Halothane-induced Cytotoxicity to Rat Centrilobular Hepatocytes in Primary Culture Is Not Increased under Low Oxygen Concentration

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Background: Halothane can be metabolized by both oxidative and reductive pathways in the liver. This anesthetic can induce direct liver injury preferentially localized in centrilobular areas, probably in relation with lower oxygen tension. The reductive pathway has been related to liver damage; however, a correlation between lower oxygen concentration in centrilobular areas, the extent of reductive metabolism of halothane, and the degree of liver injury has not yet been demonstrated. This study was designed to better evaluate the toxicity of the reduced metabolites by using centrilobular and periportal rat hepatocyte subpopulations.

Methods: Adult rat hepatocytes, either as whole cell preparations or after separation in centrilobular and periportal cell subpopulations, were placed in primary culture and exposed to either 2% or 4% halothane under various oxygen concentrations. The enriched centrilobular hepatocyte subpopulations isolated by the d-galactose-collagenase method were characterized by immunolocalization of glutamine synthetase. Three oxygen concentrations were tested: 5%, 20%, and 95%, and the main parameters measured were cell viability and fluoride ion formation.

Results: Viability of centrilobular hepatocytes was similar under 5% and 20% O2, but the unpurified hepatocyte population was more susceptible to 5% O2 (P < 0.01). Significantly higher cytochrome P-450 content was found in whole hepatocyte populations under 5% versus 20% oxygen, indicating that centrilobular hepatocytes that contained higher cytochrome P-450 monoxygenase activities were less sensitive to low oxygen concentration. Halothane toxicity to centrilobular hepatocytes was enhanced under 95% versus 20% O2 (P < 0.05). By contrast, no significant difference was observed when the cells were maintained under 5% O2, although fluoride ions, indicative of reductive metabolism of halothane, were found in much higher amounts in the culture medium. Moreover, under 20% O2, halothane toxicity was significantly greater in centrilobular versus unpurified hepatocytes (P < 0.05).

Conclusions: Isolated centrilobular hepatocytes appear to be more sensitive to halothane than their periportal counterparts in vitro. However, the authors’ results support the conclusion that increased reductive metabolism of halothane induced by decreasing oxygen concentration is not a critical parameter for the occurrence of liver damage in these cells. (Key words: Anesthesia, volatile: halothane. Liver, hepatocyte: primary culture; subpopulation. Liver: toxicity. Oxygen: tension.)

ANESTHESIA with halothane can be followed by transient liver dysfunction or, in rare cases, by a severe hepatitis. Both forms are thought to be related to metabolism of the anesthetic.1 An oxidative metabolic pathway was first demonstrated using an in vivo model,2 and, more recently, a reductive pathway that is enhanced under low oxygen tension was also identified.3 These two metabolic pathways have been confirmed, both in vitro and in halothane-anesthetized patients.4

Several studies have established a correlation between hepatic centrilobular injury and low oxygen tension after exposure of animals to halothane. The greater liver damage associated with the reductive pathway is usually linked to the formation of unstable reactive metabolites. However, although it is more metabolized through the reductive pathway, deuterated halothane has been shown to be less hepatotoxic than halothane, indicating that the relative contribution of different metabolic pathways to direct toxicity induced by halothane deserves further investigation.5-7

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A clear demonstration of a correlation between a given metabolic pathway of halothane and the degree of hepatotoxicity induced by this anesthetic is difficult. The shift toward reductive or oxidative biotransformation is mainly caused by oxygen concentrations at the active site of cytochrome P-450 (P-450). This oxygen tension can be easily estimated in microsomal preparations, but it is much more difficult to evaluate when isolated or cultured hepatocytes, or liver slices, are used.\textsuperscript{6-10} Determination of the respective importance of the oxidative and reductive pathways of halothane in whole parenchymal cell populations requires quantification of the amounts of metabolites formed, or those of specific enzymes involved in their formation. It is also difficult to determine whether nonlethal hypoxia induces variations in oxygen tension in hepatic sinusoids in animals, because different regulatory mechanisms can be involved in upstream vessels and in hepatic vessels. In addition, decreased oxygen concentrations induce marked cellular injury in isolated hepatocytes\textsuperscript{8} and liver slices.\textsuperscript{10}

Although it is established that decreased oxygen concentrations in hepatic microsomes enhance the formation of reactive metabolites responsible for lipid peroxidation, the direct role of oxygen in halothane-induced hepatotoxicity is not well elucidated.\textsuperscript{11,12} This study was designed to determine whether the sensitivity of centrilobular hepatocytes to halothane toxicity is dependent on oxygen tension, and the extent of reductive metabolism of the anesthetic. Our results support the conclusion that increased production of reduced halothane metabolites under decreasing oxygen tension is not directly correlated to the extent of cellular damage occurring in this hepatocyte subpopulation.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley rats, weighing 180–200 g, were used after acclimation in our animal facility. The animals were handled in compliance with French regulations, and were fed ad libitum.

**Cell Isolation and Culture**

Hepatocytes were isolated by the two-step collagenase perfusion method.\textsuperscript{13} Briefly, animals were first anesthetized by intraperitoneal administration of 90 mg/kg pentobarbital. Before opening the abdomen, 1,000 IU heparin was injected in the left femoral vein. The liver was cannulated through the portal vein, and was first washed with calcium-free Heps buffer, pH 7.65, for 10–15 min at a flow rate of 30 ml/min. It was then perfused with the same buffer containing 0.025% collagenase and 0.075% CaCl\textsubscript{2} for 15 min at a flow rate of 15 ml/min. After enzymatic perfusion, the organ was excised and placed in a Petri dish. The Glisson's capsule was disrupted, and the cells were dispersed in L15 Leibovitz medium containing 0.2% bovine serum albumin. The cell suspension was filtered through gauze, allowed to sediment for 20 min to eliminate cell debris, blood, and sinusoidal cells, and, finally, washed three times in Heps buffer by centrifugation at 50g. Hepatocytes were suspended in L15 Leibovitz medium containing 10% fetal calf serum, and counted. Cell viability was estimated by the trypan blue exclusion test. Cell yields ranged between 4 and 6 $\times$ 10\textsuperscript{6} hepatocytes, with a viability of 85–95%.

Hepatocytes were seeded at the density of 3 $\times$ 10\textsuperscript{6} cells per 25 cm\textsuperscript{2} flask in a humidified atmosphere composed of 95% air/5% CO\textsubscript{2}. The medium consisted of 75% minimal essential medium and 25% 199 medium buffered with 0.22% bicarbonate (w/v) and supplemented with 0.1% bovine serum albumin, 5 $\mu$g/ml bovine insulin, 133 IU penicillin, 100 $\mu$g/ml streptomycin, and 10% fetal calf serum.\textsuperscript{14} The medium, without fetal calf serum and containing 7 $\times$ 10\textsuperscript{-5} M hydrocortisone hemisuccinate, was renewed 4 h later.

**Selective Isolation of Perivenous and Periportal Hepatocytes**

Perivenous and periportal hepatocytes were prepared by the digitonin-collagenase perfusion method.\textsuperscript{15-17} The liver was connected to the perfusion system via the portal vein and the vena cava. For destruction of periportal cells, the liver was first perfused via the portal vein with Heps buffer containing 5 ml CaCl\textsubscript{2} for 2–3 min at a flow rate of 30–40 ml/min, and then with 7 mm digitonin in Heps buffer for 10–20 s at 10 ml/min. Perfusion was stopped for 10–15 s, and the flow direction was switched. Calcium-free Heps buffer was perfused via the vena cava for 10 min at 30 ml/min, and was followed by the collagenase solution. Selective destruction of centrilobular hepatocytes was obtained by the same procedure, except that flow directions were opposite, with, first, centrilobular infusion of digitonin. Digitonin treatment followed by collagenase dissociation yielded enriched periportal or centrilobular hepatocyte subpopulations that represented about
one-third of the cells obtained by the classic perfusion method.

Treatment of Hepatocyte Cultures

After medium renewal, the flasks were sealed and two needles were introduced through the cap. One was connected to the gas analyzer (Servo Gas Monitor 120; Siemens, Erlangen, Germany) to measure oxygen and anesthetic concentration gas exiting the flask. The other was connected to the bottle of gas mixtures (5% O₂/5% CO₂/90% N₂, 20% O₂/5% CO₂/75% N₂ or 95% O₂/5% CO₂) and a vaporizer chamber (Flutec 3; Ohmeda, Streetan, England) for halothane. After the different connections were established, the gases flowed into the flasks at 3 l/min until selected oxygen and halothane concentrations become stable. The linearity of halothane concentrations indicated on the analyzer compared with medium concentration has been previously checked (2%/0.37 mm and 4%/1 mm). The needles were then removed, and caps were immediately sealed with paraffin. The flasks were placed at 37°C for 12 h, and gas tightness was checked by the color of the culture medium containing phenol red. The gas concentrations are not modified at the end of incubation.

Immunocytochemistry

To identify centrilobular hepatocytes, glutamine synthetase was localized by immunoperoxidase. Cell cultures were first fixed in 3.5% paraformaldehyde buffered with PBS at 4°C for 30 min, then washed with PBS (3 × 15 min) and incubated with 0.1 m saponin for 1 h. The cultures were incubated with antiliglutamine synthetase antibodies overnight at 4°C, washed three times with PBS, and incubated with the second antibody labelled with peroxidase. The enzyme was revealed by the Graham and Karnovsky method.

Assays

Both extracellular and intracellular lactate dehydrogenase (LDH) activities were measured using the MA-.

kit Roche, LDH opt DGKC (reference 14383) and a Cobas Bio apparatus (Nutley, NJ). The values were expressed as the intracellular LDH to total LDH ratio.

Cytochrome P-450 content was assayed on whole cell homogenates after reduction with sodium dithionite according to Omura and Sato.

Determination of Fluoride Ions

Fluoride ions were quantified using a specific electrode. Ten milliliters of culture medium were harvested at the end of the experiment and diluted (1:2) in TISAB buffer (total ion-strength-adjustment buffer). This buffer was composed of 57 ml of cold acetic acid, 58 g NaCl, and 300 mg Na citrate in 500 ml ultrapure water, and was adjusted to pH 5.5. The electrode was introduced in the medium, and the concentration of fluoride ions was determined after the potential was stabilized. Concentrations expressed as μg/l were determined from a standard curve.

Statistical Analysis

The data were expressed as mean ± SD. The Mann–Whitney U test was used to compare paired groups of values. The Kruskal-Wallis test was used to compare multiple series. The values of P < 0.05 were regarded as significant.

Results

To evaluate the degree of selection of hepatocyte subpopulations by the digitonin-collagenase method, glutamine synthetase-positive parenchymal cells were looked for. As previously reported, we found positive cells to be exclusively located in centrilobular areas, forming a layer of 6–8 cells in the normal liver (not shown). After liver dissociation glutamine synthetase-positive hepatocytes were found in centrilobular hepatocyte populations; these were very scarce, if any, in perportal hepatocyte subpopulations (not shown). The degree of cell selection was similar to that obtained in previous studies.

The influence of hypoxia was evaluated on both purified and unpurified hepatocyte populations. Hepatocyte viability was estimated by light microscopy examination and measurement of the LDH ratio. Centrilobular hepatocytes incubated for 12 h under various oxygen concentrations (20% or 5%) retained a normal shape, while unpurified hepatocytes were altered under 5% versus 20% O₂ (P < 0.01). Morphologic alterations were exaggerated in enriched perportal hepatocyte populations (fig. 1). No difference in LDH ratio was evidenced with centrilobular hepatocytes exposed to either 20% or 5% O₂, but a significant loss of viability was observed in whole hepatocyte populations maintained under 5% versus 20% (P < 0.01) for 12 h (fig. 2). When total cytochrome P-450 was measured in these whole populations and expressed as pm per 10⁶ viable cells, a significantly lower level was found under 20% (122.1 ± 16.9 pm/10⁶ cells) versus 5% (159.5 ± 31.8 pm/10⁶ cells; n = 5, P < 0.05).
Fig. 1. Photomicrographs of primary cultures of centrilobular (A, B), unpurified (C, D), and periportal (E, F) hepatocytes maintained for 12 h under 20% (A, C, E) or 5% (B, D, F) O₂ atmospheres. Note the higher resistance of centrilobular hepatocytes to low oxygen concentration (×180).
When exposed to 4% halothane for 12 h, rat hepatocytes exhibited different responses. Cytotoxicity induced by a 4% concentration of the anesthetic was significantly greater in centrilobular hepatocyte cultures than in their unpurified counterparts (fig. 3). Halothane cytotoxicity to centrilobular hepatocytes varied depending on oxygen concentrations. As shown in figure 4, these cells were found to be less sensitive to low oxygen concentrations whether exposed to 2% or 4% of the anesthetic. Significant variations in cell viability were found under 95% versus 5% O₂ in the presence of 2% halothane (P < 0.05) and under 95% versus 20% O₂, as well as under 95% versus 5% O₂ in the presence of 4% halothane (P < 0.05). By contrast, no difference was observed between 20% and 5% O₂ concentrations when the cultures were exposed to either 2% or 4% halothane (fig. 4). Halothane toxicity was significant at a 2% concentration under 95% O₂ (P < 0.05) and at a 4% concentration under 95%, 20%, and 5% O₂ atmospheres compared with controls (P < 0.01, P < 0.01, and P < 0.05, respectively).

To further assess the importance of the reductive pathway of halothane metabolism in induction of cellular injury in hepatocyte cultures, fluoride ions were measured in the culture medium of centrilobular hepatocytes after a 12-h incubation with 2% halothane under various oxygen concentrations (5%, 20%, and 95%). Under 95% O₂, no formation of fluoride ions was demonstrated. When oxygen concentration was decreased, a significant production of fluoride ions was observed (P < 0.05); this production was higher under 5% versus 20% O₂ (fig. 5).

Discussion

The objective of this study was to determine whether increased hepatic biotransformation of halothane through the reductive pathway induced by decreasing oxygen tension enhanced cellular damage in centrilobular hepatocytes. Whole isolated hepatocyte populations, as well as enriched perportal and centrilobular hepatocyte subpopulations prepared by the digitonin-collagenase method, were investigated.

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Fig. 5. Fluoride ion (F⁻) levels measured in media of centrilobular hepatocyte cultures. Cells were maintained for 12 h under different oxygen atmospheres (5%, 20%, and 95%) and exposed to 2% halothane or not (control). Comparison H2% versus control; **P < 0.01; NS = not significant; n = 3.

Our results clearly show that unpurified hepatocyte populations placed in culture are sensitive to decreased oxygen concentration; this is in agreement with previous in vitro studies reporting that the extent of cellular damage was dependent on both oxygen concentration and the duration of exposure.8-10 When calculated per 10⁶ viable cells, cytochrome P-450 content was higher in cultures maintained under 5% O₂ compared with 20% O₂. This observation, also made by Van Iersel et al.,8 indicates that hepatocyte death at low oxygen concentration is restricted to a cell subpopulation, and that the remaining hepatocytes, which contain higher P-450 concentrations, are preferentially of centrilobular origin.27-29 Our study with purified hepatocyte subpopulations confirms that rat hepatocytes are differently sensitive to low oxygen concentration, depending on their intrahepatic origin. Whether placed under 20% or 5% O₂, centrilobular hepatocytes did not show any loss of viability; however, a 50% decreased viability was observed in whole cell populations after 12 h.

When exposed in vitro to halothane, centrilobular hepatocytes were found to be more sensitive than whole cell populations, whatever the oxygen concentration used. Such a greater sensitivity appears to be related to a higher metabolic capacity,27,29 linked to higher cytochrome P-450 monoxygenase activities than in their periportal counterparts.

In vivo hepatocytes are under varying oxygen tensions, depending on their intrahepatic localization. This has led many investigators to analyze halothane metabolism at different oxygen concentrations to better understand the mechanism(s) of toxicity of this anesthetic. Halothane is metabolized by an oxidative and a reductive pathway both of them involving cytochrome P-450-dependent enzymes.

The oxidative pathway leads to the formation of both trifluoroacetic acid (CF₃ COOH) and water-soluble bromide. This metabolic pathway involves formation of electrophile metabolites (R⁺) and subsequent binding to proteins.30 Although the role of covalent binding in halothane-induced direct toxicity has been demonstrated in vivo,5 no evidence for preferential centrilobular localization of the lesions has been provided in vitro.

The reductive pathway mediated by cytochrome P-450 has been demonstrated in microsomal fractions from rabbit hepatocytes incubated with NADPH. The formation of reduced metabolites is inhibited by addition of CO and enhanced by phenobarbital and 3′-methylcholanthrene treatment,31 indicating that several cytochrome P-450 enzymes are potentially able to reduce halothane in vitro.32 The reductive pathway leads to the formation of the two final metabolites 2-chloro-1,1-difluoroethylene (CDE) and 2-chloro-1,1,1-trifluoroethane (CTE). Intermediate radical metabolites (F₃C=CHCl) can follow different pathways. Additional reduction can lead to the formation of both fluoride ions and CDE.33-39 Removal of H radical from membrane-bound fatty acids responsible for lipid peroxidation before CTE formation has been reported.40-45 Both the reaction with an oxygen molecule and formation of CF₃CHClO₂⁻ can occur, and the involvement of these processes in hepatotoxicity has been reported.40,46

Clearly, the role of the reductive pathway in halothane-induced hepatic centrilobular injury remains a matter of debate. It has been possible to determine the minimum oxygen tension required to obtain reductive metabolites (CDE, CTE) in microsomes33; however, this parameter is quite difficult to evaluate in vivo. Consequently authors have turned to hepatocyte cultures and liver slices as experimental models. Again, the results obtained with these systems are questionable, because hepatocytes are sensitive to low oxygen concentrations, thereby leading to hypoxia-induced irreversible cellular damage, regardless of halothane concentration. This means that distinction between injury induced by low oxygen tension and cellular damage after halothane exposure is critical to correctly interpret the mechanism(s) involved in cellular injury induced by the anesthetic.

From our observations, it appears that halothane toxicity to rat hepatocyte maintained under low oxygen...
concentrations can be attributed both to increased production of toxic metabolites in centrilobular cells and to toxicity induced by decreased oxygen tension in periportal hepatocytes. Therefore, both toxicities are not related only to an altered metabolic profile resulting from decreasing oxygen concentration and leading to the formation of increased amounts of reduced metabolites.

Our data clearly show that the toxic effects of reductive metabolism in *in vitro* hepatocyte models are difficult to estimate. The use of centrilobular hepatocytes demonstrated that, under low oxygen concentration (5% versus 20%), halothane cytotoxicity was not enhanced, but the formation of fluoride ions was markedly augmented. These data are supported by others showing that deuterated halothane toxicity is decreased compared with that of halothane, although its reductive biotransformation is proportionally augmented.5-7

In summary, increased metabolism of halothane through the reductive pathway does not appear to enhance its toxicity to centrilobular hepatocytes *in vitro*. The higher sensitivity of these cells is, rather, related to their greater metabolic cytochrome P-450-dependent capacity, regardless of oxygen concentration.

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