

# Absence of Direct Antioxidant Effects from Volatile Anesthetics in Primary Mixed Neuronal-Glial Cultures

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**Background:** Volatile anesthetics decrease ischemic brain injury. Mechanisms for this protection remain under investigation. The authors hypothesized that volatile anesthetics serve as antioxidants in a neuronal-glial cell culture system.

**Methods:** Primary cortical neuronal-glial cultures were prepared from fetal rat brain. Cultures were exposed to iron, H<sub>2</sub>O<sub>2</sub>, or xanthine-xanthine oxidase for 30 min in serum-free media containing dissolved isoflurane (0–3.2 mM), sevoflurane (0–3.6 mM), halothane (0–4.1 mM), *n*-hexanol, or known antioxidants. Cell damage was assessed by release of lactate dehydrogenase (LDH) and trypan blue exclusion 24 h later. Lipid peroxidation was measured by the production of thiobarbituric acid-reactive substances in a cell-free lipid system. Iron and calcium uptake and mitochondrial depolarization were measured after exposure to iron in the presence or absence of isoflurane.

**Results:** Deferoxamine reduced LDH release caused by H<sub>2</sub>O<sub>2</sub> or xanthine-xanthine oxidase, but the volatile anesthetics had no effect. Iron-induced LDH release was prevented by the volatile anesthetics (maximum effect for halothane = 1.2 mM, isoflurane = 1.2 mM, and sevoflurane = 2.1 mM aqueous phase). When corrected for lipid solubility, the three volatile anesthetics were equipotent against iron-induced LDH release. In the cell-free system, there was no effect of the anesthetics on thiobarbituric acid-reactive substance formation in contrast to Trolox, which provided complete inhibition. Isoflurane (1.2 mM) reduced mean iron uptake by 46% and inhibited mitochondrial depolarization but had no effect on calcium uptake.

**Conclusions:** Volatile anesthetics reduced cell death induced by oxidative stress only in the context of iron challenge. The likely reason for protection against iron toxicity is inhibition of iron uptake and therefore indirect reduction of subsequent intracellular oxidative stress caused by this challenge. These data argue against a primary antioxidant effect of volatile anesthetics.

IN a variety of laboratory models, volatile anesthetics have been demonstrated to reduce ischemic brain injury. In a brain temperature-regulated 1-week recovery focal ischemic injury model, halothane reduced cerebral infarct size and improved neurologic injury relative to awake controls.<sup>1</sup> This is consistent with observations made in the cat that focal ischemic injury is reduced with halothane as opposed to  $\alpha$ -chloralose anesthesia.<sup>2</sup> Similarly, isoflurane reduced infarct size relative to either

awake or fentanyl-anesthetized rats subjected to focal ischemia.<sup>3</sup> In a temperature-regulated model of near-complete forebrain ischemia, isoflurane reduced injury in selectively vulnerable structures relative to controls anesthetized with fentanyl-nitrous oxide.<sup>4,5</sup> This is consistent with an early report that both halothane and isoflurane caused improvement in histologic-behavioral outcome from severe hemispheric ischemia relative to nitrous oxide-sedated controls.<sup>6</sup> Cumulatively, these results demonstrate potent effects of volatile anesthetics against ischemic mechanisms resulting in necrosis, although effects on delayed apoptotic cell death are controversial.<sup>7,8</sup>

When brain tissue is subjected to ischemia and reperfusion, reactive oxygen species are formed.<sup>9,10</sup> These products can oxidize cellular constituents, resulting in enhanced ischemic injury. Although barbiturates and propofol have chemical structures substantially different from volatile anesthetics, both of these anesthetics have been shown to serve as antioxidants.<sup>11-13</sup> To our knowledge, there has been no effort to define effects of volatile anesthetics against oxidative stress in neural tissue. Accordingly, we hypothesized that volatile anesthetics would reduce cell death in primary mixed neuronal-glial cultures subjected to a variety of forms of oxidative stress.

## Methods

All animal procedures were approved by the Duke University Animal Care and Use Committee.

### Preparation of Mixed Neuronal-Glial Cell Cultures

Mixed neuronal-glial cultures were prepared from fetal Sprague-Dawley rat brains at 18 days of gestation as previously described.<sup>14</sup> Brains were collected from 10–15 pups and dissected to separate cortex from meninges and subcortical structures using standard anatomical landmarks. Cortices were pooled and minced into 2-mm<sup>3</sup> pieces in a buffered salt solution (BSS; Hank's balanced salt solution; Life Technologies, Gaithersburg, MD) supplemented with 20 mM HEPES buffer, pH 7.4, containing 0.25% trypsin (Life Technologies). The tissue was incubated for 20 min at 37°C in a 5% CO<sub>2</sub>-95% room air atmosphere, then washed twice with ice-cold glutamine-free minimum essential medium (MEM; Life Technologies) containing 15 mM glucose, 5% fetal bovine serum (Gibco Diagnostics, Inc., Madison, WI), 5% horse serum (Gibco), and 1% DNase-I (Sigma Chemical Co., St.

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Louis, MO). Tissue pieces were dissociated by trituration through a fire-polished 9-inch Pasteur pipette. The resultant suspension was centrifuged at 50g for 10 min, the supernatant was discarded, and the pellet was resuspended in growth medium (MEM supplemented with 15 mM glucose, 5% fetal bovine serum, and 5% horse serum). The dissociated cells were plated to achieve a confluent monolayer ( $4 \times 10^5$  cells per well) on poly-D-lysine-coated, 24-well culture plates (Falcon 3047; Becton Dickinson Co., Lincoln Park, NJ). Cultures were maintained undisturbed at 37°C in a humidified 5% CO<sub>2</sub>-balance room air atmosphere for 13–16 days before use. Cultures were not fed after plating or before use in experiments. Cell types in typical 13–16-day-old cultures were determined to be  $54 \pm 4\%$  neurons and  $46 \pm 7\%$  glia using immunohistochemical staining for cell-specific cytoskeletal filaments (Neurofilament-160 [NF-160] for neurons and glial fibrillary acidic protein [GFAP] for astrocytes; see below).

#### Preparation of Volatile Anesthetic Solutions

Stock solutions of volatile anesthetics dissolved in culture medium were prepared using a modification of the method of Blanck and Thompson.<sup>15</sup> A 10-mM volatile anesthetic solution was made by injecting 100  $\mu$ l halothane (Fluothane; Ayerst Laboratories Inc., Philadelphia, PA), 130  $\mu$ l isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether; Abbot Laboratories Inc., Chicago IL), or 140  $\mu$ l sevoflurane (Ultane; Abbot Laboratories Inc.) into 103 ml of BSS in a 100-ml volumetric flask. The flask was sealed with a glass stopper so as to exclude all air from the neck of the flask. The flask was wrapped in aluminum foil, and the solution was stirred for 24 h to solubilize the anesthetic. Immediately before use, 30 ml of the concentrated stock solution was poured into a 50-ml polypropylene centrifuge tube and vortexed for 5–10 s to produce the working stock. An aliquot of the working stock was assayed by gas chromatography as described below to determine the concentration of dissolved volatile anesthetic. The working stock was then diluted with BSS to produce the desired concentration for treating cell cultures.

#### Measurement of Dissolved Volatile Anesthetic Concentration

A 200- $\mu$ l sample of media containing dissolved anesthetic was transferred to a 4.4-ml vial capped with a Teflon seal. The vial was vortexed for 1 min to equilibrate the volatile anesthetic in the gas and liquid phases. A gas-tight Hamilton syringe was used to collect a 500- $\mu$ l sample of the air space within the vial. The sample was injected onto a 6-foot Supelcoport 100/120 gas chromatography column (Sopelco, Inc., Belle Fonte, PA) coated with 3% SP2340. The anesthetic was detected by flame ionization. The detector was calibrated using gas samples taken from a anesthetic vaporizer against values

obtained with an infrared agent monitor (Model 5330, Ohmeda Inc., Louisville, CO for halothane and isoflurane; Capnomac Ultima, Datex Engstrom, Helsinki, Finland for sevoflurane).

In preliminary studies, the stability of the volatile anesthetic in solution was determined by incubating samples of the working stock under identical conditions used in treating cell cultures for 30 min. The concentration was measured before and after the incubation period. The percentage loss of anesthetic over the 30-min incubation was determined, by averaging a minimum of 10 observations, to be 27% for halothane, 48% for isoflurane, and 43% for sevoflurane. Accordingly, values presented in the tables and figures for anesthetic concentration were corrected for percentage loss by averaging the concentrations at the start and end of the exposure interval.

#### Effects of Anesthetics on Oxidative Stress

Mature cultures (13–16 days *in vitro*) were washed with Mg<sup>2+</sup>-free BSS containing 20 mM HEPES buffer, pH 7.4, and dissolved anesthetic (0–4.1 mM halothane, 0–4.1 mM isoflurane, 0–3.6 mM sevoflurane, or 0–556  $\mu$ M propofol) or the long-chain aliphatic alcohol, *n*-hexanol (0–4 mM; Aldrich Chemicals, St. Louis, MO). Volatile anesthetic concentration in the culture media was determined by gas chromatography as previously described. Treated cultures were subjected to a 30-min exposure to 300  $\mu$ M iron (ferrous sulfate:ferric chloride, 1:1) hydrogen peroxide (50  $\mu$ M H<sub>2</sub>O<sub>2</sub>), xanthine (200  $\mu$ M), xanthine oxidase (4 mU/ml, grade III, Sigma), or malonaldehyde (50  $\mu$ M, prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropan from Aldrich Chemicals). In all cases, the cultures were returned to the incubator and maintained at 37°C. Thirty minutes later, the exposure medium containing the dissolved anesthetic was removed and replaced with MEM supplemented with 20 mM glucose (with no dissolved anesthetic). Plates were returned to the incubator for 24 h, after which lactate dehydrogenase (LDH) activity (LDH release) in the media was measured as described below. In some experiments, cells were exposed to an ED<sub>90</sub> (100  $\mu$ M)<sup>1</sup> concentration of *N*-methyl-D-aspartate (NMDA).

In an independent series of studies, the effects of known antioxidant agents on neuronal–glial cell survival and lipid peroxidation were assessed under identical conditions as those used for assessing volatile anesthetic effects. Antioxidants [100  $\mu$ M probucol, 1 mM ( $\pm$ )-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox; Fluka Inc., Milwaukee, WI), 100  $\mu$ M  $\alpha$ -tocopherol, or 2 mM deferoxamine] were added 1 h before and maintained in the culture media throughout the interval of oxidative stress. The effects of treatment on LDH release were assessed.

In additional experiments, volatile anesthetics were studied in the absence of oxidative stress. Cultures were exposed to 0.3, 1.1, 1.9, or 2.7 mM halothane or 0.3, 1.2, or 3.1 mM isoflurane for 30 min. The media was then replaced with MEM supplemented with 20 mM glucose. LDH release was measured 24 h later.

#### *Measurement of Lactate Dehydrogenase Release*

Cellular injury was assessed 24 h after excitotoxic or metabolic stress by measuring the amount of LDH released into overlying medium by damaged cells.<sup>17</sup> LDH activity was determined by a modification of the method described by Amador *et al.*<sup>18</sup> A 200- $\mu$ l sample of culture medium was added to a polystyrene cuvette containing 10 mM lactate and 5  $\mu$ mol nicotinamide adenine dinucleotide in 2.75 ml of 50 mM glycine buffer, pH 9.2, at 24°C. LDH activity was determined from the initial rate of reduction of nicotinamide adenine dinucleotide as calculated using a linear least-square curve fit of the temporal changes in fluorescence signal from the cuvette (340 nm excitation, 450 nm emission) and expressed in units of enzymatic activity (nanomoles of lactate converted to pyruvate per minute). Analysis was performed on a fluorescence spectrophotometer (Perkin Elmer Model LS50B; Bodenseewerk GmbH, Uberlinger, Germany). We previously determined that NMDA-responsive neurons contribute approximately 40% of the total cellular LDH in primary mixed neuronal–glial cultures prepared according to the protocol described above.<sup>16</sup>

#### *Effects of Anesthetics on Lipid Peroxidation in a Cell-Free System*

To characterize the effect of volatile anesthetics on iron-mediated lipid peroxidation, we used a cell-free system described by Miyata and Smith.<sup>19</sup> Serum lipoprotein (high-density lipoprotein from human plasma, Sigma) was suspended in BSS at a concentration of 500  $\mu$ g protein per 3.3 ml. To induce lipid peroxidation, 25  $\mu$ M iron (ferrous sulfate:ferrous chloride, 1:1) was added to the suspension. Lipid peroxidation was assessed at the end of 1 h from the production of thiobarbituric acid-reactive substances (TBARS) in the presence of halothane (0, 1.2, or 4.1 mM), isoflurane (0 or 1.2 mM), or 100  $\mu$ M Trolox. Samples were mixed with thiobarbituric acid solution (0.3% thiobarbituric acid, 9% glacial acetic acid, pH 3.4) and heated to 95°C for 1 h. Samples were allowed to return to room temperature and centrifuged for 10 min at 10,000g. TBARS in the supernatant were measured by spectrofluorometry (Perkin-Elmer Model LS50B, excitation = 515 nm, emission = 553 nm, readings integrated over 10 s). Readings were adjusted for background fluorescence as determined using a concomitantly run reagent blank and compared against a standard curve established using freshly prepared malondialdehyde.

#### *Effects of Isoflurane Calcium and Iron Uptake after Exposure to Iron*

Calcium uptake from the extracellular space was assessed using <sup>45</sup>CaCl<sub>2</sub> (American Radiolabeled Chemicals, St. Louis, MO). Sister cultures were washed with Mg<sup>2+</sup>-free BSS containing 20 mM HEPES buffer. Dissolved isoflurane (final concentration = 1.2 mM) was added to some cultures, and the remaining wells were not treated and served as controls. <sup>45</sup>Ca was added to each well (0.28  $\mu$ Ci/ml, 0.9  $\mu$ Ci/well). Six isoflurane-treated cultures and six untreated cultures were exposed to 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> for 30 min at 37°C. Another six cultures received no iron. Thirty minutes later, the exposure medium was removed, and each well was washed three times with ice-cold Mg<sup>2+</sup>-free BSS containing 20 mM HEPES buffer to remove extracellular <sup>45</sup>Ca. Calcium uptake was determined by lysing cells with 0.2% sodium dodecyl sulfate. Radioactivity in aliquots of each lysate were measured by liquid scintillation counting in 10 mM Cytoscient (ICN, Biochemical Research Product, CA).

The effect of isoflurane on cellular uptake of added iron from the extracellular space was examined in a similar fashion using <sup>59</sup>FeCl<sub>2</sub> (American Radiolabeled Chemicals).

#### *Effect of Volatile Anesthetics and Iron on Mitochondrial Inner Membrane Polarization*

Mitochondrial membrane potential was determined with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraacetylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probe). Cells were loaded by changing culture medium to Mg<sup>2+</sup>-free BSS containing 10  $\mu$ M JC-1 and incubating at 37°C for 30 min. Thereafter, cells were washed twice with Mg<sup>2+</sup>-free BSS containing 20 mM HEPES buffer and dissolved anesthetics (0, 0.3, 1.2, or 3.1 mM isoflurane or 0.3, 1.1, 2.0, or 2.7 mM halothane, final concentration) were added. Fluorescence was measured 20 min after addition of anesthetic solutions at two sets of excitation–emission wavelengths (485–530 nm and 530–590 nm) in a BIO-TEK FL600 fluorescence plate reader (BIO-TEK Instruments, Winooski, VT). The ratio of measured intensities at both wavelengths was calculated as a measure of mitochondrial membrane polarization.

In an additional experiment, cultures were exposed to 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> at 37°C in the presence or absence of 1.2 mM isoflurane. Control cultures were not exposed to 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> or isoflurane. After 30 min, the mitochondrial membrane potential was measured. In other plates, 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> (and isoflurane if present)-containing media were removed and replaced with MEM supplemented with 20 mM glucose. Six hours later, mitochondrial membrane potential was measured as above. LDH release studies were performed in parallel

under identical treatment conditions with measurement made after 30 min of iron or iron-plus-isoflurane exposure.

#### Cell Types and Iron Sensitivity

Cell typing in the primary neuronal–glial cultures was performed using primary antibodies against cell-specific cytoskeletal filaments (NF-160 and GFAP). For these studies, cells were plated on poly-D-lysine-coated cover slips instead of plastic 24-well plates. For immunohistochemical staining, cells were washed with Hank's BSS and fixed with precooled absolute methanol for 10 min ( $-20^{\circ}\text{C}$ ). Cover slips were subsequently washed three times for 3 min in Hank's BSS + 0.1% bovine serum albumin, then exposed to primary antibodies (mouse monoclonal anti-NF-160 Clone NN18 [Sigma], 1:40 dilution; mouse monoclonal anti-GFAP Clone G-A-5 [Sigma], 1:200 dilution, 30  $\mu\text{l}$ /cover slip) and incubated 30 min at room temperature. Cover slips were washed three times for 3 min in Hank's BSS + 0.1% bovine serum albumin, then treated with secondary rhodamine-labeled antibody (antimouse immunoglobulin G (H+L)-L-rhodamine) for 30 min at room temperature, washed three times for 3 min in Hank's BSS + 0.1% bovine serum albumin, then mounted to glass slides using glycerol–Hank's BSS (1:2). Using digitized fluorescence microscopy, the percentage of NF-106 and GFAP positively staining cells were counted, respectively. Values were averaged from multiple counting sites from two different cultures.

Using the same cell-typing techniques, the types of cells injured by exposure to  $\text{FeSO}_4\text{--FeCl}_3$  were determined. Culture plates were exposed to 300  $\mu\text{M}$   $\text{FeSO}_4\text{--FeCl}_3$  at  $37^{\circ}\text{C}$  in the presence or absence of 1.2 mM isoflurane for 30 min. Control cultures were not exposed to 300  $\mu\text{M}$   $\text{FeSO}_4\text{--FeCl}_3$  or isoflurane. The media was then removed and replaced with MEM supplemented with 20 mM glucose. Twenty-four hours later, cells were stained with NF-160 and GFAP as described above. Using digitized fluorescent microscopy, the numbers of neurons and glia having normal and abnormal morphology were counted. The percentages of NF-160- and GFAP-positive cells having normal morphology (considered as alive) were calculated for each condition. In another experiment, the effect of 300  $\mu\text{M}$   $\text{FeSO}_4\text{--FeCl}_3$  with or without isoflurane was measured on the entire cell population by assessing the percentage of cells impermeable to trypan blue.

#### Statistical Analysis

Data were compared by one-way analysis of variance. When indicated by a significant F ratio, *post hoc* analysis was performed using the Scheffé F test. All data for aqueous phase  $\text{EC}_{50}$  values were analyzed using an iterative least square curve fit (KaleidaGraph; Synergy Software, Reading, PA). Values are reported as mean  $\pm$  SD. Significance was assumed when *P* was less than 0.05.

**Table 1. Effects of Volatile Anesthetics on Hydrogen Peroxide and Xanthine–Xanthine Oxidase–induced Lactate Dehydrogenase Release**

	Halothane	Isoflurane
Hydrogen peroxide (50 $\mu\text{M}$ )		
0.04 mM	101 $\pm$ 8	97 $\pm$ 7
0.8 mM	108 $\pm$ 14	103 $\pm$ 14
1.6 mM	113 $\pm$ 16*	98 $\pm$ 8
3.2 mM	123 $\pm$ 16*	100 $\pm$ 11
Xanthine–xanthine oxidase (4 mU/ml)		
0.04 mM	101 $\pm$ 8	103 $\pm$ 11
0.8 mM	109 $\pm$ 8	105 $\pm$ 6
1.6 mM	114 $\pm$ 8	102 $\pm$ 6
3.2 mM	120 $\pm$ 6*	102 $\pm$ 8
Malondialdehyde (50 $\mu\text{M}$ )		
0.8 mM	119 $\pm$ 14	100 $\pm$ 14
1.6 mM	126 $\pm$ 20*	95 $\pm$ 11
2.4 mM	125 $\pm$ 20*	98 $\pm$ 11

Concentrations of volatile anesthetic in cell culture media are given as mM values. Lactate dehydrogenase release values are mean  $\pm$  SD as percents values from cultures exposed to toxins in the absence of volatile anesthetic. N = 8 culture wells per condition.

\* *P* < 0.05 versus no treatment.

## Results

#### Effects Of Volatile Anesthetics On $\text{H}_2\text{O}_2$ , Superoxide, and Malondialdehyde-induced Cell Lysis

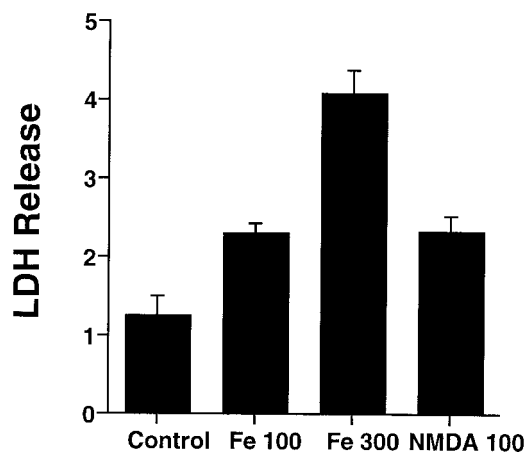
Neither halothane nor isoflurane in concentrations of up to 3.2 mM reduced LDH release caused by exposing cultures to  $\text{H}_2\text{O}_2$  (table 1). In fact, cultures treated with halothane (1.6–3.2 mM) were more damaged by  $\text{H}_2\text{O}_2$ -induced oxidative stress than cultures without halothane. No effect of isoflurane on  $\text{H}_2\text{O}_2$ -induced LDH release was observed at any isoflurane concentration (table 1). Deferoxamine (2 mM) completely protected the cultures against  $\text{H}_2\text{O}_2$ -induced cell lysis (data not shown).

Similarly, neither halothane nor isoflurane protected against cell damage induced by exposing cultures to the superoxide anion generating system, xanthine–xanthine oxidase. Halothane treatment was associated with an increased LDH release, whereas no effect was observed with isoflurane (table 1).

Exposure of the cultures to malondialdehyde caused a dose-dependent release of LDH (data not shown). Neither halothane nor isoflurane reduced malondialdehyde-induced LDH release. Halothane treatment was again associated with increased LDH release at concentrations of 1.6 mM or greater. This effect was not seen for isoflurane at doses up to 4.1 mM (table 1).

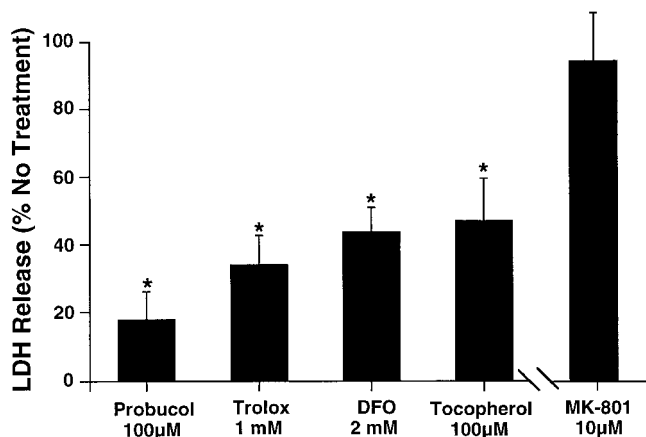
#### Iron-induced Oxidative Stress

Cultures were exposed to  $\text{FeSO}_4\text{--FeCl}_3$  (100  $\mu\text{M}$  or 300  $\mu\text{M}$ ) for 30 min and then allowed to recover for 24 h before LDH release was measured. Iron caused a dose-dependent increase in LDH release (fig. 1). Exposure of cells to 100  $\mu\text{M}$  NMDA resulted in approximately one half the severity of damage as was produced by 300  $\mu\text{M}$  iron.



**Fig. 1.** Release of lactate dehydrogenase (LDH) from neurons–glia after exposure to iron. Primary neuronal–glial cultures (13–16 days in culture) were exposed to iron (0–300  $\mu\text{M}$   $\text{FeCl}_2\cdot\text{FeSO}_4$ , 1:1) or to the neuronal excitotoxin, *N*-methyl-D-aspartate (NMDA; 100  $\mu\text{M}$ ) for 30 min in a balanced salt solution. LDH release was measured 24 h later. Values are mean  $\pm$  SD of 10 observations from two to three different cell preparations expressed in units of enzymatic activity (nanomoles lactate oxidized to pyruvate per minute at room temperature). All values were significantly different from each other ( $P < 0.001$ ), except there was no difference between Fe 100 and NMDA 100. Control = no iron–no NMDA; Fe 100 = 100  $\mu\text{M}$   $\text{FeCl}_2\cdot\text{FeSO}_4$ ; Fe 300 = 300  $\mu\text{M}$   $\text{FeCl}_2\cdot\text{FeSO}_4$ ; NMDA 100 = 100  $\mu\text{M}$  NMDA.

Iron-induced LDH release was decreased by pretreating cultures with either Trolox (100  $\mu\text{M}$ ), probucol (100  $\mu\text{M}$ ),  $\alpha$ -tocopherol (100  $\mu\text{M}$ ), or deferoxamine (2 mM), but not by the NMDA receptor antagonist, MK-801 (fig. 2).



**Fig. 2.** Protection against iron-induced lactate dehydrogenase (LDH) release. Mixed neuronal–glial cultures were treated with 100  $\mu\text{M}$  probucol, 1 mM Trolox, 2 mM deferoxamine (DFO), 100  $\mu\text{M}$   $\alpha$ -tocopherol, or 10  $\mu\text{M}$  MK-801 for 1 h before adding 300  $\mu\text{M}$  iron for 30 min. LDH release was assessed 24 h after exposure. Significant protection was observed for all antioxidants, although no effect was present for MK-801. Values are mean  $\pm$  SD of four to eight observations from one or two culture preparations expressed as a percentage of LDH release in control cultures (iron exposed–no treatment) where 100% of control = 1.09  $\pm$  0.24 units of enzymatic activity (nanomoles lactate oxidized to pyruvate per minute at room temperature). \* $P$  less than 0.01 versus no treatment.

### Prevention of Iron-induced Cell Lysis by Anesthetics

Treatment of cell cultures with media containing dissolved anesthetic during the 30-min exposure to 300  $\mu\text{M}$  iron produced dose-dependent protection against cell lysis (fig. 3). All three volatile anesthetics and propofol provided potent protection against iron-induced LDH release, albeit at different concentrations. The aqueous phase  $\text{EC}_{50}$  values (concentration producing a 50% reduction in LDH release) were calculated to be: halothane = 0.13  $\pm$  0.02 mM; isoflurane = 0.28  $\pm$  0.05 mM; and sevoflurane = 1.39  $\pm$  0.10 mM. Potency of protection by propofol was approximately two orders of magnitude greater than that of the volatile anesthetics. Elimination of calcium from the culture medium did not alter the potency of anesthetics in reducing LDH release in response to iron exposure (data not shown). Hexanol had similar potency in protecting against iron-induced LDH release (data not shown).

We performed additional studies directly comparing halothane, isoflurane, and sevoflurane treatment effects in sister cultures. Under conditions in which the cultures were treated at approximately equal aqueous concentrations for the respective anesthetics, sevoflurane was less protective against iron-induced cell lysis than either halothane or isoflurane (fig. 4A). However, when the treatment concentrations were adjusted so as to attain equal concentrations in the hydrophobic membrane space (by compensating for differences in saline–oil partition coefficients<sup>20–22</sup>), the volatile anesthetics were equally protective (fig. 4B).

We also observed a diminished protective effect of the volatile anesthetics by pretreating the cultures for 1 h and replacing the volatile anesthetic-containing medium immediately before a 30-min exposure to 300  $\mu\text{M}$  iron. In contrast, treatment with volatile anesthetic after oxidative stress resulted in an increase in LDH release (table 2).

The protection afforded by isoflurane against iron-induced injury was also apparent using trypan blue uptake as the marker of irreversible cell damage. Approximately 90% of cells (neurons + glia) were found to exclude trypan blue (indicating an intact plasma membrane) in cultures not exposed to iron. Only 28  $\pm$  4% of cells excluded the dye 24 h after exposure to 300  $\mu\text{M}$   $\text{FeSO}_4\cdot\text{FeCl}_3$  in the absence of isoflurane, whereas 58  $\pm$  6% of the isoflurane-treated, iron-exposed cells were found to exclude trypan blue. Isoflurane was found to protect both neurons and astrocytes against iron-induced damage in cultures where cells were immunolabeled with NF-160 and GFAP (table 3).

For all of the anesthetics tested, higher doses of the drugs did not protect against iron-induced LDH release. Because volatile anesthetics have been shown to interfere with mitochondrial oxidative phosphorylation *in vivo* at high concentrations,<sup>23</sup> we examined the effect of isoflurane and halothane administered in the absence of

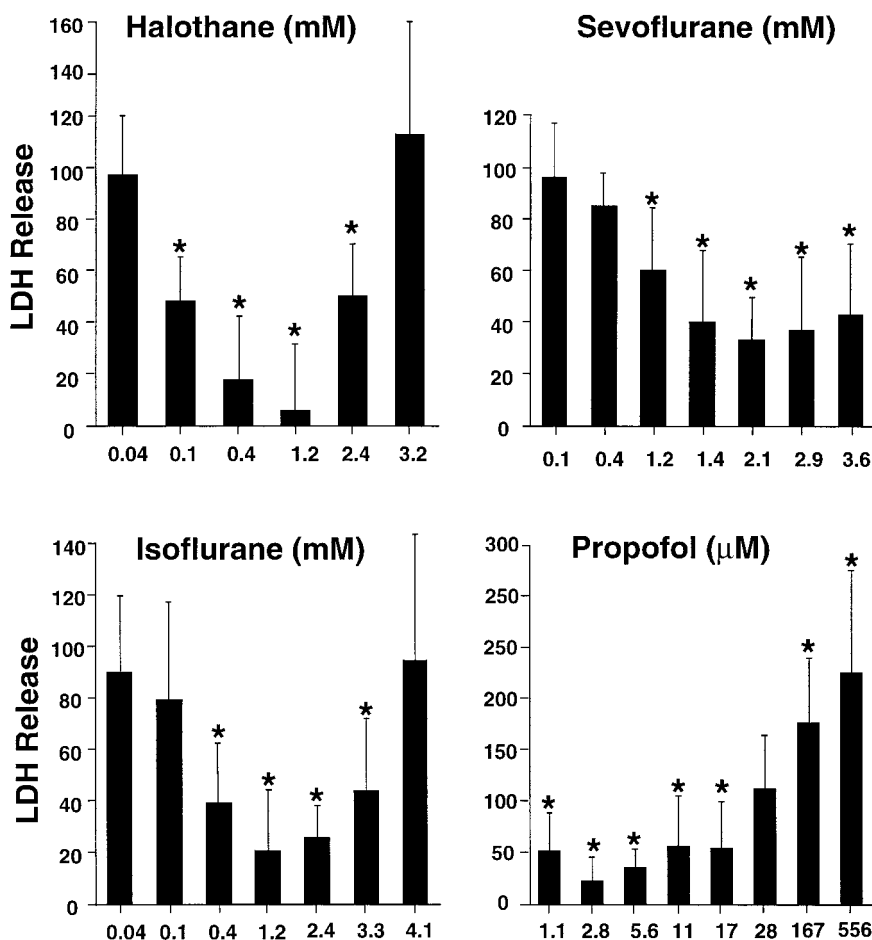


Fig. 3. Prevention of iron-induced cell lysis by dissolved anesthetics: dose response. Mixed neuronal–glial cultures were treated with anesthetic dissolved in culture medium at the indicated concentration immediately before iron exposure ( $300 \mu\text{M}$ , 30 min). All anesthetics protected against cell lysis (lactate dehydrogenase [LDH] release reported as percent of LDH release occurring in control cultures exposed to  $300 \mu\text{M}$  iron in the absence of corresponding anesthetic). Experiments were performed independently for each volatile anesthetic and its respective control (no anesthetic) group. Values are mean  $\pm$  SD ( $n = 16$ ) from at least two different culture preparations. \* $P$  less than 0.01 versus corresponding controls, where 100% control =  $1.03 \pm 0.11$  LDH activity units (halothane),  $1.29 \pm 0.32$  LDH activity units (sevoflurane),  $1.16 \pm 0.15$  LDH activity units (isoflurane), or  $0.91 \pm 0.12$  LDH activity units (propofol). LDH activity units = nanomoles lactate oxidized to pyruvate per minute at room temperature.

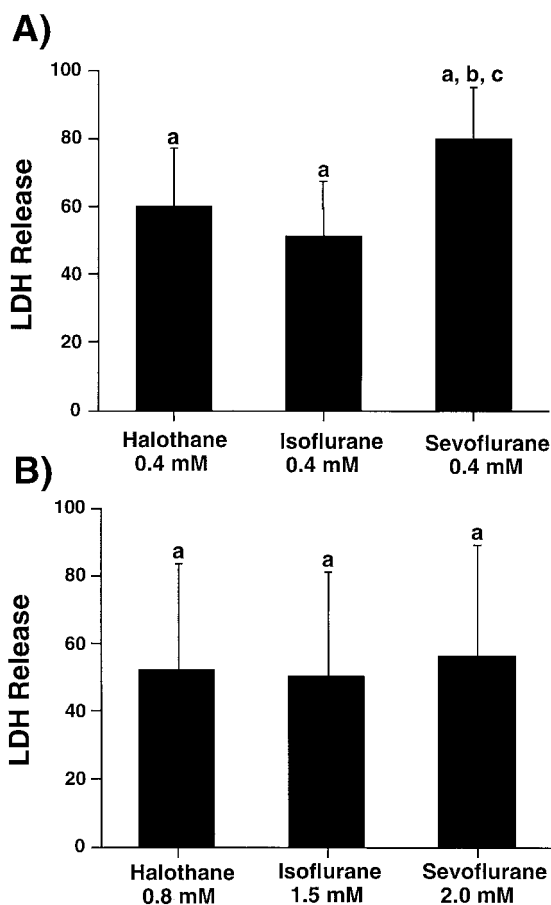
iron on mitochondrial inner-membrane polarization as a function of anesthetic concentration. We found no adverse effects of the anesthetics within the lower range of concentrations over which the anesthetics produced dose-dependent protection. However, at the higher range over which protection was progressively lost (*i.e.*, at isoflurane 3.1 mM and halothane 2.0 mM), discharge of the mitochondrial membrane potential occurred. Iron, in the concentration shown to cause cytotoxicity, also caused mitochondrial membrane discharge but only when assessed 6 h after exposure. This was inhibited by coadministration of 1.2 mM isoflurane (fig. 5). Concurrent studies showed a 40% reduction in iron-induced LDH release at 6 h by 1.2 mM isoflurane (control =  $0.71 \pm 0.12$ ;  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  alone =  $2.78 \pm 0.56$ ;  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  plus 1.2 mM isoflurane =  $1.95 \pm 1.05$  nmol lactate oxidized to pyruvate per minute at room temperature;  $P \leq 0.0001$ ;  $n = 10$  wells per condition).

To assure that effects of iron toxicity or anesthetic treatment on LDH release were not immediate and therefore lost to the medium exchange required after 30 min to halt exposure to iron and anesthetic, we examined LDH concentrations of the medium at termination of exposure to  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  in the presence or

absence of 1.2 mM isoflurane. There was no difference in LDH activity in the medium taken from these cultures versus cultures not exposed to iron or isoflurane (control =  $0.15 \pm 0.04$ ;  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  alone =  $0.13 \pm 0.05$ ;  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  plus 1.2 mM isoflurane =  $0.14 \pm 0.04$  nmol lactate oxidized to pyruvate per minute at room temperature;  $P = 0.60$ ;  $n = 10$  wells per condition), indicating that LDH release caused by iron exposure is delayed and that no artifact was introduced by the medium exchange.

#### Effects of Volatile Anesthetics on Iron-induced Lipid Peroxidation

The effects of volatile anesthetics on iron-induced lipid peroxidation were assessed in a cell-free lipid system. Iron exposure ( $25 \mu\text{M}$ ) resulted in TBARS accumulation that was completely inhibited by the water-soluble  $\alpha$ -tocopherol analog, Trolox. In this system, neither halothane (halothane 0 mM =  $216 \pm 16\%$ ; 1.2 mM =  $272 \pm 5\%$ ; 4.1 mM =  $266 \pm 16\%$ ; Trolox =  $116 \pm 12\%$  of control cultures not treated with iron or halothane) nor 1.2 mM isoflurane ( $247 \pm 11\%$  of control cultures not treated with iron or isoflurane) was found to decrease the generation of TBARS.



**Fig. 4.** Direct comparison of halothane, isoflurane, and sevoflurane in protecting against iron-induced lactate dehydrogenase (LDH) release. (A) Sister cultures were simultaneously treated with one of the anesthetics at a concentration of 0.4 mM during exposure to iron (300  $\mu$ M, 30 min). Under these conditions, sevoflurane was less protective than either halothane or isoflurane. (B) The concentrations of volatile anesthetic in the culture medium was adjusted so as to attain equal concentrations of volatile anesthetics in the cell membranes. All anesthetics conferred equivalent protection ( $P = 0.792$ ). Values are mean  $\pm$  SD ( $n = 16$ ) from at least two different culture preparations. <sup>a</sup> $P$  less than 0.05 versus controls (LDH release from untreated iron-exposed cultures); <sup>b</sup> $P$  less than 0.01 versus halothane-treated iron-exposed cultures; <sup>c</sup> $P$  less than 0.01 versus isoflurane-treated iron-exposed cultures. In (A), 100% =  $2.54 \pm 0.10$  nmol lactate oxidized to pyruvate per minute at room temperature in untreated iron-exposed control cultures. In (B), 100% =  $1.62 \pm 0.31$  nmol lactate oxidized to pyruvate per minute at room temperature in untreated iron-exposed control cultures.

#### Effects of Volatile Anesthetics on Calcium and Iron Uptake

In an effort to define the mechanism by which the anesthetics prevent iron-induced irreversible damage to neuronal-glial cultures, we assessed the effect of isoflurane (1.2 mM) on cellular uptake-internalization of extracellular calcium and iron. Neither iron exposure alone nor iron exposure in isoflurane-treated cultures resulted in a change in the rate of cellular calcium uptake ( $P = 0.34$ ; fig. 6A). In contrast, isoflurane treatment reduced cellular uptake-internalization of extracellular iron ( $P = 0.04$ ; fig. 6B).

**Table 2.** Comparison of Volatile Anesthetic Treatment Timing on Lactate Dehydrogenase Release Caused by 30-min Exposure to 300  $\mu$ M Iron

(min)	Halothane	Isoflurane
Pretreatment		
1.2	92 $\pm$ 8*	91 $\pm$ 6*
2.4	94 $\pm$ 11	92 $\pm$ 8
4.1	153 $\pm$ 11*	76 $\pm$ 6*
Posttreatment		
1.2	108 $\pm$ 11	107 $\pm$ 6*
2.4	124 $\pm$ 14*	113 $\pm$ 14*
4.1	247 $\pm$ 14*	117 $\pm$ 17*

Concentrations of volatile anesthetic in cell culture media are given as mM values. Lactate dehydrogenase release values are mean  $\pm$  SD as percents of values from cultures exposed to toxins in the absence of volatile anesthetic.  $N = 8$  culture wells per condition.

\*  $P < 0.05$  versus no treatment.

#### Discussion

The principal findings of this study were that volatile anesthetics offered no direct protection against oxidative stress induced by exposure of primary mixed neuronal-glial cultures to hydrogen peroxide or to a superoxide-generating system, xanthine-xanthine oxidase. In contrast, all three volatile anesthetics provided a dose-dependent protective effect against iron-induced cytotoxicity (for both neurons and glia) at concentrations similar to those used to produce clinical anesthesia. Potency was dependent on timing of treatment and whether aqueous phase or lipid phase anesthetic concentrations were considered. Although the anesthetics reduced iron-induced LDH release, there was no effect on toxicity from malondialdehyde, a lipid peroxidation metabolite. Furthermore, there was no effect of the volatile anesthetics on iron-induced lipid peroxidation in a cell-free system. Using isoflurane as a prototype volatile anesthetic, we found reduction in iron but not calcium uptake in cells exposed to iron. This was associated with protection against a delayed mitochondrial depolarization. Cumulatively, these data indicate that volatile anesthetics do not serve as direct antioxidants. In the unique case of iron toxicity, volatile anesthetic protection appears to be attributable to inhibition of iron uptake or stabilization of the mitochondrial membrane potential rather than direct antioxidant activity.

**Table 3.** Percentage of Alive Neurons and Glia in Cultures Exposed to 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> with or without 1.2 mM Isoflurane

	300 $\mu$ M FeSO <sub>4</sub> -FeCl <sub>3</sub> Alone	300 $\mu$ M FeSO <sub>4</sub> -FeCl <sub>3</sub> + Isoflurane
Neurons (%)	13 $\pm$ 4	55 $\pm$ 16*
Glia (%)	16 $\pm$ 8	53 $\pm$ 24*

Cell viability was defined by trypan blue exclusion. Values are mean  $\pm$  SD relative to cultures receiving neither 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> nor isoflurane.

\*  $P < 0.01$  compared with 300  $\mu$ M FeSO<sub>4</sub>-FeCl<sub>3</sub> alone.

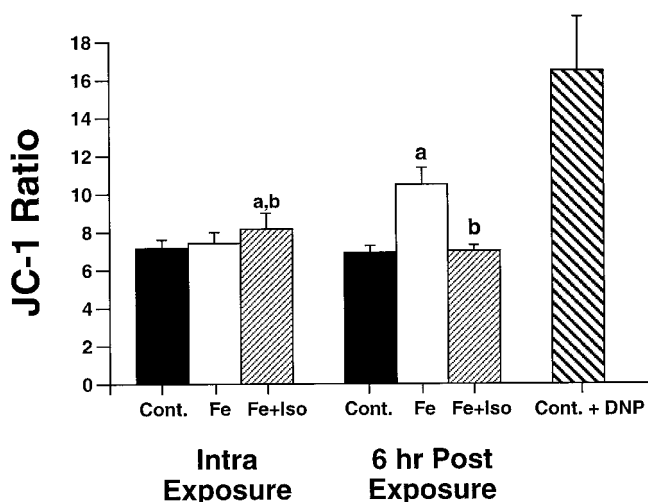


Fig. 5. Effect of exposure of cultures to iron with or without isoflurane exposure on mitochondrial membrane potential. Cultures were pretreated with  $10 \mu\text{M}$  5,5',6,6'-tetrachloro-1,1',3,3'-tetraacetylbenzimidazolylcarbocyanine iodide (JC-1) for 30 min, washed, and then exposed to iron or iron plus isoflurane. Fluorescence was measured at either 20 min after onset of iron or iron-plus-isoflurane exposure (Intra Exposure) or 6 h after completion of a 30-min exposure (6 h Post Exposure) at two sets of excitation-emission wavelengths (485–530 nm and 530–590 nm). The ratio of fluorescence intensity measured at each wavelength was calculated as mitochondrial membrane polarization. Higher ratios indicate depolarization of the mitochondrial inner membrane. Cont. = untreated control cultures; Fe =  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$ ; Fe + Iso =  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$  + 1.2 mM isoflurane. Values are mean  $\pm$  SD. <sup>a</sup>*P* less than 0.01 versus untreated controls; <sup>b</sup>*P* less than 0.01 versus Fe. N = 16 culture wells per condition. Cont. + DNP = effects of  $500 \mu\text{M}$  dinitrophenol (a potent uncoupler of oxidative phosphorylation).

Because we know of no other work that has specifically examined this issue in neural tissue, it is difficult to directly compare our work to that of others. In other organ or cellular systems, results of studies examining interactions between volatile anesthetics and free radical injury have been mixed. *In vivo*, exposure to halothane has been shown to cause free radical formation and impairment of the hepatic antioxidant defense system.<sup>24,25</sup> Inhibition of the renal antioxidant defense system has also been shown for isoflurane in guinea pigs exposed to hyperoxia as defined by attenuation of superoxide dismutase and catalase activities.<sup>26</sup> In rats, a 2-h exposure to halothane resulted in hepatic lipid peroxidation, and this was enhanced when coadministered with hypoxia.<sup>27</sup> Finally, in rats subjected to isoflurane anesthesia in the absence of any ischemic-hypoxic insult, hippocampal nitric oxide production was increased, allowing possibility that increased peroxynitrite formation could occur under conditions of enhanced superoxide production.<sup>28,29</sup> Cumulatively, *in vivo* evidence, if anything, suggests that volatile anesthetics might enhance free radical injury in ischemic-hypoxic brain.

In contrast, *in vitro* work using isolated rat hepatocytes found no evidence of halothane-induced free rad-

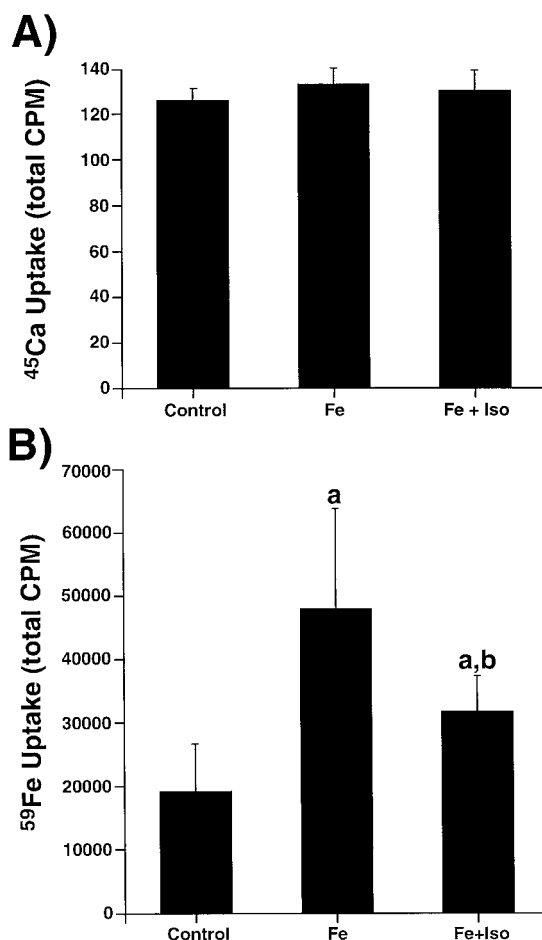


Fig. 6. Effects of isoflurane on uptake-internalization of extracellular calcium and iron in cells exposed to iron. Radioactive cations (<sup>59</sup>Fe or <sup>45</sup>Ca) were added to the culture medium immediately before exposure to  $300 \mu\text{M}$   $\text{FeSO}_4\text{-FeCl}_3$ . The amount of cation uptake was assessed after 30-min incubation at 37°C. (A) Neither iron exposure alone (Fe) or in the presence of 1.2 mM isoflurane (Fe + Iso) affected the rate of calcium uptake as compared with controls receiving no treatments (Control). (B) Iron exposure caused an increase in uptake-internalization of extracellular iron. The increase was less in cultures treated with isoflurane during iron exposure. Values are mean  $\pm$  SD (n = 16 culture wells for <sup>45</sup>Ca uptake study, n = 6 for <sup>59</sup>Fe uptake study). <sup>a</sup>*P* less than 0.05 versus untreated controls; <sup>b</sup>*P* less than 0.05 versus <sup>59</sup>Fe alone. CPM = counts/min.

ical formation, but halothane also had no effect on cell death when the hepatocytes were exposed to hypoxic conditions.<sup>30</sup> When either sevoflurane or isoflurane were examined in an isolated heart ischemia-reperfusion preparation, no enhancement of salicylate trapping (a marker of hydroxyl radical production) was observed relative to untreated controls.<sup>31</sup> However, in an isolated rat liver preparation, isoflurane significantly reduced superoxide generation on reperfusion from a hypoxic insult.<sup>32</sup> To our knowledge, no *in vitro* work has been performed in brain tissue.

Given the unique case of volatile anesthetic protection against iron-induced toxicity, we considered the possibility that the protective effects of the volatile anesthetics might be related to blockade of astrocyte gap junc-



tions but rejected this based on prior work by Blanc *et al.*,<sup>33</sup> who demonstrated that gap junction inhibitors increase neuronal vulnerability to iron toxicity. We then examined whether, instead of serving as antioxidants, volatile anesthetics might increase tolerance of the cultures to oxidant injury. Minakami and Fridovich<sup>34</sup> have shown protective effects of alcohols against cold shock injury in *Escherichia coli*. When logarithmic phase cells are subjected to cold shock (e.g., 0–15°C), weakening of cellular membranes occurs. The severity of damage is a function of the rate of temperature reduction, suggesting differential sensitivities of the inner and outer plasma-membrane to temperature. Introduction of long-chain alcohols (e.g., *n*-octanol, *n*-hexanol) caused a dose-dependent increase in bacterial respiration and survival. These alcohols are known to increase membrane fluidity similar to volatile anesthetics that might reduce membrane disruption.<sup>35</sup> Therefore, hexanol was examined in parallel to cultures treated with the volatile anesthetics during iron exposure. Indeed, similar efficacy of hexanol was found for reducing LDH release. This is consistent with the fact that anesthetic potency against iron toxicity was related to the calculated lipid partitioning of the volatile anesthetics (fig. 4).

If it was true that volatile anesthetics inhibited iron toxicity by reducing membrane disruption, it would be essential to show increased membrane permeability to material normally regulated in its flux across the plasma-membrane. Accordingly, we performed the iron and calcium uptake studies in cultures exposed to iron. There was no effect of iron toxicity on calcium uptake, but cellular iron uptake was selectively increased, and this was inhibited by isoflurane. We therefore cannot conclude that generalized volatile anesthetic effects on membrane fluidity are responsible for the protection observed against iron toxicity. Instead, we conclude that volatile anesthetics reduce iron toxicity by reducing iron uptake.

As previously stated, iron is known to catalyze conversion of hydrogen peroxide to the hydroxyl radical. Selective uptake of iron could promote intracellular hydroxyl radical formation. We did not specifically measure hydroxyl radical formation or accumulation of any reactive products. However, mitochondrial membrane polarization is known to become altered under conditions of oxidative stress, and this is accentuated by increased iron concentrations.<sup>36,37</sup> Indeed, we observed mitochondrial depolarization after iron exposure and at least partial reversal of this effect by isoflurane. We speculate that isoflurane primarily reduced iron-induced cell death by inhibiting iron uptake, which in turn reduced intracellular oxidative stress, but we cannot rule out the possibility that isoflurane also provided a direct stabilizing effect on the mitochondrial membrane potential.

It was interesting that the effect of volatile anesthetics against iron toxicity was present only at lower concen-

trations. Although the drugs caused a dose-dependent reduction in iron-induced LDH release, larger doses actually accentuated injury. The toxicity at higher doses of halothane was also observed when cells were coadministered hydrogen peroxide, xanthine-xanthine oxidase, or malondialdehyde. This caused us to examine the direct neurotoxic effects of these anesthetics. Indeed, higher doses of volatile anesthetic administered alone stimulated LDH release and mitochondrial depolarization. This may explain the reversal of protection observed against iron toxicity observed at higher doses of volatile anesthetic. However, the clinical relevance of this is likely to be small. For example, an isoflurane dose of 3.1 mM was required to induce LDH release and cause mitochondrial depolarization. This is approximately equivalent to 10 minimum alveolar concentration which greatly exceeds doses used in clinical practice.

The goal of this series of experiments was to elucidate potential mechanisms by which volatile anesthetics reduce ischemic brain injury. Damage from reactive oxygen species is a known component of ischemic brain injury. However, the study could provide no evidence that volatile anesthetics serve as primary antioxidants. The case for iron toxicity appears to be spurious and is most likely attributable to effects on iron uptake by neurons and glia. Other mechanisms must be invoked to explain the repeated observations that volatile anesthetics protect against ischemic brain damage *in vivo*. The one finding of this study that warrants further investigation is the effect of isoflurane (and presumably halothane and sevoflurane) on preventing mitochondrial depolarization during oxidative stress. Examination of this issue in a system not reliant on iron toxicity (e.g., oxygen-glucose deprivation) would be of interest.

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