Effect of Halothane on Hypoxic Toxicity and Glutathione Status in Cultured Rat Hepatocytes

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Background: In hypoxic rats, halothane causes hepatotoxicity at oxygen levels that would cause minimal hepatotoxicity in the absence of halothane. Using a model that excludes systemic and extrahepatic effects of halothane, the authors tested the hypothesis that halothane hepatotoxicity in the whole-rat model is caused by a direct hepatotoxic effect of halothane, which is mediated by halothane-derived free radicals.

Methods: Rat hepatocyte monolayer cultures were exposed to defined gas phases for 2 h. Three experimental variables were present or absent: hypoxia (1% O2), halothane (2%), and cytochrome P-450 induction (by phenobarbital). Two experimental outcomes were measured: aspartate aminotransferase release, a measure of cell death, and reduced glutathione, an endogenous free radical scavenger whose levels are decreased by physiologically significant free radical injury.

Results: As anticipated, hypoxia increased cell death. Cytochrome P-450 induction by itself increased cell death during hypoxia. However, halothane had no effect on cell death during hypoxia, with or without cytochrome P-450 induction. Halothane had no toxic effect, even when glutathione was depleted before the onset of hypoxia. Glutathione was decreased moderately by hypoxia alone. Neither halothane nor cytochrome P-450 induction had any effect on glutathione levels.

Conclusions: Halothane was not toxic, and it did not generate a physiologically significant free radical insult during hypoxia in the isolated rat hepatocyte under the experimental conditions used in testing. (Key words: Anesthetics, volatile: halothane. Enzymes: cytochrome P-450. Free radical. Glutathione. Hypoxia. Liver: toxicity. Metabolism. Methods: cell culture. Phenobarbital.)

IN clinical practice, the volatile anesthetic halothane is associated with occasional hepatotoxicity.1 In whole rat experiments, both hypoxia and cytochrome P-450 induction are required for halothane to cause hepatotoxicity.2–7 Both hypoxia and cytochrome P-450 induction enhance the reductive metabolism of halothane, generating potentially toxic free radicals. The liver’s unique dual arterial and venous supply creates areas of extremely low partial pressure of oxygen (P02).8 A small amount of reductive halothane metabolism always occurs, even at a fraction of inspired oxygen >0.5 and without cytochrome P-450 induction.4,9 Consequently, if halothane hepatotoxicity is caused by reductive metabolism, then the use of halothane, despite its marked cost advantages and potential clinical advantages,10–13 ought to be avoided.

Recommendations to avoid halothane are supported by the findings of Schieble et al.14 In their experiments, these investigators used rat hepatocyte monolayer cultures to achieve a uniform P02 and eliminate extrahepatic effects of halothane, a major advance in the study of halothane hepatotoxicity. They reported that 1.5% halothane was hepatotoxic independent of P02 and cytochrome P-450 induction, i.e., “the first evidence of a directly hepatotoxic effect of halothane in non-enzyme-induced rats.”14 However, their hepatocyte cultures had very high rates of cell death during hypoxia alone (i.e., without halothane), raising the possibility that the hepatocytes were injured before hypoxic exposure and making it difficult to interpret the response to halothane that was reported.

Furthermore, it is difficult to reconcile the findings of Schieble et al.14 with numerous whole rat studies showing that cytochrome P-450 induction is an essential precondition for halothane hepatotoxicity.2–7 Although the cardiovascular effects of halothane might
worsen hepatotoxicity during hypoxia, it is unlikely that they would ameliorate or mask any direct hepatotoxicity of halothane in the uninduced whole animal. Furthermore, isoflurane and enfurane are metabolized much less than halothane, but can cause hepatotoxicity in the hypoxic, enzyme-induced rat.\cite{2,3,15} However, Schiebel et al.\cite{14} found no toxic effect of isoflurane on hypoxic, cultured hepatocytes.

Therefore, we used hepatocyte monolayer cultures to address the question of whether halothane is hepatotoxic during hypoxia, independent of cardiovascular and other systemic factors. We derived cultures from a rat strain (Sprague-Dawley) known to exhibit halothane hepatotoxicity in vivo in a model well characterized in previous reports.\cite{2,15} Our studies with this strain, under conditions approximating the in vivo model, address the question: Is halothane hepatotoxic in the whole rat model caused by a direct hepatotoxic effect of halothane or by other, extrahepatic effects?

Because halothane-derived free radicals are generated during reductive metabolism and have been postulated to be the mechanism of halothane hepatotoxicity,\cite{16-18} we also tested the effect of halothane on intracellular reduced glutathione during hypoxia. Glutathione is required for and oxidized by innate cellular defenses against free radicals, including those generated by halothane (fig. 1).\cite{19} Glutathione deficiency worsens free radical-induced toxicity, while free radical production depletes glutathione. Decreased glutathione is an early marker of free radical insult and is evident before overt cell death occurs.\cite{20-22} Although several earlier studies investigated the effect of halothane on glutathione in hypoxic rats, using whole animals, the findings of these studies were divided on whether glutathione decreases or stays the same with halothane, and on whether interventions that alter glutathione also affect halothane hepatotoxicity.\cite{6,7,24} Those studies also were limited by the systemic effects of halothane and the inhomogeneity of cellular oxygen and nutrient supply in the whole animal. In some studies, relatively nonspecific assays for glutathione were used. We circumvented these problems by using a specific high pressure liquid chromatography assay to analyze glutathione in hepatocyte monolayer cultures. We sought to determine whether halothane administered during hypoxia decreases glutathione. We asked the specific question: Does halothane generate a physiologically significant free radical insult during hypoxia?

**Materials and Methods**

All experiments were approved by the Mayo Institutional Animal Care and Use Committee. Where indicated, the cytochrome P-450 system was induced by 0.2% phenobarbital (PB), added to the drinking water of the rats for 4 days before the animals were killed.

Culture plates were 35 x 10 mm (Primaria Falcon, Lincoln Park, NJ) and coated with collagen (Sigma rat tail, type I, Sigma, St. Louis, MO) before they were used. The collagen was dissolved in a minimal volume of 0.1 M acetic acid, then diluted with distilled water to a concentration of 0.1 mg collagen/ml. One milliliter of this solution was added to each culture plate. The plates were incubated overnight, uncovered, under germicidal ultraviolet lights, then washed well with distilled water.

Fed, male Sprague-Dawley rats from Hilltop Laboratories (Scotsdale, PA) were anesthetized with 20–25 mg thiopental via intraperitoneal injection. After their hepatocytes were harvested, the rats were killed by exsanguination. All hepatocytes used for a single experiment were taken from a single rat. Hepatocytes were harvested and cultured in a method similar to that described in published reports.\cite{25} All buffers, media, and incubators were maintained at 37°C. The liver

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Fig. 1. Glutathione (GSH) metabolism. The tripeptide GSH is synthesized from its constituent amino acids in ATP-dependent reactions. GSH reacts separately with each of the three bracketed oxidants, hydrogen peroxide (H2O2), organic hydroperoxides (ROOH), and free radicals (R). These reactions yield the three bracketed, detoxified products, as well as oxidized glutathione (GSSG). GSSG, which does not protect against free radicals or oxidants, then is recycled to its active reduced form (GSH) by NADPH-dependent GSSG reductase.

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was perfused in situ through the hepatic vein with oxygenated (100% O₂), nonrecirculating, calcium-free, perfusion buffer (consisting of 125 mm NaCl, 25 mm N-(2-hydroxyethyl) piperazone-N'-(2-ethanesulfonic acid), 1.0 mm KH₂PO₄, 1.2 mm MgSO₄, 5.0 mm KCl, 0.1 mm ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, and 20 mm glucose; pH 7.4) for 20 min at 25 ml/min. The liver then was perfused with recirculation using the aforementioned perfusion buffer, modified to contain 1.0 mm CaCl₂, no glucose, and 0.5 mg/ml collagenase (type D, Boehringer, Indianapolis, IN) for 15 min at 30 ml/min. The liver then was sliced open gently with a spatula and rinsed well with the perfusion buffer, modified to contain 1.0 mm CaCl₂, no glucose, and 2.5 mg/ml bovine serum albumin. The resultant cell suspension was poured through a 250-μ nylon mesh monofilament screening fabric (Tetko, Briarcliff Manor, NY) to eliminate cell clumps, and centrifuged in 15 ml tubes in an IEC4B Centra (Needham Heights, MA) centrifuge to 200 revolutions per minute for 90 s. Gentle centrifugation with good speed control at this stage was essential for good viability, especially for cytochrome P-450-induced cells. The pellet was resuspended in 10 ml and centrifuged twice more, first in the same buffer (containing CaCl₂ and albumin), then in the final growth medium.

At this point, trypsin blue exclusion made it evident that cells were routinely >90% viable. Aliquots of the cells were spread on culture plates at a density of 1.5 × 10⁶ cells/plate. After 2-4 h incubation, during which the cells adhered to the culture plate in a single layer, the initial medium was poured off and replaced with 1.0 ml medium. The hepatocyte monolayers were grown to confluence overnight in 94.4% air and 5.6% CO₂ in Dulbecco's modified Eagle medium supplemented with (per liter) 50 ml fetal calf serum, 0.5 g bovine serum albumin, 1.0 g niacinamide, 292 mg l-glutamine, 10 mg insulin, 50,000 U penicillin, and 50 mg streptomycin.

Immediately before exposure to experimental conditions, plates were washed twice with phosphate-buffered saline and covered with 1.5 ml of medium containing only the inorganic salts, glucose, and insulin of the previous medium to eliminate exogenous glutathione and glutathione precursors. The plates were exposed for 2 h in 9.5 × 16 × 5-cm plastic exposure chambers with inlet and outlet ports and airtight gasket fittings, submerged in the same 57°C water bath. Hypoxic gas concentrations were 5.6% CO₂, 1.0% O₂, and balance nitrogen, provided by mixtures of carbon dioxide, air, and prepurified nitrogen through a multiple flowmeter.

For every experiment, culture plates prepared at the same time from the same animal were exposed simultaneously to one of the three experimental gas phases: 20% O₂, 1.0% O₂, and 1.0% O₂ plus 2.0% halothane. The hypoxic exposures with and without halothane were performed simultaneously in the following manner: (1) The initial gas flow was split into two lines. (2) One line was passed through a halothane vaporizer (Fluotec 3, Hatfield, PA) set to deliver 2.0% halothane (verified by mass spectrometry). (3) Both lines were passed through separate plastic check valves and flowmeters (Dwyer RMA-14-TMV, Anaheim, CA) set at 500 ml/min, and coiled tubing submerged in the water bath. (4) Finally, the appropriate line was passed to one of two identical exposure chambers submerged to the same depth in the same water chamber. This arrangement, diagrammed in figure 2, was chosen so that any small variations in gas composition, temperature, among others, would be identical both with and without halothane for each experiment, except for the small

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dilution of other gases caused by the addition of halothane. All gas-supply tubing was made of Tygon, and of the same diameter and length for each exposure chamber. An open culture plate filled with exposure buffer was positioned just below the gas inlet in each exposure chamber. We found that unless all these precautions were taken to make exposures with and without halothane equivalent, spurious differences could arise easily due to different gas flow rates. The removal of the culture plate tops increased the susceptibility of the system to such artifacts, so they were kept on in all the experiments reported here. (In contrast, Schible et al. did not perform matched, simultaneous exposures of cultures with and without halothane, and left culture plate tops off during exposure to the experimental gas phase.)

Gas concentrations at the exposure chambers’ outlets were verified with a Perkin-Elmer (Norwalk, CT) model 1100 medical gas analyzer (mass spectrometer). PO2 in culture plates was documented with a mini-Clark electrode and Chemical Microsensor oxygen meter (Diamond General, Ann Arbor, MI). The electrode (3.2 mm in diameter) was placed through a tightly fitting hole in a silicone rubber stopper. The stopper was fitted tightly in a 4.8-mm diameter hole through the culture plate top, so that the tip of the electrode was flush with the bottom of the culture plate, and covered with 1.5 ml buffer. The electrode wire exited the gas exposure chamber through the gas outlet. When exposed to 1.0% O2 under the experimental conditions described, buffer PO2 decreased to 1.0% O2 in ≤17 min for buffer alone, and in ≤7 min when cells were present.

Culture media in covered plates reached equilibrium with 2.0% halothane by 20 min, as determined by gas chromatography of hexane extracts of medium. Exposure buffer equilibrated with 2.0% halothane under the described conditions contained 0.51 ± 0.02 mm halothane; the buffer-gas partition coefficient (λ) was 0.65 ± 0.02 (n = 4 separate determinations). A concentration of 2.0% halothane was chosen because total reductive halothane metabolism, and hence free radical production, is maximized by increasing halothane concentrations, while 2 minimum alveolar concentration (~2.0% for the rat) is the highest concentration at which halothane is likely to be used clinically.

In some experiments, where glutathione depletion was desired, cultures were pretreated with the superoxide generator menadione20-21 at indicated concentrations (25-50 μM) for 30 min in 20% O2 at 37°C. Menadione was dissolved in a minimal volume of ethanol before it was added to plates; control plates in the same experiment received the same volume of ethanol alone. Culture plates then were washed and covered with fresh exposure buffer and exposed to the standard experimental gas phases for 2 h. Nitrosoare derivatives, such as N,N'-bis(2-chloroethyl)-N-nitrosoare (BCNU) were not used to deplete glutathione, even though they often are used under normal oxygenation to inhibit oxidized glutathione (GSSG) reduction.27,28 The substituted nitrosoareas were denitrosated reductively by glutathione transferases29 and cytochrome P-450 reductase in the absence of oxygen,30 which presented several potential interactions during hypoxia that are not significant during normoxia, and made BCNU unacceptable as an intervention.

At the end of exposure to experimental conditions, the medium was poured off, centrifuged, and assayed for aspartate aminotransferase (AST) release using Sigma kit 505. Maximum AST was determined by freezing and thawing control plates, and the percent of cell death was calculated from the AST released as a percent of the maximum AST. Control plates were frozen and thawed for each addition (e.g., plus menadione) to account for any AST inhibition caused by the compound added.

The level of intracellular glutathione in the cells remaining on the plate was determined by a modification of the method of Newton et al.,31 using monobromobimane to form a stable, highly fluorescent derivative of glutathione. Fresh monobromobimane (1.0 ml of 2.0 mm in 50% acetonitrile, 1.0 mm (ethylenedinitrilo) tetraacetic acid, 50 mm N-ethylmorpholine; pH 8.0) was added to each plate. Samples were protected from ambient light until derivatization was complete. Cells were scraped off the plate and suspended in the monobromobimane solution, aspirated into covered test tubes, and heated at 60°C for 5 min. Samples then were centrifuged, and the supernatant was analyzed by C-18 high pressure liquid chromatography using fluorescence detection, as described.31 Recovery of added standards was >95%.

Protein was analyzed according to a method described by Lowry et al.32 Cytochrome P-450 content was analyzed by spectrophotometry of the carbon monoxide complex33 in crude extracts; six concurrently prepared culture plates were combined for each analysis. Monobromobimane was from Calbiochem (San Diego, CA). All other chemicals were from Sigma Statistical...
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analysis was performed with the program STATPAC 5.2 (Walock, Minneapolis, MN) on an IBM personal computer. To determine the effects of oxygen and halothane, analysis of variance was performed on both glutathione concentration and AST release using the different gas phases as factors. To assess the effect of cytochrome P-450 induction in the standard exposure experiment, a two-way analysis of variance was performed using gas phases as column factors and the presence or absence of cytochrome P-450 induction as row factors. When analysis of variance indicated a significant difference among groups, individual two-tailed t tests using the least significant difference correction for multiple comparisons were performed. Because of biologic variability in glutathione levels and susceptibility of cultured hepatocytes to anoxia, experimental data were reported in one of the two following ways: (1) When a large number of experiments were performed (standard exposure), the data were normalized (by dividing by the 20% O2 control level), and multiple experiments were analyzed together. (2) The data were analyzed and were presented separately for each experiment (menadione pretreatment). Each experiment contained three to five separate hepatocyte monolayer cultures for each exposure condition. All cultures for a single experiment were from a single rat. Variability is expressed as ±SD for all experiments.

Results

Cytochrome P-450 Levels

When freshly plated, before being cultured, hepatocytes prepared by our methods contained 1.1 ± 0.4 nmol cytochrome P-450 per milligram of total cell protein (n = 11) for induced preparations, and 0.26 ± 0.10 nmol cytochrome P-450 per milligram of protein (n = 27) for uninduced preparations. Overnight culturing of the hepatocytes for 18 h per our protocol reduced cytochrome P-450 levels to only 48.7% and 49.4% of original values in two separate experiments.

Effect of Hypoxia

At the level of the hepatocyte, normoxia with oxidative halothane metabolism corresponds to 4% O2, t.e., at this oxygen concentration, no limitation of mitochondrial respiration, oxidative phosphorylation, or cytochrome P-450 mediated reactions occurs. Variation in oxygen concentration from 4% to 20%, in either our exposure chamber or a regular incubator with room air, caused no change in viability or glutathione (data not shown). Exposure in 20% O2, therefore, was used as a convenient control for each experiment.

Our experiments under standard exposure conditions are summarized in figure 3. Average control glutathione levels were 13.8 ± 3.9 nmol/mg protein (without phenobarbital; −PB), and 15.3 ± 6.1 nmol/mg protein (with phenobarbital; +PB). Average control cell deaths (20% O2, 2 h in buffer) were 13.1 ± 2.8% (−PB) and 9.5 ± 4.3% (+PB). There was some variability between culture preparations in susceptibility to hypoxia. To compensate for this variability, glutathione and AST values for each experiment were normalized by dividing them by the average 20% O2 value for the respective experiment, allowing separate experiments (with three to five cultures per gas phase per experiment) to be analyzed together with greater statistical power. Normalization lessened the chance that a small effect of halothane would be obscured by random variability between cultures, and was appropriate because every experiment consisted of simultaneous exposures, using cultures prepared at the same time from the same an-
imal, to the three gas phases being tested (20% O₂, 1% O₂₁, 1% O₂₂ plus 2% halothane).

Hypoxia alone clearly increased cell death (fig. 3, upper panel; second vs. first bar). Hepatocytes induced for cytochrome P-450 (+PB) were more susceptible than uninduced cells (−PB) (1.8-fold increase −PB, 3.0-fold increase +PB in AST release compared to 20% O₂ control; P < 0.01 for both). Hypoxia also decreased glutathione (fig. 3, lower panel; second vs. first bar) to 0.7-fold of 20% O₂ control for both −PB and +PB (P < 0.01 for both).

Effect of Halothane

As shown in figure 3 (third vs. second bar), during hypoxia, halothane had no adverse effect on either cell viability or glutathione. This was true whether the −PB and +PB groups were analyzed separately or together; in all cases, P > 0.2 for any adverse effect of halothane.

Effect of Glutathione Depletion

Because halothane alone had no detrimental effect on glutathione or survival during hypoxia, we attempted to uncover any latent halothane toxicity by concomitantly depleting glutathione with the superoxide free radical generator menadione.²⁰,²¹,²⁸ Pretreatment with low levels of menadione (25 μM in one experiment and 50 μM in another; both are presented separately without normalization in fig. 4) did not affect cell viability and significantly decreased glutathione at the higher menadione concentration. Menadione pretreatment made hepatocytes more susceptible to both glutathione depletion and cell death during subsequent hypoxia; e.g., hypoxic cell death (1–20% O₂) more than doubled after 25 μM menadione, while the hypoxic decrease in glutathione (1–20% O₂) almost doubled. However, contrary to expectations based on the theory that halothane-derived free radicals play a significant role in hypoxic toxicity, halothane neither decreased glutathione nor worsened hypoxic toxicity after menadione pretreatment in either experiment.

Discussion

Effect of Halothane on Hypoxic Survival

Under our experimental conditions, halothane did not cause significant toxicity beyond that caused by hypoxia alone, either with or without cytochrome P-450 induction. Even when the free radical scavenger glutathione was depleted before halothane exposure, the anesthetic had no apparent toxic effects. The fact that halothane causes hepatotoxicity in hypoxic whole animals suggests that factors other than direct interaction between halothane and hepatocyte (e.g., cardiac output, macro- and microcirculation in the liver, modulation of the immune system, and the inflammatory response) are important in producing halothane hepatotoxicity. Because halothane was not toxic in our model, the effect of isoflurane was not studied. Isoflurane has been uniformly less hepatotoxic than halothane in every model studied.²,¹⁴,¹⁵

Our results differ markedly from those of Schieble et al.,¹⁴ who reported that halothane was directly toxic to cultured hepatocytes, even without cytochrome P-450 induction. In contrast, we applied the method of primary monolayer culture and specific glutathione analysis to the whole animal model of halothane hepatotoxicity previously established in this laboratory.⁵,¹⁵ Therefore, differences exist in rat strains, culture tech-

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Fig. 4. Effect of pretreatment with menadione, a free radical generator, for 30 min in 20% O₂. After pretreatment, cells were washed extensively, recovered with regular exposure buffer, and exposed to the gas phases indicated for 2 h under our standard conditions. The brackets at the bottom of the graph enclose all the conditions varied in a given experiment (i.e., in the first experiment, cells were +PB and received either 0 or 25 μM menadione pretreatment; in the second experiment, cells were −PB and received either 0 or 50 μM menadione pretreatment). *P ≤ 0.05 for 1% versus 20% O₂ (no halothane). ŠP = 0.004 for −halothane versus +halothane (1% O₂). ŠŠP ≤ 0.03 for −menadione versus +menadione pretreatment.
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Techniques, and exposure methods between our experiments and those of Schieble et al. However, the findings reported here are consistent with known properties of the cytochrome P-450 and glutathione systems.

**Cytochrome P-450 Levels**

Cytochrome P-450 levels decline moderately during hepatocyte culture. We chose not to add dexamethasone and 5-aminolevulinic acid to maintain cytochrome P-450 during cell culture, in contrast to Schieble et al. These nonspecific inducers not only affect proteins other than cytochrome P-450 but also may alter the distribution of cytochrome P-450 isozymes. In addition, these inducers may not coordinate the synthesis of cytochrome P-450 reductase, which also is required for halothane metabolism. Other investigators have been unable to maintain cytochrome P-450 levels in cultured hepatocytes with dexamethasone and 5-aminolevulinic acid, and have found that apparent maintenance of cytochrome P-450 expressed per milligram of protein is an artifact because of "accelerated protein degradation in cultures exposed to [dexamethasone]." It is unlikely that, if direct halothane metabolism-mediated toxicity exists, cytochrome P-450 values declined so radically as the result of our culture protocol that our cells no longer metabolized halothane enough to cause toxicity. Our initial uninduced cytochrome P-450 levels (1.1 ± 0.4 nmol cytochrome P-450 per milligram of protein) compare favorably to others reported for whole cell extracts (0.23, 0.17 nmol cytochrome P-450 per milligram protein). Furthermore, our increase on cytochrome P-450 induction, 4.2-fold, was greater than the 3.1-fold increase reported by Schieble et al. Our plates had >2 mg total protein per plate, so even after being cultured for 18 h and undergoing a 50% decrease in cytochrome P-450, each induced plate had at least 1.1 nmol cytochrome P-450, and each uninduced plate had at least 0.26 nmol cytochrome P-450. These values are 7–9-fold higher than those reported by Schieble et al. (0.12 nmol cytochrome P-450 per induced plate [2.5 nmol cytochrome P-450 per milligram of microsomal protein × 0.046 mg microsomal protein per plate] and 0.037 nmol cytochrome P-450 per uninduced plate), even though Schieble et al. cultured twice as many cells per plate as we did (3 x 10⁶ vs. 1.5 x 10⁶ cells per plate).

Cytochrome P-450 induction worsened hypoxic survival, independent of halothane, both in our experiments and those of Schieble et al. This finding supports the contention that our cultured cells retained enough cytochrome P-450 for potential adverse effects to be revealed. The fact that cytochrome P-450-induced cells had no more glutathione depletion, despite poorer hypoxic survival (fig. 3), suggests that cytochrome P-450 induction may exert its harmful effects by means other than free radical production, and that the metabolism of halothane is not central to the toxicity seen in the hypoxic rat model. In fact, PB exposure causes significant intracellular changes besides increases in cytochrome P-450 (e.g., induction of intracellular proteases, alteration of hormonal receptors and signal transduction mechanisms).

**Effect of Rat Strain**

The Fisher 344 rat, used by Schieble et al., is more susceptible to halothane hepatotoxicity in the whole animal model. However, it is not established in which system this greater susceptibility arises (e.g., hepatic vs. circulatory). In fact, although the Fisher 344 rat exhibited more liver toxicity after halothane and hypoxia, its production of volatile reductive metabolites of halothane was not greater than that of the Sprague-Dawley rat. We chose the Sprague-Dawley rat because it is the strain best characterized in our laboratory in the whole animal model. If halothane hepatotoxicity can be demonstrated in the whole animal of any strain, but not in hepatocyte culture of the same strain, the result is clear-cut: A direct hepatotoxic effect of halothane cannot be the sole cause of liver damage in the whole animal.

**Adequacy of Hypoxic Exposure**

The measured oxygen concentration achieved with our apparatus (1.0% in 17 min) was comparable to that reported by Schieble et al. (1% in 20 min). There are two reasons why we tested only one hypoxic oxygen concentration (1%). First, our findings were sufficient to disprove the hypothesis that halothane is always toxic in some degree to the hypoxic hepatocyte, regardless of cytochrome P-450 induction. We thought it more important to replicate this important finding than to study a variety of oxygen concentrations with fewer replications. Second, our results with 1% O₂ correlated much better with those of the whole rat model of halothane hepatotoxicity than those reported by Schieble et al. A key feature of the whole rat model is that the degree of hypoxia is sufficient to produce
halothane hepatotoxicity, but only minimal toxicity due to hypoxia alone. The mild degree of cell death we observed during 2 h of hypoxia alone (9.9% uninduced for cytochrome P-450 and 18.7% induced for cytochrome P-450, above normoxic controls) more closely resembles the in vivo model than does the high cell death (~35% uninduced, ~70% induced) reported by Schieble et al. during hypoxia alone, in the absence of halothane.

The lesser sensitivity of our hepatocytes to 1% O2 is also much more consistent with published reports from other laboratories than that reported by Schieble et al. Jones et al. in two separate reports found that isolated hepatocytes from fed Sprague-Dawley rats had 5–10% cell death after 2 h at 0% O2 in buffer. Lemasters et al. reported that 5 mm cyanide (chemical anoxia) alone caused no change in cell morphology or viability of hepatocytes from fed Sprague-Dawley rats when the hepatocytes were exposed to the solution for periods of over an hour. Anundi and deGroot using a sophisticated oxystat system that measures and adjusts Po2 directly in the experimental buffer, found that hepatocytes from Wistar rats who had fasted had only ~10% more cell death than hepatocytes from control rats after 2 h at 0.3 mmHg O2 (cf. 1% O2 ~7 mmHg O2). Von Ruecker et al. reported ~10% cell death in hepatocytes from fed Sprague-Dawley rats exposed to 0.5% O2 for 4 h. The initial resistance of hepatocytes to hypoxia is due in part to endogenous glycop and is not affected by exogenous glucose, which is not effectively used by hepatocytes during hypoxia. Some variability in glycop stores from culture to culture is expected and was observed in our experiments, but this variability is unlikely to account for the extreme sensitivity to hypoxia reported by Schieble et al. Until the factors leading to the unusual hypoxic sensitivity of their hepatocytes are better characterized, it is wise to interpret their results with caution, and to avoid implicating halothane as “directly hepatotoxic.”

Statistical Power

Statistical power can be excluded as a reason for our failure to observe halothane toxicity, compared to Schieble et al. For each of the three gas phases in our experiments, 39 separate culture plates were analyzed, but Schieble et al. analyzed only 21 plates at normoxia without halothane, 27 plates at 1% O2 without halothane, and 27 plates at 1% O2 with halothane. Although Schieble et al. analyzed more plates in other gas phases, e.g., normoxia with halothane, inspection of figure 3 in Schieble et al. suggests that those plates contributed minimally to their conclusion that halothane was toxic during hypoxia. Furthermore, our use of simultaneous exposures of cultures prepared at the same time from the same animal, and subsequent normalization to the control values prior to analysis, decreased the effect of random biologic variation between cultures, and lessened the chance that a small effect of halothane would be obscured by random variability between cultures.

Effect of Hypoxia on Glutathione

Our observation that hypoxia alone decreases glutathione was unexpected, given that glutathione is a reduced compound, and that the hypoxic cell has an excess of reducing equivalents (e.g., [NADH] > [NAD+]). However, since reducing GSSG to glutathione requires NADPH rather than NADH, and since transfer of reducing equivalents from NADH to NADPH requires ATP, a decrease in glutathione with hypoxia is plausible. Two other reports confirm our observation of a moderate decrease in glutathione with hypoxia, one incidental to investigating the effect of radiation on hypoxic Chinese hamster ovary cells, and the other to investigating the effect of added oxidant on hypoxic rat hepatocytes.

Our experiments were not designed to address the mechanism of hypoxic glutathione depletion, but several mechanisms are plausible (fig. 1). Glutathione consumption may be increased during hypoxia because of hydrolysis of its peptide bonds, transport out of the cell, or oxidation by free radicals generated from the over-reduced mitochondrial electron transport chain. Glutathione regeneration by oxidized glutathione reductase will be slowed by the decreased NADPH level during hypoxia, while de novo glutathione synthesis from its constituent amino acids is ATP-dependent and is decreased in hepatocytes during hypoxia. Further investigation of the mechanism of hypoxic glutathione depletion may prove fruitful in deciphering the mechanisms of cell damage during hypoxia.

Effect of Halothane on Glutathione

Glutathione levels did not change during hypoxia when halothane was added, even if cytochrome P-450...
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was induced, or if glutathione was depleted before hypoxic halothane exposure, showing that halothane-derived free radicals are qualitatively insignificant compared to the free radical scavenging capacity of the normal glutathione system. There is no question that hepatocytes generate free radicals from halothane during hypoxia and that a small portion of these free radicals may escape detoxification by glutathione and react with cellular lipids and proteins.5,9,16,17 The significant question is whether free radical production from halothane is quantitatively significant enough to deplete glutathione and allow uncontrolled reaction of free radicals with vital cellular components.

Adequacy of Glutathione Concentration as a Measure of Free Radical Damage

The fact that free radical reaction with cellular constituents is detectable with sensitive assays does not automatically imply that the source of those free radicals is toxic. Free radicals are continuously generated from oxygen during normal cellular metabolism, particularly by membrane bound oxygen-dependent enzymes, including cytochrome P-450 and cytochrome oxidase.5,56,57 These free radicals are lethal at high concentrations because they react covalently with vital cellular components faster than the cell can repair them. However, at low free radical levels, during normal metabolism, oxygen is obviously not toxic. This is because: (1) defense systems against oxygen-dependent free radical products (superoxide dismutase, glutathione, glutathione peroxidase, α-tocopherol) scavenge many of the free radicals before they can react with anything else;19,55–58 and (2) cells possess numerous repair systems whereby proteins, DNA, and lipids covalently altered by free radicals (and other agents) are degraded and replaced.59–61 Thus, previous demonstrations16,17 that halothane-derived free radicals react covalently with various cellular constituents do not establish that halothane is toxic; i.e., that halothane is causing damage beyond that which the cell normally encounters during aerobic metabolism and which the cell can repair without difficulty.

Glutathione levels are a well-documented, quantitative measure of free radical flux, even when the free radicals are generated by membrane bound enzymes such as cytochrome oxidase and cytochrome P-450, because when there is a sufficient quantity of free radicals to injure the cell irreparably there is also a sufficient quantity of free radicals to deplete glutathione.

In contrast, when free radical generation is occurring at a finite but nontoxic level, glutathione is not depleted. Numerous studies have documented that decreases in glutathione precede lethal cell injury from free radical generators.20–22,62

Our data, obtained with a normal glutathione-regenerating system, do not exclude the possibility that animals or patients treated with agents (e.g., BCNU chemotherapy)27,28 that cause severe deficiencies in glutathione synthesis or GSGG reductase could be unable to detoxify halothane-derived free radicals, which would then contribute to hepatotoxicity. Our data do exclude a role for halothane-related glutathione consumption at the level of the isolated hepatocyte in the standard rat model of halothane hepatotoxicity, and suggest that the reductive halothane metabolism which may be observed in almost all patients,4,9 probably due to intrahepatic variations in F0,s8 is benign.

In conclusion, under the conditions investigated here, halothane does not have a direct hepatotoxic effect during hypoxia, and does not deplete glutathione.

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