

Effects of Halothane on Surfactant Biosynthesis by Rat Alveolar Type II Cells in Primary Culture

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Background: Pulmonary surfactant, which is synthesized by alveolar type II cells (ATII cells) almost exclusively, plays a major role in maintaining alveolar homeostasis by reducing surface tension at the fluid-gas interface. Phosphatidylcholine (PC), the main surfactant lipid component, is largely responsible for this surface activity. The effects of halothane on the phospholipid metabolism of the pulmonary surfactant by ATII cells are unknown, even though these cells are exposed directly to volatile anesthetics during anesthesia and even though any alteration in surfactant biosynthesis by anesthetics may have deleterious effects on lung function and thereby facilitate postoperative pulmonary complications. In the current study, the effects of halothane exposure on surfactant synthesis by rat ATII cells in primary culture were investigated.

Methods: ATII cells were isolated from adult rat lungs and used for the experiments after 24 h in primary culture. The ability of ATII cells to synthesize surfactant was assessed by the incorporation of radioactive precursors in PC. Cytotoxicity was measured by the rate of lactate dehydrogenase release into the culture medium, and the lactate metabolism was taken as an index of glycolytic metabolism. All metabolic measurements were made after 24 h in primary culture. Effects of var-

ious halothane concentrations (1, 2, 4, and 8%) exposure for 4 h were studied, as were the effects of 2% halothane for various durations of exposure (2, 4, 8, and 12 h). The reversibility of halothane effects on PC synthesis was assessed after a 2% halothane exposure for 4 h. PC secretion and adenosine triphosphate cellular content were also measured for 4 h exposure at the various halothane concentrations.

Results: During a 4-h exposure, PC synthesis was reduced by 10, 24, 29 and 36% for 1, 2, 4, and 8% halothane respectively when compared with control values. At 2% halothane concentration, the observed decreases in PC synthesis were 12, 24, 31 and 34% for 2, 4, 8, and 12 h exposure, respectively. The inhibitory effect of halothane was completely reversed 2 h after the end of exposure. PC secretion was unaffected by increasing halothane concentrations during a 4-h exposure. Halothane did not produce cell damage except for the longest exposure durations (8 and 12 h) at 2% vapor concentration. Whatever the exposure conditions, lactate production by ATII cells exposed to halothane was greater than production by unexposed cells.

Conclusions: These results indicate that halothane decreases the biosynthesis of pulmonary surfactant by ATII cells in primary culture and alters the high energy phosphate metabolism of these cells. (Key words: Anesthetics, volatile: halothane. Lungs, alveolar type II cells: metabolism. Surfactant: biosynthesis; phosphatidylcholine.)

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HALOTHANE and other volatile anesthetic agents are known to affect cellular metabolism.¹ Most studies regarding the metabolic effects of halothane have been performed in liver, reflecting clinical concern about the potential hepatotoxic effects of the drug or its metabolites. In contrast, although this agent is introduced and then principally eliminated through the lungs, little is known about its effects on lung metabolism. Several studies reported the inhibitory effects of halothane on protein synthesis in perfused rat lungs and in primary cultures of mixed lung cells² or in rabbit pulmonary macrophages.³ However, the effects of halothane on lipid metabolism and specifically on pulmonary surfactant phospholipid metabolism have not been studied in spite of its fundamental role in maintaining normal lung function. Pulmonary surfactant, which is synthesized by alveolar type II cells (ATII cells) almost ex-

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clusively, lines the alveolar surfaces of the lung and reduces surface tension at the fluid-gas interface providing mechanical stability to the lung and therefore preventing alveolar collapse at end-expiration. Pulmonary surfactant also prevents pulmonary edema, decreases the work of breathing and plays a role in pulmonary defense system by removing foreign particles from airways and by stimulating the phagocytic and bactericidal capacity of alveolar macrophages.⁴ Therefore, any alteration in the surfactant synthesis during anesthesia may have deleterious effects on lung function and may facilitate the appearance of postoperative pulmonary complications. The current study was designed to investigate the *in vitro* effects of halothane on surfactant phospholipid metabolism by rat AII cells in primary culture. We studied the biosynthesis and the secretion of phosphatidylcholine (PC), the main lipid component of surfactant and largely responsible for its surface activity.⁵ Halothane effects on AII cells glycolytic metabolism and cellular adenosine triphosphate (ATP) content were also measured as index of cellular high energy phosphate metabolism.

Materials and Methods

Handling procedures as recommended in the guide for the use of Laboratory Animals were followed throughout.

Materials

Adult male pathogen-free Sprague-Dawley rats (200–220 g) were obtained from Iffa-Credo (St-Germain-sur-L'Arbresle, France) and were used within 2 days of delivery. The sources of materials used in this work were as follows: [³H-methyl]-choline chloride (75.9 Ci/mmol) (Amersham, UK); porcine pancreas elastase (Worthington Biochemicals, Freehold, NJ); fetal bovine serum, N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (Hepes) buffer, antibiotics, L-glutamine, and Dulbecco's modified Eagle's medium (DMEM) (Flow Labs, Irvine, Scotland); phosphate-buffered saline (PBS) (Gibco, Grand Island, NY); halothane (Trofield, England); lactate PAP kit and lactate dehydrogenase (LDH) Enzyline kit (BioMerieux, Marcy-l'Etoile, France); protein assay dye reagent (Bio-Rad, München, Germany); luciferin-luciferase reagent (ATP monitoring reagent, LKB, Finland); plastic ware (Costar, Cambridge, MA); and other biochemicals of the highest grade available (Sigma, St. Louis, MO).

Cell Cultures

Two balanced salt solutions were prepared for harvesting type II cells. Solution 1 contained (mmol/l): 140 NaCl, 5 KCl, 2.5 PBS, 10 Hepes, 6 D-glucose, and 0.2 ethylene-glycol bis (β -aminoethyl ether)-N,N'-tetraacetic acid, pH 7.40 at 22°C. Solution 2 was composed of (mmol/l) 140 NaCl, 5 KCl, 2.5 PBS, 10 Hepes, 2 CaCl₂, and 1.3 MgSO₄. Elastase solution was prepared in solution 2 (40 U/ml). AII cells were isolated from adult Sprague-Dawley rats by enzymatic dissociation as described by Dobbs *et al.*⁶ with minor modifications as previously reported.⁷ The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and heparin sodium (1 U/g). After a tracheotomy was performed, the animal was exsanguinated. Solution 2 was perfused through the air filled lungs *via* the pulmonary artery to clear blood from the vascular space. The lungs were removed from the thorax and lavaged to total lung capacity (~ 10 ml) five times with solution 1 and two times with solution 2 to remove macrophages. Then lungs were filled with 10 ml of elastase solution and incubated in a water bath for 10 min at 37°C, after which additional elastase (10 ml) was instilled for another 10-min incubation. After enzymatic digestion, the trachea and large airways were discarded. Each lung was minced in the presence of deoxyribonuclease I (to help prevent cell clumping) and 5 ml fetal bovine serum were added to stop the elastase effect. Tissue pieces were removed by sequential filtration through 150- and 25- μ m nylon mesh (Scrynel, ZBF, Switzerland). The filtrate was centrifuged at 250 \times g for 10 min, and was resuspended in DMEM. The cell suspension was purified by plating onto bacteriologic plastic dishes. This differential adherence technique permitted selective removal of macrophages that adhere rapidly to plastic. After 1 h incubation at 37°C in a 5% CO₂/95% air atmosphere, the unattached cells were recovered and centrifuged at 250 \times g for 10 min at 4°C. The cell pellet was then resuspended in DMEM containing 25 mM glucose, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and plated at a density of 10⁶ cells/ml, 1 ml/well in 24-well cell culture dishes. The cells were then returned to the incubator. Cell isolation yield and cellular viability (assessed by the Trypan blue exclusion test) were 30 \pm 1 \times 10⁶ cells/rat and 94.4 \pm 0.6% (means \pm SEM, n = 29) respectively. After 24 h, nonadherent cells were removed by gentle washing with PBS and adherent cells were used for surfactant phospholipid biosynthesis experiments. At this time,

90 ± 3% of adherent cells were ATII cells as assessed by the characteristic fluorescence with phosphine 3R.⁸ Adherent cells viability always exceeded 98%. Contaminating cells were essentially macrophages.

Halothane Exposure and Experimental Design

Exposure of culture plates to halothane was performed using a 12-liter air-tight Lwoff chamber (Lequeux, Paris, France) with inflow and outflow valves as previously described.⁹ The humidification in the exposure chamber was performed by one open Petri dish (100 mm). This dish remained filled with 15 ml sterile distilled water and the chamber sealed continuously between each experiment to keep the chamber atmosphere saturated with H₂O. The chamber was kept continuously at 37°C. In preliminary experiments, we observed that over a 12-h period, PC synthesis by ATII cells cultured in a standard incubator with free gas flow or in the sealed chamber flushed with a 95% air–5% CO₂ mixture was similar. Thus, these different culture conditions did not influence PC synthesis by ATII cells.

Halothane vapor was provided by directing a 95% air–5% CO₂ mixture at 5 l/min through two Fluotec Mark II vaporizers placed at the entrance to the chamber. The calibration of these vaporizers was verified by mass spectrometry. Halothane flowed in from the top of the chamber onto the surface of the Petri dish filled with sterile water that was placed on the top shelf. The resulting turbulence caused a uniform gas flow over culture plates placed on lower shelves. A halogen monitor (Capnomac Datex, Helsinki, Finland) determined the concentration of the anesthetic exiting the chamber, which was sealed when the desired concentration was obtained.

After incubation, halothane concentrations in the culture medium were checked by gas-phase chromatography.¹⁰ For each halothane point, a control point was obtained with cells issued from the same pool of isolated cells but cultured in a standard 95% air–5%CO₂ incubator without halothane exposure. The effects of halothane exposure on ATII cells metabolism were assessed during a 4-h exposure of cells at various halothane concentrations (1, 2, 4, and 8%) and during a 2% halothane exposure for increasing durations (2, 4, 8, and 12 h). Finally, the reversibility of halothane effects on PC synthesis was assessed over the 1st and the 2nd h after a 4-h 2% halothane exposure.

Assays

Surfactant Phospholipid Synthesis in Purified Type II Cells. The ability of isolated type II cells to

synthesize surfactant was assessed by the incorporation of a radioactive precursor, [³H-methyl]-choline, in PC, as previously described.¹¹ Twenty-four hours after isolation, the cells monolayers were washed three times with DMEM. Then, 2 μCi/ml of [³H-methyl]-choline chloride were included in fresh DMEM (1 ml/well), and the culture plates were returned to the incubator for the desired time. After halothane exposure, the cells were washed again three times with DMEM containing 40 mM Hepes and 3.0 mg/ml bovine serum albumin (to remove unincorporated [³H-methyl]-choline) and one time with PBS. The cells were sonicated and the lipids were extracted by the method of Bligh and Dyer¹² after removing an aliquot for protein determination. In preliminary experiments, thin-layer chromatography analysis¹³ indicated that 94.4 ± 0.2% (means ± SEM, n = 3) of the lipid associated label was in PC as previously reported.^{14,15} Similar results were obtained when cells were incubated with a 2% halothane concentration for 4 h (97 ± 0.2% [n = 3]). Recovery of radioactivity from plates was > 98% in both experiments. Because separation from the other lipids was therefore unnecessary, routinely the radioactivity in the total lipid fraction was determined. Radioactivity was quantified by scintillation counting (Rack Beta, LKB Wallac, Gaithersburg, MD) with an automatic quenching correction. The values were expressed as disintegrations per minute per microgram intracellular protein. Protein in cell suspensions were measured by the method of Bradford using the differential color change of Coomassie blue dye in response to various concentrations of protein.¹⁶ To test the reversibility of halothane effects, ATII cells were exposed for 4 h to 95% air–5%CO₂ (control cells) or 2% halothane (halothane-exposed cells), and then fresh DMEM was replaced and 2 μCi/ml of [³H-methyl]-choline chloride was added. The PC biosynthesis was assessed over the 1st and 2nd h, with the cells incubated in 95% air–5%CO₂ in a regular incubator.

Surfactant PC Secretion. [³H]-PC was used as a marker for surfactant secretion.¹⁷ Freshly isolated cells were plated at a density of 10⁶ cells/ml, 1 ml/well in 24-well cell culture dishes in DMEM containing 10% fetal bovine serum, glutamine, antibiotics, and 2 μCi/ml of [³H-methyl]-choline chloride. After 24 h incubation the cells were washed three times with fresh DMEM containing 40 mM Hepes and 3.0 mg/ml bovine serum albumin (to remove unincorporated [³H-methyl]-choline and unattached cells) and one time with PBS. Then, 1 ml fresh DMEM was added, and the cells were

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incubated with halothane (1, 2, 4, or 8%) or without halothane (control cells). After a 4-h period, the medium was gently collected from each well, quickly centrifuged to pellet loose cells and the supernatant was recovered. Adherent cells were sonicated. Phospholipids were extracted from both the media and the cells by the method of Bligh and Dyer. PC secretion was expressed as a percentage, calculated as the amount of [^3H]-PC in the medium relative to the amount of [^3H]-PC present in both cells and medium: $[\text{dpm}_{\text{medium}}/(\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}})] \times 100$, where dpm = disintegrations per minute.

Lactate Dehydrogenase Release. To determine whether halothane exposure had a cytotoxic effect on ATII cells, the rate of LDH release into the cell culture medium was measured to assess cellular integrity¹⁸ on the same culture plates used for PC synthesis measurements but on different wells. After exposure to halothane, the medium was gently collected from each well, quickly centrifuged at $200 \times g$ for 10 min to pellet loose cells, and a supernatant sample was removed for extracellular LDH assay. A sample of sonicated cells suspension was used for determination of Intracellular LDH. LDH activity was assayed spectrophotometrically by monitoring the NADH-dependent conversion of pyruvate to lactate at 340 nm.¹⁹ LDH release was expressed as a percentage of total cellular LDH activity: $(\text{extracellular LDH}/[\text{intracellular LDH} + \text{extracellular LDH}]) \times 100$.

Lactate Production. The metabolism of glucose *via* glycolysis by AT II cells was estimated by measuring the lactate production. After halothane exposure, lactate concentration was determined in the cell culture supernatant by an enzymatic method.²⁰ Results were expressed as μmol of lactate per 10^6 cells initially plated in each well.

Measurement of Cellular Adenosine Triphosphate Content. To evaluate if halothane has an effect on the ATII cells energy metabolism, ATP cellular content was measured. ATP cellular contents were measured on cells exposed to various halothane concentrations in separate experiments. After halothane exposure, monolayers were washed three times with PBS and then sonicated into 1 ml ice-cold PBS. Aliquots were removed for protein assay. Samples from each well cell suspension were introduced into conical microtubes containing ice-cold 3 M perchloric acid added with 7 mM EDTA. The samples were mixed thoroughly for 5 min at 4°C to complete the protein extraction and centrifuged at $20,000 g$ for 10 min at 4°C . The

supernatants were removed, neutralized with 3 M KOH, and the centrifugation was repeated. Finally, supernatants were frozen in liquid nitrogen and stored at -80°C until ATP assay. The luciferin-luciferase assay of ATP was adapted from Boulekbache *et al.*²¹ Bioluminescence was measured using a LKB 1250 Lumino-meter with a temperature controlled measuring chamber (25°C). The light emission was calibrated using standardized 10^{-8} to 10^{-7} M ATP solutions. No inhibitory effect of halothane on the luciferin-luciferase reaction was observed. All determinations were made in triplicate.

Data Analysis

Numeric data are presented as the mean \pm SEM derived from at least $n = 3$ independent experiments, each done in triplicate. For each experiment, cells isolated from four rats was pooled and each experimental datum was accompanied by a control datum derived from the same pool of isolated cells. A Paired *t* test was used when one mean value for cells exposed to halothane was compared with its appropriate control value. Regression analysis was used to determine the significance of relation between the measured values and halothane concentration or duration of exposure. In case of significance, the linearity hypothesis was verified. For PC synthesis and cellular ATP content, basal values varied from one cell preparation to another and no control values were common for all the experiments; thus for the regression analysis, values were expressed as percentage of appropriate control values. Statistical significance was accepted with $P < 0.05$.

Results

Phosphatidylcholine Synthesis

A 4-h exposure to halothane reduced PC synthesis by ATII cells for all halothane concentrations except 1% (fig. 1). PC synthesis was reduced by 24, 29 and 36% for 2, 4 and 8% halothane concentrations respectively when compared with controls. The inhibitory effect of halothane appeared to be dose-dependent when PC synthesis was calculated as a function of halothane concentration using a regression analysis of [^3H -methyl]-choline incorporation ($P = 0.006$, $r = 0.52$, standard error of the estimate = 13.44).

The increase in the incorporation rate of [^3H -methyl]-choline into PC over a 12-h period was linear either in controls or after a 2% halothane exposure (corre-

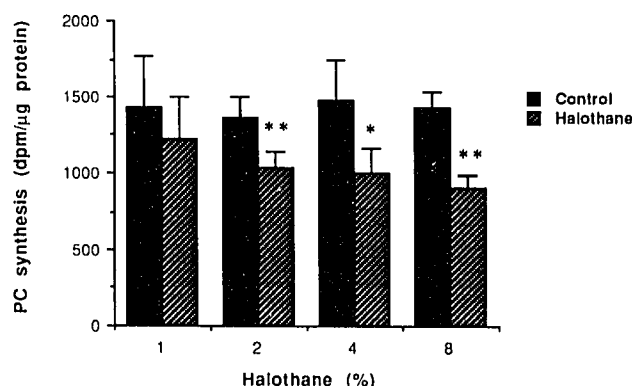


Fig. 1. Effect of increasing halothane concentrations on phosphatidylcholine (PC) synthesis by alveolar type II cells exposed 4 h to halothane. PC synthesis was measured as the incorporation of [3 H-methyl]-choline into PC and was expressed as disintegrations per minute per microgram intracellular protein. Columns = mean \pm SEM of five to eight experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$ versus respective control.

lation coefficients: 0.940 and 0.937 respectively). PC synthesis in ATII cells was reduced by 12, 24, 31 and 34% when cells were exposed to a 2% halothane concentration during 2, 4, 8, and 12 h, respectively (fig. 2). When compared with unexposed cells, PC synthesis tended to be reduced ($P = 0.07$) after 2 h of halothane exposure. The reduction became significant after 4, 8 and 12 h halothane exposure (fig. 2). When PC synthesis was calculated as a function of halothane duration exposure by regression analysis, a time-dependent inhibition of surfactant PC synthesis was noted ($P = 0.008$, $r = 0.52$, standard error of the estimate = 12.67).

As shown in table 1, a rapid reversibility of the halothane induced inhibition of PC synthesis was observed: PC synthesis reached 89% of the control group rate ($P = 0.06$) 60 min after discontinuation of a 2% halothane exposure for 4 h, and further increased to 97% over the 60–120-min interval.

Phosphatidylcholine Secretion

Eight percent halothane increased PC secretion from $2.34 \pm 0.34\%$ (control cells) to $2.78 \pm 0.38\%$ (halothane-exposed cells) ($P < 0.05$) (fig. 3). After exposure to 1, 2, and 4% halothane, PC secretion was not different from control.

Cytotoxicity

When compared with unexposed cells, LDH release from ATII cells exposed to halothane was only in-

creased when cells were exposed for 8 and 12 h to a clinical concentration (2%) (fig. 4). For shorter exposure duration (4 h), the LDH release was not affected by increasing halothane concentrations even by the highest concentration (8%) (table 2). The same results were found when the percentage of specific release of cell-associated ^{51}Cr was used to measure cellular death (data not shown).²² LDH release was affected by halothane exposure duration because when this index was expressed as a function of 2% halothane exposure duration using a regression analysis, a time-related relation was observed ($P = 0.0003$, $r = 0.74$, standard error of the estimate = 1.1).

Lactate Production

The metabolism of glucose *via* glycolysis was estimated by measuring the production of lactate. A 4-h halothane exposure increased lactate production by ATII cells at all the halothane concentrations that we tested, but this increase reached statistical significance only for 2% and 8% concentrations (table 3). When ATII cells were exposed to 2% halothane for increasing periods, we observed that for each interval, lactate production was higher in exposed cells than in control cells (fig. 5). Δ Lactate, which expressed the difference between lactate formation in 2% halothane-exposed cells and appropriate control cells, reached the increasing values of 0.13, 0.41, 0.63 and 0.81 $\mu\text{mol}/10^6$ cells for 2, 4, 8, and 12 h of 2% halothane exposure respectively; a time-dependent increase in this difference was observed ($P = 0.0001$, $r = 0.79$, standard error of the estimate = 0.21). To determine if the increase in lactate concentration in cell culture medium

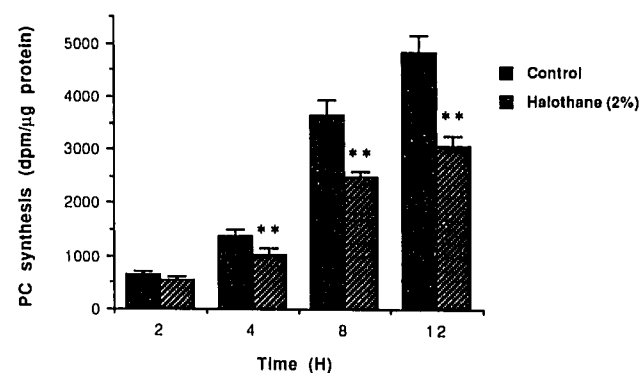


Fig. 2. Time effect on phosphatidylcholine (PC) synthesis by alveolar type II cells during a 2% halothane exposure. Columns = mean \pm SEM of five to eight experiments performed in triplicate. ** $P < 0.01$ versus respective control.

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Table 1. Reversibility of Halothane Effect on Phosphatidylcholine (PC) Synthesis

	0–60 min		60–120 min	
	Control	Halothane	Control	Halothane
PC synthesis (dpm/ μ g protein)	153 \pm 11	136 \pm 11	132 \pm 47	128 \pm 52

To test reversibility, ATII cells were exposed for 4 h to 95% air–5% CO₂ (control cells) or 2% halothane (halothane exposed cells), then 2 μ Ci/ml of [methyl-3H] choline chloride was added to fresh DMEM. The PC biosynthesis was assessed over the next first (interval 0–60 min) and second hour (interval 60–120 min), the cells being incubated in 95% air–5% CO₂ in a regular incubator. PC synthesis was not statistically different in both groups during the two intervals of measurement. Values are mean \pm SEM of four experiments in triplicate.

could have altered PC synthesis by ATII cells, we measured the PC synthesis of ATII cells cultured 4 h with 1, 2 or 3 mM exogenous lactic acid or without exogenous lactic acid (controls). One millimolar lactic acid increased PC synthesis ($P < 0.05$) whereas 2 and 3 mM lactic acid had no effect (data not shown).

ATP Cellular Content

ATP cellular content was reduced in ATII cells exposed to 8% halothane for 4 h when compared with its respective control (fig. 6). With lesser halothane concentrations, reduction in ATP cellular content in exposed cells did not reach statistical significance.

Discussion

The main results of the current study can be summarized as follows. (1) Halothane exposure of rat ATII

cells decreases PC synthesis, the later being dependent of both halothane concentration and exposure duration; (2) this effect is rapidly reversible; (3) surfactant secretion is unaffected by halothane at clinical concentration; (4) cytotoxicity measured by LDH release is mild and appears related to the exposure duration; and (5) halothane-exposed cells exhibit increased glycolytic metabolism reflected by enhanced lactate production, possibly in response to a reduction in cellular ATP levels.

In the current study, the use of ATII cells in primary culture allowed us to measure the direct effects of halothane in well-defined, reliable, and reproducible experimental conditions on the cell population that synthesizes and secretes pulmonary surfactant *in vivo*. Because ATII cells constitute only 15% of adult lung cells, it is impossible to derive any conclusions regarding surfactant metabolism and ATII cells functional alterations during halogenated exposure from studies using whole lungs or mixed-cell cultures. Initial separation of ATII cells is mandatory for study of the me-

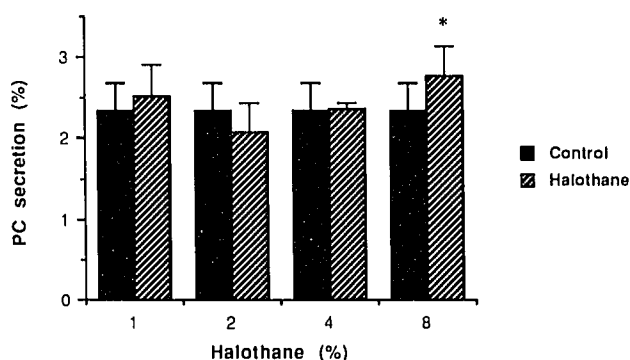


Fig. 3. Effect of increasing halothane concentrations on phosphatidylcholine (PC) secretion by alveolar type II cells exposed 4 h to halothane. PC secretion was measured by the release of [³H]-PC and was expressed as a percentage, calculated as the amount of [³H]-PC in the medium relative to the amount of [³H]-PC present in both cells and medium: $[\text{dpm}_{\text{medium}} / (\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}})] \times 100$, where dpm = disintegrations per minute. Columns = mean \pm SEM of four experiments performed in triplicate. * $P < 0.05$ versus respective control.

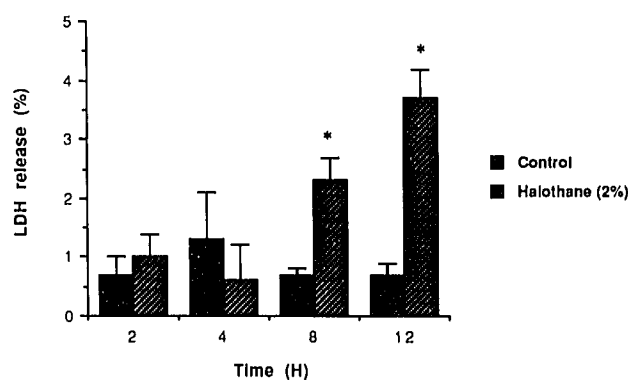


Fig. 4. Time effect on lactate dehydrogenase (LDH) release by alveolar type II cells during a 2% halothane exposure. LDH release is expressed as a percentage of total cellular LDH activity. Columns = mean \pm SEM of three or four experiments performed in triplicate. * $P < 0.05$ versus respective control.

Table 2. Effect of Increasing Halothane Concentrations on LDH Release by ATII Cells Exposed 4 Hours to Halothane

Halothane Concentration (%)	LDH Release (%)		n
	Control	Halothane	
1	2.9 ± 1.4	2.6 ± 0.2	3
2	1.3 ± 0.8	0.6 ± 0.6	3
4	1.9 ± 0.9	2.7 ± 0.6	5
8	1.8 ± 0.8	1.6 ± 1.0	4

Values are mean ± SEM of n experiments performed in triplicate. LDH release was not statistically different in controls and halothane exposed cells for each halothane concentration.

tabolism of surfactant lipids and its regulatory mechanisms.^{2,3} In addition, with cell separation and culture in monolayers, exposure of cells to volatile anesthetics is much easier under conditions approaching those in the bronchoalveolar space.

In this study we used the incorporation of labelled choline into PC as an index of lung surfactant biosynthesis as previously reported by others.⁴ However, because phospholipids are membrane cell constituents, this index may also reflect general membrane synthesis in addition to surfactant synthesis.^{2,3} As we and others^{14,15} observed, more than 90% of the lipid associated label was in PC. When compared with cell membranes, surfactant contains unusually high proportions of saturated PC (60% of PC).^{2,3} Betenbourg *et al.*¹¹ showed that in rat ATII cells the percentage of PC radioactivity found in desaturated PC was 74%. This result agrees with the finding that in freshly isolated ATII cells, the pattern of incorporation of radioactive precursors into various phospholipid classes is quite similar to the relative proportion of phospholipids by content.^{2,3} Therefore, [³H-methyl]-choline incorpora-

Table 3. Effect of Increasing Halothane Concentrations on Lactate Production by ATII Cells Exposed 4 Hours to Halothane

Halothane Concentration (%)	Lactate Production (μmol/16 ⁶ cells)		n
	Control	Halothane	
1	1.74 ± 0.31	2.23 ± 0.26	5
2	1.41 ± 0.26	1.83 ± 0.29*	8
4	1.32 ± 0.35	1.59 ± 0.39	6
8	1.12 ± 0.19	1.49 ± 0.24*	7

Values are mean of n experiments performed in triplicate

* *P* < 0.05 versus respective control.

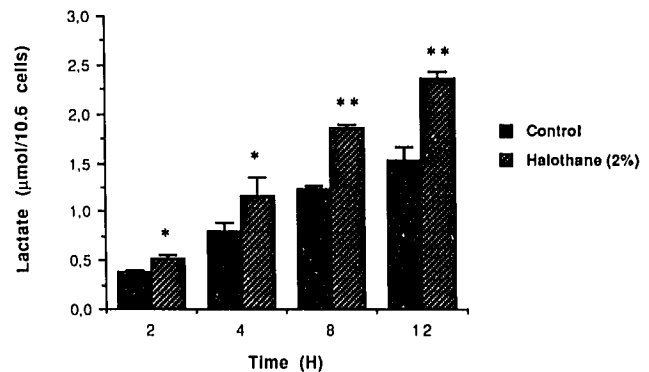


Fig. 5. Time effect on lactate production by alveolar type II cells during a 2% halothane exposure. Columns = mean ± SEM of four to eight experiments performed in triplicate. **P* < 0.05; *P* < 0.01 versus respective control.**

tion into PC reflects primarily the incorporation into desaturated PC. This makes the incorporation of [³H-methyl]-choline into PC a valid index of surfactant biosynthesis, even though it is not an absolutely specific index.

The incorporation of [³H-methyl]-choline into ATII cell PC was inhibited by halothane exposure in a dose- and time-dependent fashion. The maximal PC synthesis inhibition was observed for halothane concentrations surpassing those of clinical relevance. However, PC synthesis tended to decrease in cells treated with concentration as low as 1%, and reached statistical significance for 2% halothane. The MAC value for halothane in the rat (0.9–1.15%) is somewhat higher than for

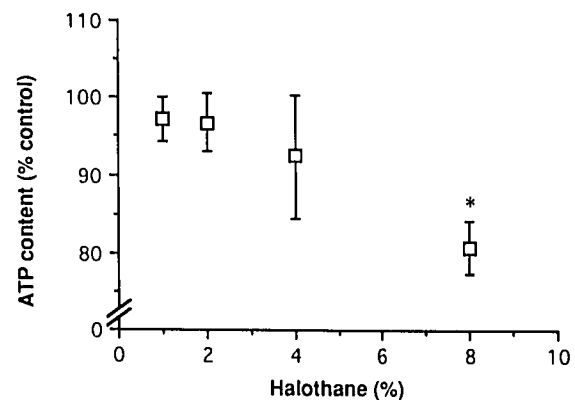


Fig. 6. Effect of increasing halothane concentrations on cellular content of adenosine triphosphate (ATP). Alveolar type II cells were exposed 4 h to halothane. ATP was expressed as a percentage of the respective control. Values are the mean ± SEM of four to six experiments performed in triplicate. **P* < 0.05 versus respective control.

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humans, therefore 1% represents approximately 0.9–1. MAC²⁴ Furthermore, at clinical concentration (2%), PC synthesis was already reduced by 24% in cells exposed to halothane for only 4 h. This inhibitory effect of halothane was rapidly and fully reversible. Indeed, the rate of PC synthesis in cells previously exposed to halothane did not differ significantly from those of unexposed cells on the 0–120-min interval after the anesthetic delivery was stopped.

The mechanisms by which halothane induces a decrease in PC biosynthesis in ATII cells remain to be elucidated. Several possibilities can be discussed. A cytotoxic effect of halothane may account, at least in part, for the reduced PC synthesis observed for 8 and 12 h halothane exposure. Indeed a larger LDH release from cells exposed to halothane than from control cells was noticed for these long incubation periods (8 and 12 h). In contrast, for shorter incubation periods (4 h) the dose-related effect of halothane exposure on PC synthesis was not associated with cytotoxicity even for the highest halothane concentrations (8%), as assessed by both the release of the intracellular enzyme LDH and the release of cell associated ⁵¹Cr (data not shown). In addition, the complete and rapid reversibility of the halothane effects on PC synthesis after 4 h exposure at 2% halothane, supports the minor role of a direct cytotoxic effect of the drug. Exposure to the higher halothane concentrations tested involved dilution of the O₂ and CO₂ in the carrier gas. We do not believe that this phenomenon could account for the reduction of PC synthesis by ATII cells, because Fisher and colleagues demonstrated that radiolabeled choline incorporation into lung PC was not altered by severe CO hypoxia (5%).²⁵

Another mechanism that could account for the reduction of the radio-labelled PC measured in type II cells would be an increase in surfactant secretion by ATII cells under halothane exposure. In our study, surfactant secretion was not affected by increasing halothane concentration except by the highest concentration (8%). However, the increase in surfactant secretion measured at this concentration and for 4-h halothane exposure was very mild and could not explain the decrease in surfactant biosynthesis that we observed.

Changes in cellular energy metabolism under halothane may also be discussed. ATII cells in culture exhibit predominantly glycolytic metabolism as assessed by the enhanced lactate production. Glucose consumption by ATII cells produces lactate, pyruvate, and CO₂; the ratio of lactate production to pyruvate pro-

duction is about two in basal culture conditions.²⁶ In the current study, ATII cells exposed to halothane produced more lactate than did the respective control cells. Furthermore, a time-dependent increase in lactate production was observed in both control and halothane-exposed cells but the rate of increase was higher in halothane-exposed cells. This results may reflect direct consequences of halothane exposure on the cellular energetic metabolism. Glycolytic metabolism is induced in response to a decrease in ATP concentration, referred to as the Pasteur effect.²⁷ Therefore, the increase in lactate formation under halothane exposure could reflect the activation of glycolysis concurrently to a decrease in ATP production. It has been shown that halothane alters mitochondrial function by inhibiting directly certain reactions of electron transport, particularly the stepwise transfer of fuel-derived electrons to O₂ that releases sufficient energy to support the bulk of normal cellular ATP production (oxidative phosphorylation).^{28,29} Cellular ATP content in halothane-exposed cells decreased with increasing halothane concentrations. Consequently the increase in glycolytic metabolism in halothane-exposed cells is related at least in part to a reduction in cellular ATP levels. However the cellular ATP depletion was mild at clinically relevant halothane concentrations and reached statistical significance only for the highest halothane concentration that we tested (8%). Thus it is unlikely that cellular ATP depletion plays a major role in the reduction of PC synthesis by ATII cells under halothane exposure. The decrease in PC biosynthesis with halothane could also be related to the metabolic effects of halothane by themselves. The halothane effect on surfactant was not duplicated by lactic acid alone, excluding an indirect effect of halothane. Finally, other mechanisms, such as an effect of halothane upon the cellular membrane³⁰ or intracellular calcium metabolism,³¹ may also be involved in the observed reduction in PC synthesis.

The possible consequences of type II cells exposure to halothane *in vivo* remain to be described. Pulmonary surfactant synthesized by type II alveolar cells plays a major role in maintaining normal lung function by decreasing the surface tension in the alveolar space; and thus preventing alveolar collapse at end expiration. This surface tension reducing properties of surfactant are related primarily to the phospholipid components of surfactant and much more specifically to PC.⁴ Therefore, halothane alteration in surfactant phospholipid metabolism may be of clinical relevance although the

observed dose-response relation suggested that the inhibition of PC synthesis would be small at halothane concentrations equivalent to those used for maintenance of clinical anesthesia (1–2%). These data emphasize the need for more extensive investigations of the volatile anesthetic effects and their mechanisms on type II alveolar cells and surfactant metabolism.

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References

- Finck BR, Kenny GE: Metabolic effects of volatile anesthetics in cell culture. *ANESTHESIOLOGY* 29:505–516, 1968
- Wartell SA, Christopherson R, Watkins CA, Rannels DE: Inhibition of synthesis of lung proteins by halothane. *Mol Pharmacol* 19:520–524, 1981
- Hammer JA III, Rannels DE: Effects of halothane on protein synthesis and degradation in rabbit pulmonary macrophages. *Am Rev Respir Dis* 124:50–55, 1981
- Van Golde LMG, Batenburg JJ, Robertson B: The pulmonary surfactant system: Biochemical aspects and functional significance. *Physiol Rev* 68:374–455, 1988
- Kikkawa Y, Smith F: Cellular and biochemical aspects of pulmonary surfactant in health and disease. *Lab Invest* 49:122–139, 1983
- Dobbs LG, Gonzalez R, Williams MC: An improved method for isolating type II cells in high yield and purity. *Am Rev Respir Dis* 134:141–145, 1986
- Crestani B, Rolland C, Petiet A, Colas-Linhart N, Aubier M: Cell surface carbohydrates modulate neutrophil adherence to alveolar type II cells in vitro. *Am J Physiol* 264 (Lung Cell Mol Physiol 8):L391–L400, 1993
- Mason RJ, Williams MC, Greenleaf RD, Clements JA: Isolation and properties of type II alveolar cells from rat lung. *Am Rev Respir Dis* 115:1015–1026, 1977
- Malledant Y, Siproudhis L, Tanguy M, Clerc C, Chesne C, Saint-Marc C, Guillouzo A: Effects of halothane on human and rat hepatocyte cultures. *ANESTHESIOLOGY* 72:526–534, 1990
- Heusler H: Quantitative analysis of common anaesthetic agents. *J Chromatogr* 340:279–319, 1985
- Batenburg JJ, Longmore WJ, Van Golde LMG: The synthesis of phosphatidylcholine by adult rat lung alveolar type II epithelial cells in primary culture. *Biochim Biophys Acta* 529:160–170, 1978
- Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917, 1959
- Gilfillan AM, Chu AJ, Smart DA, Rooney SA: Single plate separation of lung phospholipids including desaturated phosphatidylcholine. *J Lipid Res* 24:1651–1656, 1983
- Chander A: Regulation of lung surfactant secretion by intracellular pH. *Am J Physiol* 257 (Lung Cell Mol Physiol 1):L354–L360, 1989
- Mettler NR, Gray ME, Schuffman S, Lequire VS: β -Adrenergic induced synthesis and secretion of phosphatidylcholine by isolated pulmonary alveolar type II cells. *Lab Invest* 45:575–586, 1981
- Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
- Dobbs LG, Gonzalez RF, Marinari LA, Mescher EJ, Hawgood S: The role of calcium in the secretion of surfactant by rat alveolar type II cells. *Biochim Biophys Acta* 877:305–313, 1986
- Rice WR, Whitsett JA: Inhibition of surfactant release from isolated type II cells by compound 48/80. *Biochim Biophys Acta* 805:261–267, 1984
- Wroblewski F, Ladue JS: Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* 90:210–213, 1955
- Shimojo N, Naka K, Nakajima C, Yoshikawa C, Okuda K, Odako K: Test-strip method for measuring lactate in whole blood. *Clin Chem* 35:1992–1994, 1989
- Boulekbache H, Bastin J, Andriamihaja M, Lefebvre B, Joly C: Aging of fish oocytes: Effects on adenylic nucleotides content, energy charge and viability of carp embryo. *Comp Biochem Physiol* 93B:471–476, 1989
- Crim C, Simon RH: Effects of oxygen metabolites on rat alveolar type II cell viability and surfactant metabolism. *Lab Invest* 58:428–437, 1988
- Dobbs LG: Isolation and culture of alveolar type II cells. *Am J Physiol* 258 (Lung Cell Mol Physiol 2):L134–L147, 1990
- Quasha AL, Eger EI II, Tinker JH: Determination and applications of MAC. *ANESTHESIOLOGY* 53:315–334, 1980
- Fisher AB, Dodia C, Chander A: Energy dependence of lung phosphatidylcholine biosynthesis studied with CO hypoxia. *Am J Physiol* 249 (Endocrinol Metab 12):E89–E93, 1985
- Fisher AB, Furia L, Berman H: Metabolism of rat granular pneumocytes isolated in primary culture. *J Appl Physiol* 49:743–750, 1980
- Tang MJ, Suresh KR, Tannen RL: Carbohydrate metabolism by primary cultures of rabbit proximal tubules. *Am J Physiol* 256 (Cell Physiol 25):C532–C539, 1989
- Hall GM, Kirtland SJ, Baum H: The inhibition of mitochondrial respiration by inhalational anaesthetic agents. *Br J Anaesth* 45:1005–1009, 1973
- Harris RA, Munroe J, Farmer B, Klim KC, Jenkins P: Action of halothane upon mitochondrial respiration. *Arch Biochem Biophys* 142:435–444, 1971
- Franks NP, Lieb WR: What is the molecular nature of general anaesthetic target sites? *Trends Pharmacol Sci* 8:169–174, 1987
- Iaizzo PA, Seewald MJ, Powis G, Van Dyke RA: The effects of volatile anesthetics on calcium mobilization in rat hepatocytes. *ANESTHESIOLOGY* 72:504–509, 1990