic the clinical scenario in patients. Further studies are needed to compare the potency of three commonly used inhalational anesthetics on intracellular calcium homeostasis and apoptosis in animals and patients in the future.

Taken together, our findings suggest that the commonly used inhalational anesthetic isoflurane may induce apoptosis in lymphocytes by significantly decreasing ER calcium and subsequently increasing cytosolic and mitochondrial calcium, which was associated with an excessive activation of IP_3R. Sevoflurane and desflu-

rane have significantly less potency to induce calcium release from the ER and apoptosis in these lymphocytes.

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