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Halothane and Isoflurane Increase Pulmonary Artery Endothelial Cell Sensitivity to Oxidant-mediated Injury

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Volatile anesthetics inhibit phagocytic cell function, yet little is known about their effects on target tissues or on the target tissue response to stimulated phagocytes. Experiments were performed to determine how exposure to halothane and isoflurane changes rat pulmonary artery endothelial cell (RPAEC) viability in response to the toxic oxygen metabolites produced by stimulated phagocytic cells. RPAECs were grown in monolayer culture. The monolayers were treated with phorbol myristate acetate (PMA) –stimulated human neutrophils at an effector-to-target ratio of 20:1 after equilibration with 0.4% or 1.7% halothane or 0.7% or 2.8% isoflurane. As measured by percent-specific release of incorporated ^{51}Cr label (mean ± SE), cytotoxicity in the presence of 1.7% halothane (75.3 ± 3.4%) was significantly greater ($P < 0.02$) than cytotoxicity in 5% CO_2 in air (44.7 ± 3.3%) and in 0.4% halothane (57.3 ± 4.7%). Also, cytotoxicity in 1.7% halothane was significantly greater than in 0.4% halothane ($P < 0.02$). The authors found that RPAECs incubated in isoflurane exhibited significantly greater release of ^{51}Cr than cells incubated in the MAC equivalent concentrations of halothane: 78.2 ± 2.6% in 0.7% isoflurane ($P = 0.0004$) and 83.8 ± 1% in 2.8% isoflurane ($P = 0.005$). Because early neutrophil cytotoxicity has been found to be mediated primarily by hydroxyl radical (HO·) and hydrogen peroxide (H_2O_2), the authors measured H_2O_2 production by similar numbers of PMA-stimulated neutrophils under similar exposure conditions. In carrier gas, PMA-stimulated neutrophils produced 20.5 ± 1.3 nmol $H_2O_2 \cdot 10^6$ cells$^{-1} \cdot h^{-1}$. At the higher concentrations of halothane, H_2O_2 production actually was inhibited in comparison with carrier gas (15.4 ± 1.4 nmol $H_2O_2 \cdot 10^6$ cells$^{-1} \cdot h^{-1}$ in 1.7% halothane and 16.8 ± 0.8 in 2.8% halothane), but the degree of inhibition did not reach statistical significance. In isoflurane, however, H_2O_2 production was not different from that seen in carrier gas. In other experiments, the monolayers were treated with 0, 200, 500, and 1,000 μM H_2O_2 after equilibration with 0.4%, 1.7%, and 2.8% halothane or 0.7%, 2.8%, and 5% isoflurane in 5% CO_2 in air. Efficiency of replating was used to measure degree of injury. Both halothane and isoflurane enhance the sensitivity of the RPAEC monolayers to injury by H_2O_2. The sensitizing effect of halothane was reversed by removing the anesthetic. Halothane and isoflurane thus enhance RPAEC sensitivity to injury by both H_2O_2 and PMA-stimulated neutrophils. In increasing RPAEC sensitivity to injury by oxygen metabolites, halothane and isoflurane may be inhibiting processes involved in intracellular antioxidant defenses. Use of volatile anesthetics in patients at risk for oxidant-mediated end-organ injury thus may enhance the magnitude of such injuries. (Key words: Anesthetics, volatile: halothane; isoflurane. Hydrogen peroxide: cytotoxicity. Lung: pulmonary artery endothelium. Superoxide.)

VOLATILE ANESTHETICS have been shown to have profound effects on the functions of immune effector and phagocytic cells of all types. Studies early in this century described inhibition of neutrophil phagocytic function and bacterial killing by such drugs as ether, chloroform, and alcohol.[1,2] More recent investigations have similarly demonstrated that inhalational anesthetics inhibit neutrophil bactericidal function and respiratory burst activity.[3–6] These studies, however, deal only with the effects of anesthetic agents on phagocytic cell function. The question of how the responses to inflammation of end organs and tissues themselves are affected by anesthetic agents remains to be addressed.

Both rat and bovine pulmonary artery endothelial cells are susceptible to injury from hydrogen peroxide (H_2O_2) and from neutrophils stimulated with a phorbol ester, phorbol myristate acetate (PMA).[7,8] In previous work using the *ex vivo* perfused rabbit lung, we found that inhalational anesthetics potentiate the pulmonary vascular pressor response to the organic oxidant *tert*-butyl-hydroperoxide.[9] Additionally, in lungs treated with inhalational anesthetics and challenged with *tert*-butyl-hydroperoxide, significant alterations occur in the arachidonate mediator profile, with increased production of thromboxane A_2, and in the endothelial barrier function, with a decreased rate of pulmonary edema formation, compared with lungs ventilated with carrier gas.[9,10] Therefore, we hypothesized that, if inhalational anesthetics potentiate pulmonary vasoreactivity and mediator production and preserve endothelial barrier function in oxidant-injured, isolated whole lungs, then inhalational anesthetics will alter the response of rat pulmonary artery endothelial cells (RPAECs) *in vitro* to oxidant-induced injury. Experiments were conducted to test this hypothesis by use of cultured RPAEC monolayers exposed to H_2O_2 or to PMA-stimulated human neutrophils. The results suggest that the combination of anesthetic plus oxidant may be more damaging to tissues and end organs than is oxidant alone.

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Materials and Methods

Endothelial Cells

After institutional approval was obtained, RPAECs were isolated in the laboratory of Dr. Una S. Ryan (University of Miami School of Medicine) by a method described elsewhere.¶,**

Briefly, the pulmonary artery, left ventricle, and trachea of the pentobarbital-anesthetized rat were cannulated after the animal was anticoagulated with heparin (500 U/kg) and killed by exsanguination. The lungs were inflated with 20 ml air administered through the tracheal cannula. The lungs were perfused with warm (37° C) phosphate-buffered saline until they were cleared of blood. Subsequently, at a rate of 15 ml/min, the lungs were perfused with 0.02% EDTA in phosphate-buffered saline containing approximately 600 microcarrier beads/ml (40–80 μm). The direction of perfusion was then reversed, and fractions of effluent-containing microcarriers were collected, washed, and plated in 25-cm^2 culture flasks in Ryan Red medium supplemented with 20% fetal calf serum.

After subsequent steps were performed that are required to obtain a pure culture of endothelial cells, the cells were characterized by their high angiotensin-converting enzyme activity ($3.2 \times 10^4 - 1.9 \times 10^5$ molecules per cell). This assay was performed using the Ventrex converting enzyme activity radioassay system (Ventrex Laboratories, Portland, ME), with [^{3}H]Benzoyl-phe-ala-pro as the substrate, and by their affinity for antibodies to Factor VIII by indirect immunofluorescence using antihuman Factor VIII–related antigen (Atlantic Antibodies, Scarborough, ME) and fluorescein-conjugated rabbit antigoat IgG (DAKO Corporation, Santa Barbara, CA) as the secondary antibody, just before shipment to our laboratories. Cells were subcultured into contact-inhibited monolayers with the typical "cobblestone" appearance by phase-contrast light microscopy. Once in our laboratories, the cells were discarded after a maximum of three to four additional passages. The day before the experiment, the RPAEC monolayers were released into single-cell suspension using 0.3% trypsin in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}), washed twice with Minimum Essential Medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Whittaker Bioproducts, Walkersville, MD) (MEM-FCS), and plated in 24-well cluster plates (Costar, Cambridge, MA) at a density of approximately 10^5 cells per well. At this density, the monolayers grew to 90–95% confluence after an overnight incubation.

Delivery of Anesthetic Vapor

Rat pulmonary artery endothelial cell monolayers were exposed to volatile anesthetic by equilibration of the wells containing culture medium with anesthetic vapor in carrier gas. Five percent CO_2 in air (carrier gas) was drawn across agent-specific vaporizers containing liquid anesthetic used in clinical practice, through a manifold, and thence into an air-tight exposure chamber in a water bath heated to 37° C (fig. 1). The multiwell plates were placed in a layer of water within the airtight exposure chamber to ensure adequate temperature control of the medium and cells in the wells. The volume of medium per well never exceeded 1 ml, and the depth was less than 5 mm. The concentration of vapor delivered was ascertained by sampling the chamber atmosphere on both the inflow and the outflow sides and measuring the anesthetic concentration by gas chromatography (GOW-MAC Instruments, Madison, NJ). In other experiments described below, the anesthetic concentration in buffer in the wells of the multiwell plates also was determined by gas chromatography, after extraction of the aqueous phase by *n*-heptane.

In previous work, we have demonstrated that anesthetic concentration in 5 ml medium completely equilibrates with atmospheric vapor in 10 min.[11] In the experiments that we describe here, the equilibration period lasted 40 min. This pretreatment duration of exposure was chosen because, with use of the particular method that we describe, anesthetic concentration in the buffer (measured by gas chromatography after *n*-heptane extraction) was

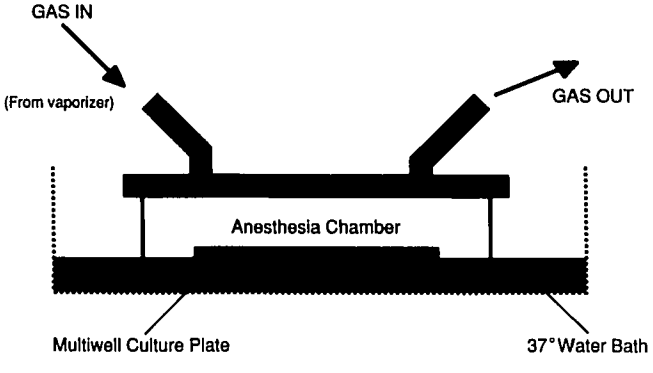

FIG. 1. The anesthesia exposure chamber. The chamber consists of an airtight Plexiglas® box, divided into two separate chambers, so that two anesthetic concentrations can be administered simultaneously. The multiwell plates are maintained at 37° C by a layer of water at the bottom of the exposure chamber. The anesthetic and CO_2 concentrations are intermittently determined at both the gas inflow and outflow ports.

¶ Ryan US, White L: Microvascular endothelium isolation with microcarriers: Arterial, venous. Journal of Tissue Culture Methods 10: 9–13, 1986.

** These cells were provided by Dr. Una S. Ryan of the University of Miami School of Medicine by the 19th passage. Because they were all of the same cell line isolated from the same lung, many passages were required to obtain enough cells with which to work.

found to equilibrate completely with atmospheric vapor after 30–40 min.

Neutrophil-induced Cytotoxicity Assays

In experiments using PMA-stimulated neutrophils, RPAEC cytotoxicity was determined by specific release of ^{51}Cr.[7] The goal of these experiments was to determine whether halothane and isoflurane modulate the interaction between RPAEC monolayers and stimulated neutrophils, which injure endothelial cells through toxic oxygen metabolites.[7] Recent studies have documented that cytotoxicity based on measurement of specific ^{51}Cr release accurately assesses cell death but, in comparison with replating efficiency, slightly underestimates the extent of injury.[12–14]

On the day of the experiment, 60 ml heparinized peripheral venous blood was obtained from a healthy human volunteer donor. Neutrophils were isolated by Ficoll centrifugation and dextran sedimentation and suspended in Hank's Balanced Salt Solution (GIBCO) supplemented with 0.02% bovine serum albumin (HBSS-BSA) (Sigma Chemical Company, St. Louis, MO).[15] The cell suspension was kept on ice until needed, but never longer than 2 h. With the use of this method, populations of cells consisting of 94% neutrophils with 98% viability by Trypan blue dye exclusion typically are obtained. We usually isolated $15–20 \times 10^7$ neutrophils from a single donor.

Rat pulmonary artery endothelial cell monolayers were incubated for 18 h in 24-well plates. Each well in the plate contained 1 ml MEM-FCS, to which 4 μCi $Na[^{51}Cr]O_4$ (DuPont-New England Nuclear, Wilmington, DE) was added.

On the day of the experiment, the RPAEC monolayers were washed three times with fresh HBSS-BSA to remove residual radioactivity. HBSS-BSA plus the appropriate reactants were then added to each well to achieve a final volume of 1 ml per well. To obtain spontaneous ^{51}Cr release, only HBSS-BSA was added to the well; and, to obtain total release, 0.1% (final concentration) Triton X-100 was added. To determine the effect of the reactants alone (not in combination) on ^{51}Cr release, either PMA (50 nM final concentration) or neutrophils at an effector-to-target ratio of 20:1 were added to the well. At the time the neutrophils were added, each well on the plate contained a monolayer consisting of approximately 2×10^5 cells. A period of 40 min was provided after addition of neutrophils to allow the neutrophils to settle on and adhere to the RPAEC monolayers; this process is essential for proper neutrophil–RPAEC interaction.[16,17] During this period, the multiwell plates were equilibrated with carrier gas; halothane, 0.4% or 1.7% in carrier gas; or isoflurane, 0.7% or 2.8% in carrier gas.

After addition of 50 nM PMA to the appropriate wells, mixtures were incubated in their respective atmospheres for a total of 6 h from the time PMA was added. This incubation period was chosen because previous work by Gannon *et al.* showed that, after 4 h, the specific ^{51}Cr release by RPAEC monolayers treated with PMA-stimulated neutrophils is measurable.[8] All conditions were replicated six times for each experiment. At the end of the incubation period, 0.8 ml buffer was removed from each well and centrifuged at 1,500 rpm for 5 min. The supernatant was then transferred to counting vials and the radioactivity measured in a gamma counter. The release of ^{51}Cr attributable to RPAEC cytotoxicity was determined with the following formula:

$$\% \text{ specific } ^{51}Cr \text{ release} = \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}\right) \cdot 100$$

Stimulated Neutrophil Production of H_2O_2

To determine whether halothane and isoflurane affected neutrophil H_2O_2 production, we measured the concentration of H_2O_2 in HBSS-BSA by the method described by Thurman *et al.*[18] In this assay, the ability of H_2O_2 to oxidize Fe^{2+} to Fe^{3+} is used to form a ferrithiocyanate complex, which is colored and exhibits peak absorbance at 480 nm. This assay can detect H_2O_2 concentrations less than 10 μM. Neutrophils were obtained from a single donor (JRS), as described previously.

In siliconized glass culture tubes, 4×10^6 cells were suspended in 0.8 ml HBSS-BSA containing 2 mM sodium azide to inhibit endogenous catalases. The culture tubes were sealed with specially fitted gas-tight caps and subsequently gassed with 0%, 0.4%, 1.7%, or 2.8% halothane or with 0%, 0.6%, 2.5%, or 4% isoflurane, in carrier gas, for 12 min. In each experiment, two sets of duplicate or triplicate tubes were gassed with each anesthetic concentration, one anesthetic per experiment. The neutrophil suspensions were allowed to warm to 37° C for 30 min. Then, 50 nM (final concentration) PMA was added to one set of tubes and additional buffer to the other; the incubation at 37° C was continued for 1 h. At the end of this period, 100 μL 50% trichloroacetic acid (for a final concentration of 5%) was added to each tube to stop all reactions, and the tubes were centrifuged at 2,000 rpm for 10 min. The supernatant was then transferred to 12×75 mm clean glass test tubes. Ferrous ammonium sulfate was added to each test tube, for a final concentration of 1.5 mM, followed by potassium thiocyanate (both from Sigma), for a final concentration of 250 mM. The absorbance of the red ferrithiocyanate complex was measured in a Gilford Model 250 spectrophotometer. Before each experiment, a standard curve was constructed using di-

lutions of stock 30% reagent H_2O_2, the concentrations of which were verified by measuring absorbance at 240 nm. Unknown H_2O_2 concentrations were calculated on the basis of the standard curve for that day.

H_2O_2-INDUCED CYTOTOXICITY ASSAYS

To assess the effects of halothane and isoflurane on the sensitivity of RPAEC monolayers to injury by direct application of H_2O_2, we measured the efficiency of replating after injury. Replating efficiency is assumed to be directly proportional to cell viability—that is, the greater the number of viable cells, the greater the efficiency of replating. Over the time course of these experiments (70 min), we did not find that ^{51}Cr was released from labeled cells after treatment with H_2O_2; ^{51}Cr release did not become detectable until 4–6 h after injury. On the day before the experiment, 10^5 cells in MEM-FCS were transferred to each well of 24-well plates and incubated for 18 h at 37° C in 5% CO_2 in air. On the day of the experiment, old medium was removed and replaced with fresh sterile MEM-FCS, and monolayers were first equilibrated for 40 min with carrier gas (5% CO_2 in air) alone; halothane (0.4%, 1.7%, and 2.8%) in carrier gas; or isoflurane (0.7%, 2.8%, and 5%) in carrier gas. Reagent H_2O_2 (Sigma), obtained as a 30% solution, was then added to the wells to obtain final concentrations of 0, 200, 500, and 1,000 μM.

All wells were then incubated in the equilibrating gas mixture for another 30 min after the addition of 0, 200, 500, or 1,000 μM H_2O_2 to triplicate wells. At the end of this period, 100 U bovine liver catalase (Sigma) was added to each well to remove excess H_2O_2. The medium was removed from the wells, and trypsin was added to release adherent RPAEC monolayers into cellular suspension. The cell suspension was transferred to 12 × 75 mm glass test tubes containing 1 ml fresh MEM-FCS. These test tubes were then centrifuged at 1,500 rpm for 10 min, after which time the supernatant was decanted and replaced with 1 ml fresh MEM-FCS. After thorough mixing of the cell suspension, the fresh medium was transferred to the wells of 24-well cluster plates and incubated overnight at 37° C in 5% CO_2 in air.

The following day, the wells were washed once with fresh medium to remove unattached cells, and the monolayers were resuspended with 0.5 ml trypsin. Detachment of cells from the wells was verified by phase contrast microscopic examination. In all wells examined, virtually 99–100% of the monolayer was removed. The number of cells in each well was determined with an electron particle counter (Coulter Corporation, Hialeah, FL). Specific cytotoxicity was calculated with the following formula:

$$\%\ \text{cytotoxicity} = \left(\frac{\text{control well cell count} - \text{experimental well cell count}}{\text{control well cell count}}\right) \cdot 100$$

A family of dose-response curves for the effects of halothane and isoflurane on H_2O_2-mediated cytotoxicity was thus obtained. Each condition was replicated three times in each experiment. For both anesthetics, experiments were repeated three times, with similar results each time.

REVERSIBILITY OF HALOTHANE-ENHANCED RPAEC SUSCEPTIBILITY TO H_2O_2-INDUCED INJURY

To determine whether the sensitizing effect of halothane is reversible, we measured the change in replating efficiency of H_2O_2-injured endothelial cells over time after removal of halothane from the atmosphere. Three different groups of RPAEC monolayers were used: cells never exposed to halothane (carrier gas only—group 1), cells exposed only to halothane in carrier gas (group 2), and cells equilibrated with halothane in carrier gas but subsequently incubated in carrier gas alone (group 3). In each group, monolayers were treated with either 0 or 400 μM H_2O_2. Group 1 monolayers were divided into two subgroups, one treated with 400 μM H_2O_2 immediately after an initial 40-min period of equilibration, and the second treated with 400 μM H_2O_2 after a 90-min delay. Group 2 cells were treated with H_2O_2 after equilibration with 4% halothane in carrier gas for 40 min and immediately replaced in the halothane-containing atmosphere for the additional 30-min incubation period. We elected to use a high concentration of halothane in this group of experiments because we wanted the assurance of a maximal anesthetic. In group 3, monolayers all were equilibrated initially with 4% halothane in carrier gas for 40 min but then removed from the halothane-containing atmosphere for 1, 2, 5, 10, 60, and 90 min before challenge with H_2O_2. Group 3 monolayers were treated in duplicate rather than triplicate (as in all other experiments), primarily because of the space limitations of the 24-well plates. At the end of the H_2O_2 challenge, monolayers were treated according to the H_2O_2 cytotoxicity assay protocol, and the percentage of cytotoxicity was determined. These experiments were repeated three times, with similar results each time.

STATISTICAL ANALYSIS

Data are expressed as mean ± SE of the data from replicate wells. To determine the main effects of injury

and anesthetic exposure on cell viability, we used two-way analysis of variance (ANOVA). If the two-way ANOVA demonstrated a statistically significant main effect of anesthetic on cell viability ($P < 0.05$), and more than two doses of anesthetic were used, we used a one-way repeated measures ANOVA to examine within-group differences and Scheffé's test for multiple comparisons. For between-group multiple comparisons, we used the Bonferroni-corrected t test. $P < 0.05$ was considered to be statistically significant throughout.

Results

Effect of Halothane and Isoflurane on Neutrophil-induced RPAEC Cytotoxicity

Rat pulmonary artery endothelial cell injury was enhanced after equilibration with 0.4% and 1.7% halothane or 0.7% and 2.8% isoflurane, compared with injury in monolayers treated with carrier gas (fig. 2). Isoflurane 0.7% induced a significantly greater enhancement ($P = 0.0004$) of neutrophil-mediated injury than did the equivalent MAC concentration of halothane (0.4%). Similarly, the enhancement of neutrophil-mediated injury induced by 2.8% isoflurane was greater than that seen in the presence of 1.7% halothane ($P = 0.005$). In uninjured monolayers, either anesthetic increased specific ^{51}Cr release to no more than 4%. In all cases, spontaneous release was approximately 10% of total release. PMA alone had virtually no effect on ^{51}Cr release, and neutrophils alone never increased specific ^{51}Cr release to more than 6%. At equilibrium, buffer concentration of halothane was 691 μM in the 1.7% atmosphere and 190 μM in the 0.4% atmosphere; buffer concentration of isoflurane was 367 μM in the 2.8% atmosphere and 127 μM in the 0.7% atmosphere.

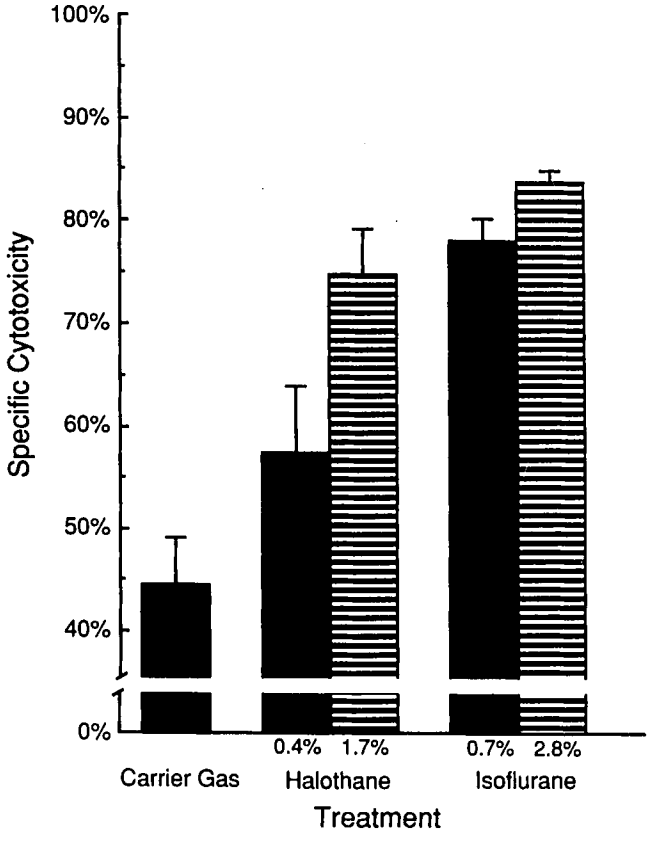

FIG. 2. Effect of two different doses of halothane and isoflurane on neutrophil-mediated cytotoxicity. Incubation of RPAEC monolayers in a halothane-containing atmosphere increases their sensitivity to injury by PMA-stimulated neutrophils at a concentration of 1.7% but not at 0.4%. Neutrophil-mediated cytotoxicity in isoflurane, however, is enhanced at both the 0.7% and 2.8% concentrations to a greater degree than in halothane. 0.7% isoflurane greater than 0.4% halothane, $P = 0.0004$; 2.8% isoflurane greater than 1.7% halothane, $P = 0.005$; 1.7% halothane greater than 0.4% halothane, $P < 0.02$. Vertical bars denote standard error.

Effect of Halothane and Isoflurane on H_2O_2 Production by PMA-stimulated Neutrophils

On the basis of these results, we wanted to determine whether halothane and isoflurane affected the neutrophil or target cell (endothelium) primarily. Because stimulated neutrophils exert their early cytotoxic activity by production of hydroxyl radicals and H_2O_2, we measured H_2O_2 production by similar numbers of PMA-stimulated neutrophils in the same buffers equilibrated with similar concentrations of halothane and isoflurane (table 1). In carrier gas, neutrophils, when stimulated, were found to produce 20.5 ± 1.3 nmol $H_2O_2 \cdot 10^6$ cells$^{-1} \cdot$ h^{-1} (n = 16); however, unstimulated neutrophils did not produce H_2O_2 in concentrations detectable by the assay method used. Stimulated neutrophils, when equilibrated with halothane (0.4%, 1.7%, and 2.8%) in carrier gas (n = 7), actually were found to produce less H_2O_2 than neutrophils in carrier gas alone, especially at the highest halothane concentrations. This inhibition of H_2O_2 production did not reach statistical significance. When equilibrated with isoflurane (0.7%, 2.5%, and 3.8%) in carrier gas (n = 9), H_2O_2 production by stimulated neutrophils was not appreciably different from production by neutrophils equilibrated with carrier gas alone. Thus, endothelial cells exhibit greater sensitivity to neutrophil-mediated injury when exposed to halothane and isoflurane, despite production of similar quantities of H_2O_2 by neutrophils in carrier gas and in volatile anesthetic.

TABLE 1. Hydrogen Peroxide Production (nmol · 10^{-6} cells · h^{-1}) by PMA-stimulated Neutrophils in Varying Concentrations of Volatile Anesthetics

Concentration (MAC)	Anesthetic	
	Halothane (n = 7)	Isoflurane (n = 9)
0	19.3 ± 1.8	21.5 ± 2.1
0.5	20.2 ± 1.5	23.3 ± 2.3
2	15.5 ± 1.4	21.8 ± 1.6
3	16.8 ± 0.8	22.6 ± 1.9

Data are expressed as mean ± SE.

0 represents 5% CO_2 in air (carrier gas). Concentrations of halothane are 0.4% (0.5 MAC), 1.7% (2 MAC), and 2.8% (3 MAC) and of isoflurane are 0.7% (0.5 MAC), 2.5% (2 MAC), and 4% (3 MAC), all in carrier gas.

Effect of Halothane and Isoflurane on H_2O_2-induced RPAEC Injury

On the basis of previous results, and on the assumption that the RPAEC injury induced by PMA-stimulated neutrophils results from reactions mediated by the hydroxyl radical (HO·), we hypothesized that a similar injury pattern would appear in RPAECs treated directly with H_2O_2. Figure 3 demonstrates the morphologic features of RPAEC monolayers after replating.

Halothane enhances RPAEC susceptibility to peroxide-induced injury at the highest halothane concentration (fig. 4). It is interesting that endothelial cell replating efficiency in 0.4% halothane exceeded replating efficiency in carrier gas by 40% and 18% at the 500 μM and 1,000 μM concentrations of H_2O_2, respectively. This difference suggests a protective effect of low halothane concentrations on H_2O_2-mediated injury. In 2.8% halothane, significantly increased H_2O_2-induced injury occurs, with virtually complete loss of replating ability at the 500 μM and 1,000 μM doses of H_2O_2. In contrast with the pattern of injury seen with halothane, however, RPAEC injury is enhanced on exposure of monolayers to isoflurane at all concentrations studied in comparison with monolayers not exposed to agent (fig. 5). The anesthetics themselves had no effect on replating efficiency in monolayers not treated with H_2O_2.

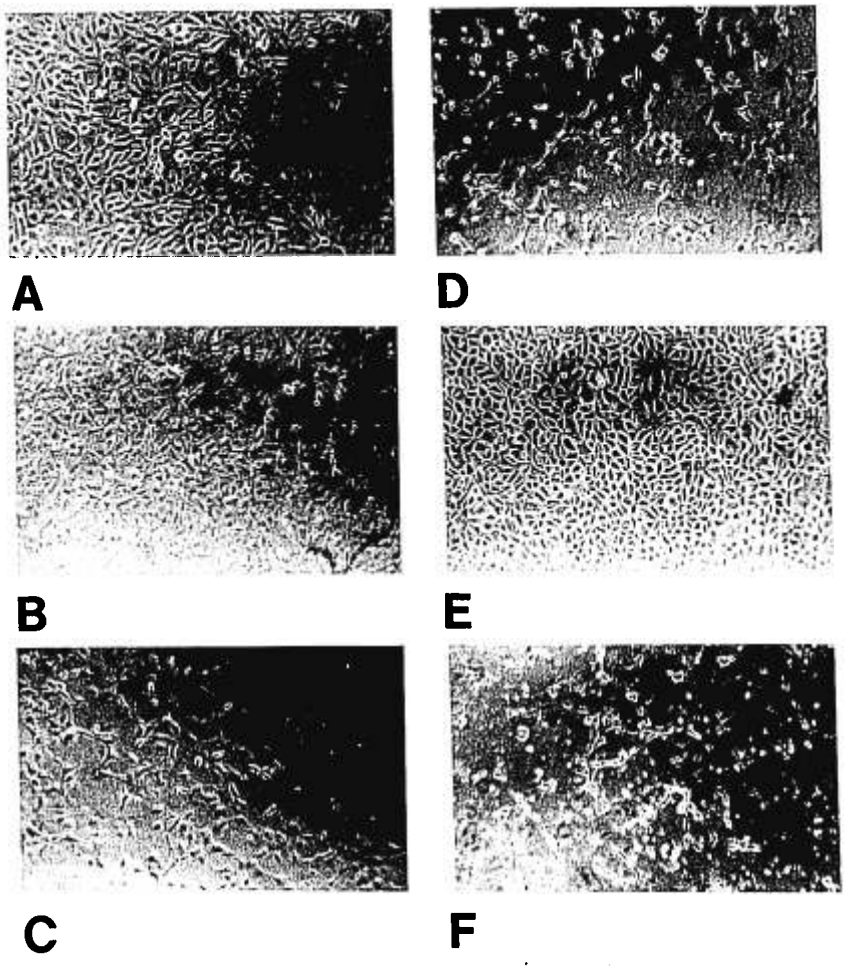

FIG. 3. Phase-contrast light photomicrographs of RPAEC monolayers after replating. *A:* Control monolayer, uninjured, incubated in air/CO_2. Note typical cobblestone appearance and near confluence of cells. *B:* Monolayer injured with 1,000 μM H_2O_2 while in air/CO_2. Note cellular debris and decreased density of monolayer compared with *A*, implying cell death. *C:* Monolayer equilibrated with isoflurane 0.7% and then injured with 1,000 μM H_2O_2. Compared with *B*, this monolayer exhibits a lower cell density. *D:* Monolayer equilibrated with 2.8% isoflurane, and then injured with 1,000 μM H_2O_2. Note paucity of attached cells compared with *B* and *C* and plentiful cellular debris. *E:* Monolayer equilibrated with 5% isoflurane, but uninjured. This monolayer shows nearly complete cellular confluence with minimal debris. Compare with *A*. *F:* Monolayer equilibrated with 5% isoflurane and then injured with 1,000 μM H_2O_2. Note almost total absence of attached cells, which are partially obscured by highly reflective cellular debris. Magnification ×100 for *A–F*.

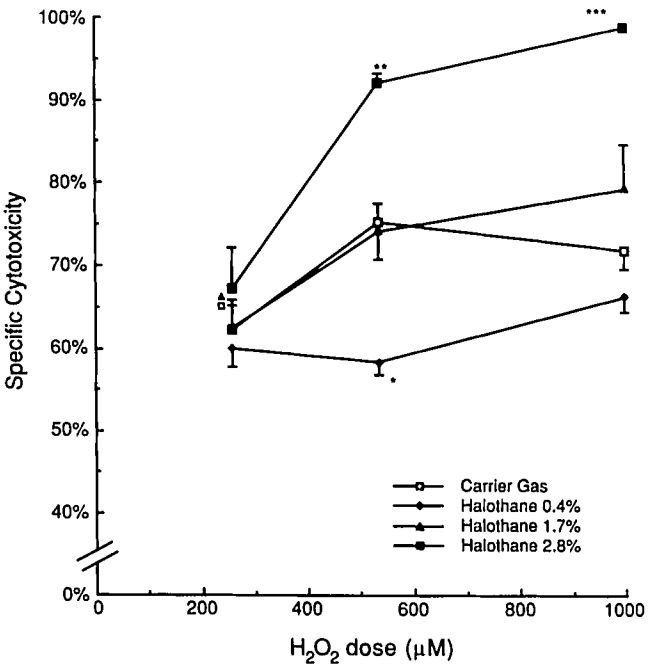

FIG. 4. The effect of halothane on H_2O_2-induced RPAEC injury. Cells equilibrated with low (0.5 MAC) concentrations of halothane actually appear to be protected against injury by H_2O_2. This protective effect disappears as the halothane concentration is increased. $N = 9$ for each data point. *Less than carrier gas, $P = 0.0041$; **greater than carrier gas, $P = 0.015$; and ***greater than carrier gas, $P = 0.0001$. Vertical bars denote standard error. The triangle to the left of the 200-μM H_2O_2 point represents the top of the 1.7% halothane standard error bar; the square beneath the triangle represents the top of the carrier gas standard error bar.

Reversibility of Halothane-Enhanced RPAEC Susceptibility to H_2O_2-Induced Injury

Our results (fig. 6) indicate that RPAEC sensitization to H_2O_2 associated with halothane exposure is reversed readily with increasing duration of time after removal of halothane from the gas phase. When H_2O_2 is added to monolayers 1 min after removal from halothane, H_2O_2-induced cytotoxicity is nearly equal to that seen with monolayers never exposed to halothane (group 1). At 2, 5, and 10 min after removal of the monolayers from halothane, cytotoxicity after H_2O_2 treatment appears to be less than that of group 1 monolayers. At 60 and 90 min after removal of the monolayers from halothane, however, cytotoxicity returns to values similar to those seen in the group 1 monolayers. These results are consistent with our finding (fig. 4) of a biphasic concentration-dependent response of RPAECs to halothane.

In additional experiments, we determined the residual halothane concentration in 1-ml aliquots of Hank's Balanced Salt Solution after equilibration with 4% halothane for 40 min and subsequent exposure to carrier gas alone at 37° C for 0, 1, 2, 5, 10, 60, and 90 min by gas chromatography after extraction of the medium with *n*-heptane; then we compared these values with those obtained from medium equilibrated with 1.7% (691 μM) and 0.4% (190 μM) halothane for 40 min. At time 0, halothane concentration was 1,051 μM; at 1 min after removal of anesthetic from the gas phase, 1,061 μM; at 2 min, 1,048 μM; at 5 min, 740 μM; at 10 min, 636 μM; at 60 min, 325 μM; and at 90 min, 85 μM. By 5 min after removal of halothane from the gas phase, the concentration of the anesthetic in Hank's Balanced Salt Solution approached the concentration found in medium equilibrated with 1.7% halothane.

Discussion

The data from these experiments suggest the following: 1) both halothane and isoflurane enhance injury mediated by PMA-stimulated neutrophils (with isoflurane exerting a greater effect than halothane) but do not alter the pro-

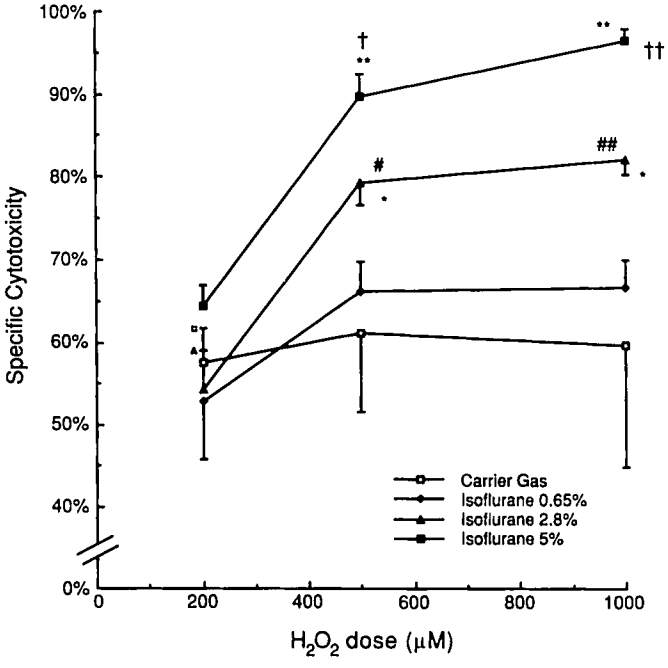

FIG. 5. The effect of isoflurane on H_2O_2-induced RPAEC injury. Unlike the cells equilibrated with halothane, cells equilibrated with isoflurane exhibit evidence of increased injury in all the concentrations of isoflurane used. The degree of cytotoxicity does not become statistically significant, however, until 2.8% isoflurane is used. $N = 9$ for each data point. *Greater than 200 μM H_2O_2, $P < 0.001$; **greater than 200 μM H_2O_2, $P < 0.0001$; #greater than carrier gas, $P = 0.002$; ##greater than carrier gas, $P = 0.002$; †greater than carrier gas, $P = 0.002$; and ‡‡greater than carrier gas, $P = 0.0003$. Vertical bars denote standard error. The triangle to the left of the 200-μM H_2O_2 point represents the top of the isoflurane 2.8% standard error bar; the square above the triangle represents the top of the carrier gas standard error bar.

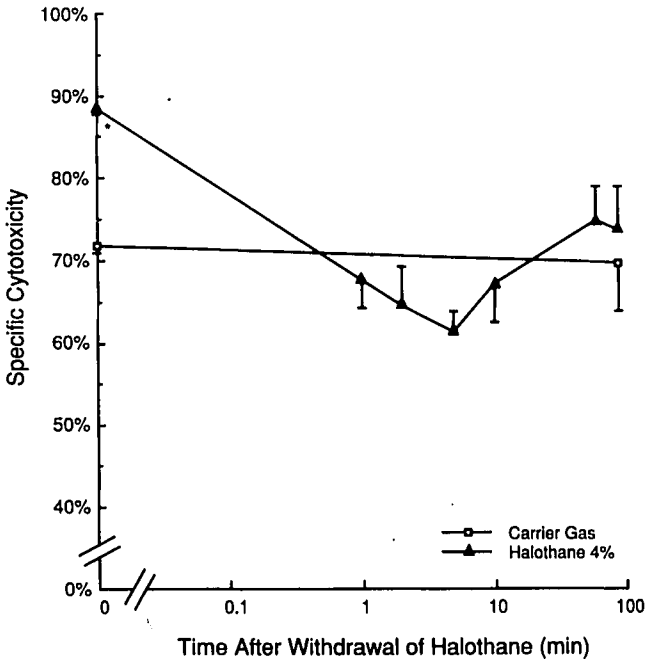

FIG. 6. Reversibility of halothane effect of RPAEC sensitivity to H_2O_2-induced injury. In these experiments, we show that the sensitizing effect of high concentrations of halothane on H_2O_2-induced cytotoxicity, as determined by the replating efficiency assay, is reversed by removal of the cells from the anesthetic. Five minutes after removal from halothane, RPAEC monolayers show significantly less cytotoxicity ($P = 0.003$) than monolayers exposed only to carrier gas. $N = 9$ each for group 1 and 2 monolayers; $n = 6$ for group 3 monolayers. *Greater than carrier gas, $P < 0.05$. Vertical bars denote standard error.

duction of H_2O_2 by stimulated neutrophils in a statistically significant manner; 2) both halothane and isoflurane increase the sensitivity of RPAECs to direct H_2O_2-mediated injury; and 3) the halothane effect is readily reversible.

At first glance, the enhancement by volatile anesthetics of endothelial cell cytotoxicity induced by PMA-stimulated neutrophils may seem to be at variance with other published results, in which volatile anesthetics have been shown to inhibit neutrophil bactericidal function,[4] oxidative activity,[5] and superoxide production,[6] as well as H_2O_2 production by peripheral blood monocytes.[19] Our data, however, demonstrate that H_2O_2 production by PMA-stimulated neutrophils is affected little by halothane and isoflurane. Although H_2O_2 production in the presence of halothane showed a tendency to decrease, the difference from H_2O_2 production in the presence of carrier gas alone was not statistically significant. In the presence of isoflurane, H_2O_2 production remained virtually unchanged from that in the presence of carrier gas alone.

On the basis of these findings, we conclude that halothane and isoflurane enhance RPAEC sensitivity to injury because of an anesthetic-mediated diminution of the ability of the target cell (the RPAEC) to withstand oxidative stress. This conclusion was supported by the experiments performed using direct H_2O_2 injury, in which exposure of RPAEC monolayers to halothane and isoflurane enhanced the injurious effect of H_2O_2.

Because the activity of endogenous catalase tends to bring about a rapid decline in H_2O_2 concentrations in the presence of cells, the duration of exposure to cytotoxic levels of H_2O_2 in these experiments was probably briefer than that in the neutrophil experiments. The initial concentrations of H_2O_2 that we used (200, 500, and 1,000 μM), however, are within the range of final peroxide concentrations measured by us and by other investigators during PMA stimulation of neutrophils.[20]

The injury enhancement associated with halothane exposure was readily reversed by removal of halothane from the gas phase. The reason the endothelial cell monolayers did not exhibit a sustained enhancement of injury during the first few minutes after removal of halothane from the gas phase (fig. 6) was that the multiwell plates holding the monolayers remained in a halothane-free atmosphere during the injury phase of the experiment. By 30 min after removal of halothane from the gas phase, the buffer concentration of halothane (530 μM) was less than one third the initial concentration. In the H_2O_2 cytotoxicity experiments, this buffer concentration was insufficient to generate an enhanced response to injury. The reversibility of the effect of high concentrations of halothane indicates that injury enhancement may be a pharmacologic, rather than a cytotoxic, effect of this drug. The lack of significant release of ^{51}Cr by RPAEC monolayers exposed either to halothane or to isoflurane alone—plus absence of diminished replating efficiency in uninjured monolayers exposed to isoflurane and halothane compared with carrier gas—lends additional support to this concept.

Adherence interactions between neutrophils and endothelial cells do not appear to be a critical factor in changing RPAEC vulnerability to injury in the presence of halothane and isoflurane, because RPAEC sensitivity to injury was enhanced both in the system using neutrophils and in the system using H_2O_2 alone. Other neutrophil-related factors may be important, however, because of the clear differences in the injury patterns of halothane-exposed monolayers treated with a bolus dose of H_2O_2 in comparison with those treated with stimulated neutrophils.

In other related work, we have demonstrated volatile anesthetic-mediated alterations in the function of the endothelial barrier in the pulmonary vascular bed. In the isolated *ex vivo* rabbit lung, perfused without blood, pretreatment of the lung with halothane is associated with enhanced output of thromboxane B_2 in the effluent perfusate in response to oxidant challenge.[9,10] On the mi-

croscopic level, we have shown that direct injury of RPAEC monolayers equilibrated with halothane or isoflurane by H_2O_2 is associated with greater loss of cell–cell contact, greater loss of cell surface area, and a greater degree of vimentin intermediate filament disaggregation and redistribution away from the peripheral areas of the cell cytoplasm toward the perinuclear region, compared with monolayers treated with peroxide alone in the absence of anesthetic agent.[21] These additional data provide added support for the supposition of a volatile anesthetic-mediated perturbation in target cell function that tends to increase sensitivity to oxidative stress-related damage.

Our experimental data do not address directly the mechanisms of changes in target cell functions on exposure to anesthetics, nor do they address which functions are changed. Based on these studies, as well as reports concerning oxidant-mediated injury to the vascular endothelium and volatile anesthetic effects on other end organs, such as liver, certain hypotheses about mechanisms can be proposed.

H_2O_2 and HO· are the major oxygen metabolites involved in endothelial cell cytotoxicity induced by activated neutrophils.[7,8,22] The mechanism of injury may involve stimulation of HO· production by the endothelial cell itself by Fenton kinetics.[23] This increased intracellular oxidant load can arise through two separate, but related, pathways. In one pathway, superoxide anion is generated as an end product of purine reductive metabolism accompanying conversion of internal stores of xanthine dehydrogenase to xanthine oxidase by an as-yet unclear mechanism initiated during attack by stimulated neutrophils.[7,8,22,24] In the other, more complex, pathway, receptor-mediated stimulation of a G protein activates phospholipase C, which, in turn, releases diacylglycerol and inositol triphosphate (IP_3) from membrane-associated phosphatidylinositol.[25] The free diacylglycerol induces protein kinase C activation, and IP_3, Ca^{2+} mobilization.[23] One of the results of this step is activation of phospholipase A_2, and resultant release of free arachidonate, which is rapidly metabolized by cyclooxygenases and lipoxygenases to various eicosanoid mediators.[23] Many intermediates in these arachidonate pathways are unstable lipid peroxides or epoxides, which release superoxide in the spontaneous process of breaking down to more stable lipid mediators.[26,27] Superoxide anion rapidly undergoes dismutation, and, in the presence of Fe^{2+}, the H_2O_2 produced in superoxide dismutation appears as short-lived HO· (Haber-Weiss reaction).[8] These oxygen metabolites not only neutralize antiproteases and enhance neutrophil elastase-induced cellular injury,[28] but also attack DNA, inducing strand breaks, stimulation of poly(ADP-ribose) polymerase, and subsequent depletion of intracellular energy stores.[29]

If we assume that the target cell, the RPAEC, displays evidence of increased injury because of a greater oxidant load accrued in the presence of halothane and isoflurane, what processes could be inhibited or stimulated to produce this greater oxidant load? The tendency of highly lipophilic volatile anesthetics to dissolve in hydrophobic cell membranes makes membrane-associated processes the most likely to be affected by halothane and isoflurane. The glutathione redox cycle constitutes such an intimately membrane-associated intracellular antioxidant system. The relationship between antioxidant profiles and endothelial cell vulnerability to oxidant injury is well established.[30] Inhibition of glutathione redox cycling, or hexose monophosphate shunt activity, enhances H_2O_2-mediated injury to endothelial cells.[31–33] In fasted rats, halothane and possibly enflurane, but not isoflurane, have been shown to deplete hepatic stores of reduced glutathione.[34,35] Thus, at least in the case of halothane, increased RPAEC sensitivity to injury may result from inhibition of protective antioxidant systems.

Similarly, the processes involved in the protein kinase C-Ca^{2+} pathway are also primarily membrane associated. Hyslop *et al.* have shown that H_2O_2-mediated injury is related to disturbances in intracellular Ca^{2+} homeostasis, with enhanced mobilization of Ca^{2+} from sequestered internal stores in the presence of H_2O_2.[36] We recently demonstrated similar changes in Ca^{2+} homeostasis in single endothelial cells.[37] Our data also show that halothane and isoflurane enhance the H_2O_2-mediated release of Ca^{2+} from sequestered internal stores, with isoflurane exerting a greater effect than the MAC-equivalent concentration of halothane.[37] Because IP_3 plays a key role in regulation of intracellular Ca^{2+} exchange, these results indicate that halothane and isoflurane may exert an enhancing effect on the regulation of inositol polyphosphate turnover. The intimate association of arachidonate with inositol regulation[23] may indicate, in turn, that increased release of free arachidonate and arachidonate-derived mediators constitutes a potential source of oxidant loading in anesthetic-treated endothelial cells. Indirect support for this hypothesis is derived from our work demonstrating augmented release of thromboxane B_2 from oxidant-injured isolated lungs ventilated with halothane.[9]

Another possible source of intracellular oxidant loading may involve enhanced conversion of xanthine dehydrogenase to xanthine oxidase in the presence of volatile anesthetics. The paucity of information regarding the mechanism of this pathway, however, leads to difficulties in developing support for this hypothesis. A third source of intracellular oxidant loading may be related indirectly to regulation of intracellular *p*H gradients. Halothane is known to collapse *p*H gradients across membrane-bound cell compartments.[38] This disturbance in the homeostasis

of intracellular *p*H may increase availability of membrane-associated Fe^{2+} for participation in the Haber-Weiss reaction, thus increasing opportunities for the dismutation of superoxide and the appearance of H_2O_2 and $HO\cdot$.

Our experimental data demonstrate that oxidant RPAEC injury is increased during exposure of the cells to volatile anesthetic agents because of an as-yet undefined anesthetic-mediated effect on the target cell. The increase in sensitivity to oxidant injury is reversible with removal of the anesthetic. Possible mechanisms of the increase in cytotoxicity are proposed. Although substantial research has been conducted focusing on the alterations in phagocytic cell function accompanying anesthetic exposure, few studies have examined changes induced by anesthetics in the responses of end organs or tissues, other than liver, to inflammation or injury. On the basis of our observations on the effects of halothane and isoflurane on RPAEC responses to oxidants, and recognizing that these observations were made on an *in vitro* model, we tentatively suggest that exposure to volatile anesthetics may increase risk of lung injury edema, and perhaps other end-organ damage, in patients who already incur risk by virtue of concomitant disease processes (*e.g.*, sepsis, intraoperative cardiac arrest, or prolonged exposure to high oxygen concentrations).

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