

Differential Effects of Halothane and Thiopental on Surfactant Protein C Messenger RNA In Vivo and In Vitro in Rats

Catherine Paugam-Burtz, M.D.,* Serge Molliex, M.D., Ph.D.,† Bernard Lardeux, Ph.D.,‡ Corinne Rolland, T.A.,§, Michel Aubier, M.D.,|| Jean-Marie Desmonts, M.D.,# Bruno Crestani, M.D., Ph.D.**

Background: Pulmonary surfactant is a complex mixture of proteins and phospholipids synthesized by alveolar type II cells. Volatile anesthetics have been shown to reduce surfactant phospholipid biosynthesis by rat alveolar type II cells. Surfactant-associated protein C (SP-C) is critical for the alveolar surfactant functions. Our goal was to evaluate the effects of halothane and thiopental on SP-C messenger RNA (mRNA) expression *in vitro* in rat alveolar type II cells and *in vivo* in mechanically ventilated rats.

Methods: *In vitro*, freshly isolated alveolar type II cells were exposed to halothane during 4 h (1, 2, 4%) and 8 h (1%), and to thiopental during 4 h (10, 100 μ M) and 8 h (100 μ M). *In vivo*, rats were anesthetized with intraperitoneal thiopental or inhaled 1% halothane and mechanically ventilated for 4 or 8 h. SP-C mRNA expression was evaluated by ribonuclease protection assay.

Results: *In vitro*, 4-h exposure of alveolar type II cells to thiopental 10 and 100 μ M increased their SP-C mRNA content to 145 and 197%, respectively, of the control values. In alveolar type II cells exposed for 4 h to halothane 1, 2, and 4%, the SP-C mRNA content increased dose-dependently to 160, 235, and 275%, respectively, of the control values. *In vivo*, in mechanically ventilated rats, 4 h of halothane anesthesia decreased the

lung SP-C mRNA content to 53% of the value obtained in control (nonanesthetized, nonventilated) animals; thiopental anesthesia increased to 150% the lung SP-C mRNA content.

Conclusions: These findings indicate that halothane and thiopental used at clinically relevant concentrations modulate the pulmonary SP-C mRNA content in rats. *In vivo*, the additive role of mechanical ventilation is suggested. (Key words: Lung; pneumocytes, volatile anesthetics.)

PULMONARY surfactant is a complex mixture of proteins and phospholipids synthesized by alveolar type II (ATII) epithelial cells. It reduces lung alveolar surface tension, maintains alveolar fluid balance, and possibly exhibits host-defense properties. Although the phospholipid makeup of surfactant is critical to its functional integrity, surfactant-associated proteins, particularly the hydrophobic surfactant-associated protein C (SP-C), have been recognized increasingly as conferring the properties of rapid surface adsorption and surface-tension reduction to phospholipids during dynamic compression.^{1,2} SP-C, which is synthesized exclusively by ATII cells,³ facilitates the adsorption and the spreading of the phospholipids to form the surfactant monolayer^{2,4} and enhances *in vitro* the lipid uptake in ATII cells.⁵ *In vivo* in animal models of surfactant deficiency^{6,7} and in preterm infants with respiratory distress syndrome,⁸ bovine-derived exogenous surfactants that contain SP-C are more effective at decreasing the rates of barotrauma and mortality.

During inhalation anesthesia, lung alveolar epithelium is exposed directly to volatile anesthetics. Recently, potentially deleterious effects of halothane on the surfactant metabolism of ATII cells have been described. Our group has shown that halothane at clinically relevant concentrations reduces surfactant phospholipid biosynthesis by rat ATII cells *in vitro*,⁹ whereas intravenous anesthetic agents such as thiopental have no effect on phospholipid surfactant metabolism.¹⁰ These results highlight differential effects of anesthetic agents on

* Associate Professor of Anesthesiology, Service d'Anesthésie-Réanimation Chirurgicale, Hôpital Bichat, and Unité INSERM 408, Faculté X. Bichat.

† Professor of Anesthesiology, Unité INSERM 408, Faculté X. Bichat.

‡ Physical Doctor, Unité INSERM 327, Faculté X. Bichat.

§ Research Associate, Unité INSERM 408, Faculté X. Bichat.

|| Professor of Pneumology, Unité INSERM 408, Faculté X. Bichat.

Professor of Anesthesiology, Service d'Anesthésie-Réanimation Chirurgicale, Hôpital Bichat.

** Professor of Pneumology, Unité INSERM 408, Faculté X. Bichat.

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Address reprint requests to Dr. Paugam-Burtz: Department of Anesthesiology, Hôpital Bichat, 46, Rue Henri Huchard, 75877 Paris cedex 18. Address electronic mail to: catherine_paugam@hotmail.com

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physiologic functions of ATII cells. Whether or not pulmonary SP-C is affected by volatile anesthetics remains to be determined. The present study was designed to assess the effects of halothane and thiopental on surfactant SP-C messenger RNA (mRNA) expression, *in vitro* in freshly isolated ATII cells and *in vivo* in mechanically ventilated rats.

Materials and Methods

Animals were treated according to the guidelines of the French Institut National de la Santé et de la Recherche Médicale (INSERM) for laboratory care of animals.

Materials

Adult male pathogen-free Sprague-Dawley rats (200–220g) (Charles Rivers Breeders, St Aubin les Elbeuf, France) were used within 4 days of delivery. Animals were killed by exsanguination under general anesthesia. Halothane was purchased from Trofield (London, United Kingdom). Sodium thiopental was purchased from Spécia (Paris, France). TEMED, ammonium persulfate, and guanidine thiocyanate were from Sigma (La Verpillère, France). [α - 32 P]UTP (128×10^{11} Bq/mmol) was from Amersham (Les Ulis, France). RNase-free DNase I and ribonucleases A and T1 were supplied from Boehringer (Mannheim, Germany). Acrylamide-bisacrylamide, phenol, urea, and proteinase K were from Appligene (Illkirch, France). BSU36I restriction enzyme was from New England Biolabs (Beverly, MA). Tissue culture media, supplements, and fetal bovine serum were from Gibco BRL Life Technologies (Cergy Pontoise, France). Tissue culture plastic was from Costar (Cambridge, MA).

In Vitro Study

Alveolar type II cells were isolated from rat lungs by elastase dissociation and purified by differential adherence to plastic as previously described.^{9,11} Cell isolation yield per rat and cellular viability (assessed by the Trypan blue exclusion test) were 25 ± 1.10^6 and $95 \pm 5\%$ (mean \pm SD, $n = 30$). In this preparation, about 60% of the cells are ATII cells.¹¹ For each experiment, freshly isolated cells from four different rats were pooled and immediately plated in 10-cm diameter cell culture plastic dishes (20×10^6 cells/dish) with 20 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10^5 U/l penicillin, 100 mg/l streptomycin, and 0.25 mg/L amphotericin B; the cells then were exposed to the different experimental conditions (control, halo-

thane, or thiopental). Each experiment was performed three times.

Freshly isolated ATII cells were exposed to halothane using a 12-l airtight Lwoff chamber (Lequeux, Paris, France) as previously described.^{9,12} The chamber atmosphere was kept continuously saturated with water at 37°C. In preliminary experiments, we observed that over an 8-h period the different culture conditions used in our experiments did not influence SP-C mRNA expression by ATII cells, the expression being similar in standard incubator and in the sealed chamber flushed with a 95% air-5% CO₂ mixture. Halothane was vaporized by directing a 95% air-5% CO₂ mixture at 5 l/min through a calibrated Fluotec Mark II vaporizer (Abbott Paris, France) placed at the entrance of the chamber. Halothane concentration was monitored at the chamber exit port using a halogen monitor (Capnomac Datex Helsinki, Finland). The effects of halothane on SP-C mRNA expression of ATII cells were assessed during 4-h exposure with various halothane concentrations (1, 2, and 4%) and during 8-h exposure with 1% halothane.

Exposure of freshly isolated ATII cells to thiopental was achieved by addition of sodium thiopental (molecular weight: 264) to the culture medium (final concentrations: 10 and 100 μ M). The cell plates were returned to a standard 95% air-5% CO₂ incubator for the desired period of time (4 or 8 h). We chose thiopental concentrations consistent with those that may be observed in plasma in clinical practice.^{13,14}

For each exposure condition, a control point was obtained simultaneously with ATII cells cultured in a standard 95% air-5% CO₂ incubator without anesthetic exposure. At the end of each experiment, supernatants and adherent cells were frozen immediately in liquid nitrogen and stored at -80°C until RNA extraction.

In Vivo Study

In all animals (adult rats weighting 200–220 g), anesthesia was induced initially with ether vapor, and then the trachea was cannulated with a 14-gauge catheter. Animals were assigned randomly for maintenance of anesthesia to inhaled 1% halothane or intraperitoneal injection of thiopental. Halothane was vaporized at 1% inspired concentration (one minimal alveolar concentration for rats)¹⁵ monitored by a halogen monitor (Capnomac Datex). Rats then were ventilated for 4 and 8 h ($n = 6$ animals for each duration). Thiopental-anesthetized rats received a first intraperitoneal injection of thiopental (12.5 mg/100 g weight). Three animals ventilated

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during 8 h needed extra doses of thiopental (3 mg/100 g weight) at the 6th h of ventilation.

Ventilator strategies were identical in all animals and consisted of a fraction of inspired oxygen of 0.21, a tidal volume of 3 ml, and a respiratory rate of 60 breaths/min without positive end-expiratory pressure. A rectal temperature probe was positioned for core temperature monitoring, and temperature was maintained with a heating pad. After 4 or 8 h of ventilation, rats were killed by aorta transection, and the lungs were removed from the thorax and immediately frozen in liquid nitrogen and stored at -80°C until total pulmonary RNA extraction.

Six rats anesthetized with ether vapor and immediately killed by section of the abdominal aorta after trachea cannulation without additional anesthesia or mechanical ventilation, were used as a control group.

Ribonuclease Protection Assay

Total RNA from lung and cultured ATII cells was isolated using RNAPlus (Bioprobe, Montreuil, France) according to the instructions of the manufacturer. SP-C gene expression by rat ATII cells was evaluated by ribonuclease protection assay on total RNA solubilized in guanidine thiocyanate.¹⁶ Total RNA was cohybridized with β -actin and SP-C RNA probes. A 243-nucleotide SP-C riboprobe was obtained from SP-C complementary DNA inserts subcloned into PGEM 4Z phagemid vector.¹⁷ Riboprobe synthesis was performed in the presence of [α - ^{32}P]UTP (128×10^{11} Bq/mmol, 5×10^4 Bq) and T7 RNA polymerase after linearization of the vector by BSU36I digestion. The 144-nucleotide β -actin riboprobe was synthesized from a BSU36I rat complementary DNA insert into PGEM 3Z.

For hybridization in guanidine thiocyanate, 10 μl of appropriate dilution of RNA preparations was mixed with 2 μl of each labeled riboprobe (10^5 counts/min/ μl). After overnight hybridization at room temperature, the samples were treated with ribonucleases A and T1 and exposed to proteinase K. After extraction with phenol:chloroform:isoamylalcohol, the protected RNA:RNA hybrids were precipitated and loaded on a 6% acrylamide-bisacrylamide urea denaturing gel. Quantitative analysis of radioactive protected bands was performed by direct counting of the gel (InstantImager, Packard, Groningen, The Netherlands). To control for variations in RNA loading, the ratio of the SP-C mRNA signal and the corresponding β -actin mRNA signal was calculated for each sample.^{16,18} Expression of β -actin mRNA has been shown to be constant under various conditions, including halothane and thiopental exposure.¹⁸ Relative changes

were expressed versus control (nonexposed) cells or control (nonanesthetized, nonventilated) animals.

Statistical Analysis

Results are presented as the mean \pm SD. *In vivo* results are derived from six animals in each group. For *in vitro* experiments, cells isolated from four rats were pooled and each experimental datum was accompanied by a control datum derived from the same pool of isolated cells. *In vitro* results are derived from at least three independent pools of ATII cells. Comparisons between groups were made using a Kruskal-Wallis nonparametric analysis of variance, followed in case of significance by a Mann-Whitney U test. $P < 0.05$ was accepted as significant.

Results

In Vitro Study

Exposure of rat ATII cells to halothane at 1, 2, and 4% for 4 h increased the SP-C: β -actin mRNA ratio compared with ATII cells cultured in standard conditions for 4 h. The ratio was increased to $160 \pm 48\%$, $232 \pm 53\%$, and $275 \pm 110\%$ with 1, 2, and 4% halothane ($P < 0.05$ for each comparison *vs.* control value) (fig. 1). The stimulating effect of halothane appeared to be dose-dependent if the SP-C: β -actin mRNA ratio was calculated as a function of halothane concentration using a regression analysis ($P = 0.032$, $r = 0.59$, SE of the estimate = 15.35).

The culture of ATII cells with thiopental (10 and 100 μM) for 4 h increased the SP-C: β -actin mRNA ratio to

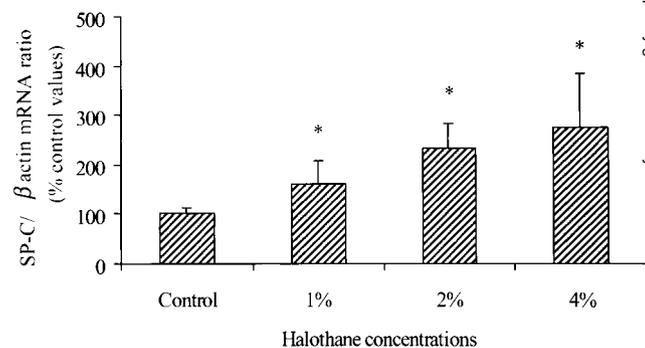


Fig. 1. *In vitro* effects of 4-h halothane exposure at various concentrations on surfactant-associated protein C: β -actin messenger RNA ratio in rat alveolar type II cells (percentage of control values, $n = 3$ per condition, mean \pm SD). * $P < 0.05$ versus control values. (Alveolar type II cells cultured in standard conditions.)

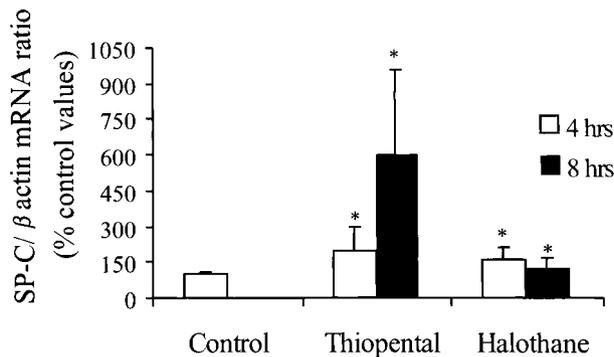


Fig. 2. *In vitro* effects of exposure of alveolar type II cells to halothane (1%) or thiopental (100 μ M) for 4 and 8 hours on surfactant-associated protein C: β -actin messenger RNA ratio (percentage of control value, $n = 3$ per condition, mean \pm SD). * $P < 0.05$ versus control values. (Alveolar type II cells cultured in standard conditions.)

145 \pm 48% and 197 \pm 97%, respectively, compared with cells cultured in standard conditions ($P < 0.05$).

In some experiments, we evaluated the effects of higher durations of exposure. ATII cells were cultured 4 and 8 h with 1% halothane or 100 μ M thiopental (fig. 2). After 4- and 8-h exposure to halothane, the SP-C: β -actin mRNA ratio in ATII cells increased to 160 \pm 48% and 121 \pm 43%, respectively ($P < 0.05$). A stronger increase in the SP-C: β -actin mRNA ratio was noted in ATII cells incubated with thiopental for 4 and 8 h (197 \pm 97% and 598 \pm 359% of the control values; $P < 0.05$).

In Vivo Study

The lung SP-C: β -actin mRNA ratio of halothane-anesthetized rats decreased to 47 \pm 5% of the control value in animals anesthetized for 4 h ($P < 0.05$) and to 61 \pm 26% in animals anesthetized for 8 h ($P < 0.05$), com-

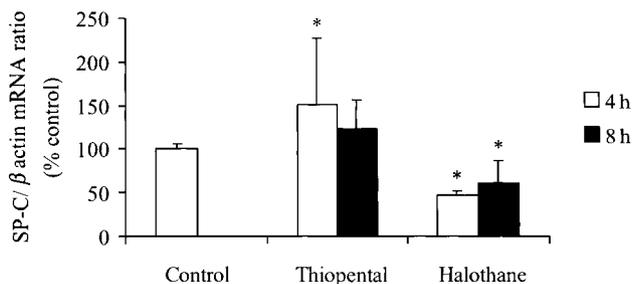


Fig. 3. *In vivo* effects of halothane and thiopental anesthesia on lung surfactant-associated protein C: β -actin messenger RNA ratio (percentage of control value, mean \pm SD) in rats mechanically ventilated during 4 and 8 hours ($n = 6$ for each duration). * $P < 0.05$ versus control values. Control values are derived from nonanesthetized and nonventilated animals.

pared with (nonventilated, nonanesthetized) control animals (fig. 3). By contrast, in animals anesthetized with intraperitoneal sodium thiopental, the SP-C: β -actin mRNA ratio increased to 150 \pm 77% of the control value after 4 h ($P < 0.05$). After 8 h, there was a trend toward an increase of the SP-C: β -actin mRNA ratio (124 \pm 33%), which was not statistically significant.

Discussion

Our study demonstrates that *in vitro*, halothane or thiopental used at clinically relevant concentrations and over a short time period increase the SP-C mRNA content in purified rat ATII cells. By contrast, *in vivo* in mechanically ventilated rats, we observed that inhalation of 1% halothane for 4 h was associated with a marked decrease in the SP-C mRNA content, whereas thiopental anesthesia induced a moderate increase in the SP-C mRNA content.

Before the interpretation of these findings, methodologic considerations must be discussed. In the current study, we measured SP-C mRNA content using ribonuclease protection assay. This method of solution hybridization has been shown to be easier than Northern blot and an excellent method to quantify relatively abundant mRNA.¹⁹

We exposed freshly isolated ATII cells, which are about 60% pure, to anesthetic agents. Because expression of SP-C is specific to ATII cells,³ this probably did not influence our results, particularly if halothane or thiopental did not influence the release of factors from these other cells that could have affected the SP-C metabolism. *In vitro* experiments allowed us to approach the *in vivo* conditions of alveolar epithelial cell exposure to inhaled anesthetics. This model has been validated by previous studies by our group.^{9,10,12} Similarly, ATII cells were exposed to thiopental concentrations consistent with those measured in plasma during anesthesia after thiopental administration.^{13,14} These concentrations, however, are probably higher than the actual free thiopental concentrations. Moreover, thiopental concentrations in alveolar space during anesthesia are unknown.

In vivo, we studied anesthetized and mechanically ventilated rats. We used ether vapor to perform tracheotomy in rats. The effects of ether on surfactant metabolism *in vivo* have not been studied. All animals, however, were exposed in similar manners to ether vapor. We believe that our *in vivo* experimental model repro-

duces some of the usual circumstances of anesthesia in humans. One percent inspired concentration of halothane has been shown to be the minimum alveolar concentration for rats.¹⁵

In our study, thiopental induced a significant increase in SP-C mRNA content in both the *in vivo* and the *in vitro* experiments. The effects of thiopental on surfactant have not been studied extensively. Ward and Nicholas²⁰ have shown *in vivo* in rats that thiopental anesthesia inhibits surfactant phospholipid release in the alveolar compartment, leading to an increase in surfactant synthesis. Marota *et al.*¹⁸ have observed that thiopental *in vivo* in rats increases the cerebral content of jun-B mRNA after 120 min of anesthesia. In that study, thiopental did not modify the cerebral β -actin mRNA content. *In vitro*, thiopental administration is associated with a significant increase in mRNA synthesis in hepatocytes.²¹ Phenobarbital also has been shown to increase *in vivo* the hepatic α_1 -acid glycoprotein mRNA content. This effect was related to a transcriptional regulation of the gene through a specific phenobarbital responsive element.²² We speculate that thiopental can increase SP-C mRNA expression through a transcriptional regulation. The variation in SP-C protein lung content induced by the variation in SP-C mRNA content, however, remains undetermined.

In vitro, we observed that halothane increased the SP-C mRNA content of ATII cells. Only a few studies have previously reported the effects of halothane on specific mRNA expression. Inhibition of RNA transcription in lymphocytes exposed to halothane for 6 hours has been described *in vitro*.²³ *In vivo*, halothane anesthesia in rats decreases the cerebral jun-B mRNA content,¹⁸ whereas c-fos mRNA content is unchanged and decreases the lung macrophage inflammatory protein-2 mRNA content after an inflammatory challenge.²⁴ These results highlight a differential effect of halothane on the expression of various mRNAs that could be related to transcriptional or posttranscriptional changes. To our knowledge, however, precise mechanisms of action of halothane have not been elucidated. Surprisingly, we observed opposite findings between *in vivo* and *in vitro* expression of SP-C mRNA exposed to halothane. Halothane produced a significant decrease in lung SP-C mRNA content in mechanically ventilated rats, whereas *in vitro* halothane exposure of ATII cells increased the SP-C mRNA content. Results from *in vivo* experiments reflect effects of both anesthetic agent and mechanical ventilation. Our *in vivo* experimental model does not allow us to differentiate these two effects clearly. Exper-

imental models designed to ventilate mechanically animals without anesthesia are not available. In preliminary experiments, we observed that spontaneously breathing rats anesthetized with halothane and thiopental died within 2 hours because of alveolar hypoventilation and major lung atelectasis. Experimental models of perfused and ventilated isolated lungs could have been useful to individualize the effects of mechanical ventilation on SP-C. These models, however, do not allow for ventilation of the lungs for 4 or 8 hours. Data on effects of mechanical ventilation on surfactant are scarce. Wirtz and Dobbs²⁵ have shown that the mechanical stretch of ATII cells increases the cytosolic calcium concentration and stimulates the secretion of surfactant phospholipid. In a similar model, Gutierrez *et al.*²⁶ have demonstrated that mechanical distension of ATII cells resulted in a 20% decrease in the mRNA content of SP-C and a 35% decrease of the surfactant-associated protein B mRNA content. The decrease appeared related to changes at both transcriptional and posttranscriptional levels. These data suggest that mechanical distension induced by mechanical ventilation can reduce the SP-C mRNA content in ATII cells. This effect could explain why halothane induced a mild increase in the SP-C mRNA *in vitro* whereas SP-C mRNA content decreased during halothane anesthesia in mechanically ventilated rats. It also could explain why the increase in the lung SP-C mRNA content after thiopental anesthesia was much less important than the increase observed after *in vitro* exposure of ATII cells to thiopental. Behavior of isolated cultured ATII cells are likely to be different from *in vivo* cells because of loss of cellular interactions *in vitro*. These changes also could account for the discrepancy in results between our two experimental conditions.

The results of this study shows that mechanical ventilation under halothane anesthesia decreases the SP-C mRNA pulmonary content in rats, but thiopental anesthesia in the same conditions increases the SP-C mRNA content. Ventilation-induced injury such as alveolar edema has been recognized recently as one of the potential complications of mechanical ventilation. Surfactant inactivation has been suggested to be one component of various events occurring during ventilation-induced lung injury.²⁷ Moreover, halothane can alter phosphatidyl secretion in ATII cells.⁹ Taken together, these experimental findings suggest potentially additive deleterious effects of volatile anesthetics and mechanical ventilation on the homeostasis of the alveolar space. This might be particularly relevant in patients with acute lung injury. In these particular clinical conditions, surfactant

metabolism is already impaired.^{28,29} It remains to be determined whether the variations in SP-C mRNA content induced by anesthetics translate into similar variations in SP-C protein.

Our results suggest a positive effect of halothane and thiopental on the pulmonary SP-C mRNA content in rats A₁II cells *in vitro*. By contrast, *in vivo* during anesthesia and mechanical ventilation, thiopental and halothane have different effects. Further studies are needed to address the clinical significance of these effects.

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