ISOFLURANE REDUCES THE SYNTHESIS OF SURFACTANT-RELATED PROTEIN A OF ALVEOLAR TYPE II CELLS INJURED BY H_2O_2

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SUMMARY

The influence of isoflurane (Iso) on the synthesis of surfactant-related protein A (SP-A) of alveolar type II (AT II) cells in primary culture and after injury by H_2O_2 was investigated. AT II cells were isolated and purified from adult Sprague-Dawley rats and used for experiments after 32 h in primary culture. The cell cultures were randomized to six groups (n = 8 in each group): control group (no treatment), 0.28 mM Iso group, 2.8 mM Iso group, 75 μM H_2O_2 group, 75 μM H_2O_2 + 0.28 mM Iso group, and 75 μM H_2O_2 + 2.8 mM Iso group. Each group was continuously incubated for 3 h after administration of Iso and/or H_2O_2. The intracellular SP-A and the SP-A of the culture medium were measured with an enzyme-linked immunosorbent assay (ELISA). Iso significantly decreased the intracellular SP-A content and that of the culture medium, and aggravated the decrease of SP-A content induced by H_2O_2. These findings suggest that Iso itself may decrease SP-A synthesis of AT II cells *in vitro*, and aggravate the damage to AT II cells under peroxidation conditions.

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KEY WORDS

isoflurane, alveolar type II cells, surfactant related protein A, hydrogen peroxide, *in vitro*

INTRODUCTION

Surfactant-related protein A (SP-A) synthesized by alveolar type α (ATα) cells is a major constituent of pulmonary surfactant. Pulmonary surfactant is a mixture of phospholipids and proteins, and reduces the surface tension within the alveoli of the lung and maintains alveolar fluid balance. Isoflurane (Iso) is frequently used as a volatile anesthetic agent (including Iso, halothane, desflurane and sevoflurane). During Iso anesthesia, ATα cells are directly exposed to isoflurane. Our previous study reported that Iso itself could inhibit the synthesis of phosphatidycholine (PC) of AT II cells *in vitro*, and further damaged the cells' function under peroxidation conditions /1/. In previous *in vitro* studies, it has been reported that in AT II cells exposed to halothane, the surfactant protein C (SP-C) mRNA content increased in a dose-dependent manner /2/. On the other hand, *in vivo*, in mechanically ventilated rats, halothane anesthesia decreased the lung SP-C mRNA content to 53% of the value obtained in control non-anesthetized and non-ventilated animals /2/. Recent studies found that Iso /3/ and sevoflurane /4/ induced the generation of reactive oxygen species (ROS). It has been demonstrated that ROS destroys surfactant protein /5/. Whether Iso influences SP-A synthesis of AT II cells has not been investigated. The goal of the current study was to evaluate the influence of Iso on SP-A synthesis of AT II cells.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: isoflurane from Abbott Company (Chicago, USA), and elastase from Difico Laboratories (Detroit, USA). DMEM, DNAse I and all chemicals of the highest grade available were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Cell culture

This study was approved by the Committee of Scientific Research and the Committee of Animal Care of the Third Military Medical University, Chongqing, China.

Alveolar type II cells were isolated from the lungs of adult Sprague-Dawley rats. Pooled cells from 20 rats (200 ± 22 g) were prepared as described by Dobbs *et al.* /6/. Rats were weighed and anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital, and injected intravenously with 4000 U/kg heparin sodium. After tracheotomy, the rats were exsanguinated via the abdominal aorta, and the lungs were perfused via the pulmonary artery with solution II containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1.3 $MgSO_4$, 2.5 PBS, pH 7.4, at 22°C, and the lungs were ventilated several times via the trachea. The heart and lungs were removed, and the lungs were lavaged 4-6 times via the trachea with solution I containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 0.2 EGTA, 6-D-glucose, 2.5 PBS, pH 7.4, at 22°C to remove macrophages. The lungs were washed twice with solution II and filled with 10 ml of elastase solution, and then incubated and digested in a water bath in air at 37°C for 20 min, after which an additional 10 ml of elastase solution was instilled after an interval of 10 min. The lungs were cut in a DNAse I (0.25 mg/ml) solution and digestion was stopped with 5 ml newborn calf serum. The tissue suspension was filtered through 120 μm and 200 μm stainless steel mesh, and the filtrate was centrifuged at 130 *g* for 8 min at 4°C. The cell pellet was suspended in 10 ml DMEM (containing 10% newborn calf serum, 100 U/ml penicillin, 100 U/ml streptomycin), and transferred to rat IgG-coated culture dishes at 37°C in a 5% CO_2 incubator for 1-2 hours to remove macrophages, lymphocytes and neutrophils by differential adherence. The unattached cells were centrifuged again at 130 *g* for 8 min at 4°C, and the cell pellet was resuspended with DMEM. The resulting cells (>90% purity and >95% viability) were plated at a density of 5 x 10^5/ml in culture dishes and incubated in 5% CO_2/95% O_2 at 37°C for 24 hours. The AT II cells were identified with a tannic and polychrome stain.

Enzyme-linked immunosorbent assay (ELISA)

AT II cells were cultured for 24 hours in primary culture and digested with elastase. The cells were gently plated at a density of

10^6/ml in 96-well culture dishes and further cultured for 8 hours. The cells were washed with DMEM and randomly divided into six groups (n = 8 in each group). These included the control group (no treatment), 0.28 mM Iso group, 2.8 mM Iso group, 75 μM H_2O_2 group, 75 μM H_2O_2 + 0.28 mM Iso group, and 75 μM H_2O_2 + 2.8 mM Iso group. After adding the relevant experimental factors, each group of cells was cultured at 37°C in a 5% CO_2 incubator for 3 hours, and the SP-A of the culture medium and cell lysates was immediately measured with ELISA. Firstly, 0.5 μg of rabbit anti-rat SP-A polyclonal antibody in 100 μl of 0.1 M $NaHCO_3$ was adsorbed overnight onto wells of microtiter immunoassay plates. The wells were washed and then blocked with 5% skim milk/1% Triton X-100/PBS. Samples from the culture medium and cell lysates, and SP-A standards (0 to 50 ng), were added and incubated for 1 h at 37°C. After washing the wells, 2 μg of horseradish peroxidase-conjugated rabbit anti-rat SP-A polyclonal antibody was added and further incubated for 90 min at 37°C. The cells were next washed with 1% Triton X-100/PBS. Substrate and buffer (10 mg *O*-phenylenediamine dihydrochloride, 10 μl of 30% H_2O_2 in 10 ml of 0.1 M citrate buffer, pH 4.6) were added, and color development was allowed to proceed for 5-10 min in the dark. The reaction was stopped by adding 2 N H_2SO_4. Product formation was quantified by measuring absorbance (OD) at 490 nm using an automated microplate reader (Synergy HT, Bio-Tec Instruments, USA).

Data analysis

All data are presented as means ± SD. Statistical analysis was performed using SPSS 12.0 software. Differences between the groups were analyzed by analysis of variance (ANOVA). Statistical significance was attributed to p values lower than 0.05.

RESULTS

Exposure to 0.28 mM or 2.8mM Iso significantly decreased SP-A content both in the culture medium and AT II cells, as compared with the normal control group ($p < 0.05$). 75 μM H_2O_2 markedly decreased the SP-A content of the culture medium and the AT II cells, as compared with the normal cell groups ($p < 0.01$). Exposure to Iso

significantly aggravated the decrease in SP-A content induced by H_2O_2 both in the culture medium and AT II cells, and in the H_2O_2-treated control cells group compared with 0.28 mM Iso group ($p < 0.05$) and 2.8 mM Iso group ($p < 0.01$). No difference was found in the effect of Iso on SP-A content between the 0.28 and 2.8 mM groups. Details are shown in Tables 1 and 2.

TABLE 1

Effect of isoflurane (Iso) on the surfactant-related protein A (SP-A) content of alveolar type II cells (n = 8)

	SP-A (ng)	
	Normal cells	**H_2O_2-treated cells**
Control group	121.33 ± 8.56	59.32 ± 6.88 [a]
0.28 mM Iso group	86.79 ± 6.52 [b]	40.25 ± 5.66 [a,c]
2.8 mM Iso group	79.47 ± 7.44 [b]	34.14 ± 5.32 [a,d]

[a] $p < 0.01$ compared with normal cells (control or 0.28 mM Iso or 2.8 mM Iso group).
[b] $p < 0.05$ compared with control (normal cells) group.
[c] $p < 0.05$ compared with control (H_2O_2-treated cells) group.
[d] $p < 0.01$ compared with control (H_2O_2-treated cells) group.

TABLE 2

Effect of isoflurane (Iso) on surfactant-related protein A (SP-A) content of the culture medium (n = 8)

	SP-A (ng)	
	Normal cells	**H_2O_2-treated cells**
Control	79.57 ± 8.45	39.22 ± 4.15 [a]
0.28 mM Iso group	62.35 ± 7.75 [b]	20.42 ± 3.54 [a,c]
2.8 mM Iso group	54.65 ± 6.54 [b]	17.88 ± 0.03 [a,d]

[a] $p < 0.01$ compared with normal cells (control or 0.28 mM Iso or 2.8 mM Iso group).
[b] $p < 0.05$ compared with control (normal cells) group.
[c] $p < 0.05$ compared with control (H_2O_2-treated cells) group.
[d] $p < 0.01$ compared with control (H_2O_2-treated cells) group.

DISCUSSION

In the present study, we found that both 0.28 mM and 2.8 mM Iso significantly decreased the SP-A content of the culture medium and AT II cells, as compared with the normal control group. 75 μM H_2O_2 markedly decreased the SP-A content of the culture medium and AT II cells, as compared with normal cell groups, Iso aggravated the decrease of SP-A content induced by H_2O_2 in both the culture medium and AT II cells. It has been reported that 0.28 mM and 2.8 mM isoflurane is equal to blood concentration of 1 minimal alveolar anesthetic concentration (MAC) and 10 MAC, respectively, in clinical studies /7/.

The mechanism(s) by which Iso decreases the SP-A content of the culture medium and AT II cells remains unknown. Osanai *et al.* /8/ examined the synthesis, transport, and localization of SP-A in primary cultures of ATα cells, and found newly synthesized SP-A was mainly secreted into the culture medium, and SP-A was taken up by the ATα cells and incorporated into lamellar bodies (LBs) from the extracellular pool. Thus the decrease of SP-A content may be the result of Iso inhibition of SP-A synthesis. It has been demonstratedthat halothane inhibits protein synthesis in primary cultures of mixed lung cells in a dose-dependent manner, and halothane exposure inhibits the incorporation of [^{14}C]phenylalanine (the precursor amino acid) into lung proteins /9/. The decrease of SP-A content may result from an indirect action of Iso on intracellular ATP. Molliex *et al.* /10/ demonstrated that clinically relevant concentrations of halothane were capable of causing metabolic dysfunction and reduced ATP content in ATα cells. Isoflurane is a halogenated anesthetic similar to halothane, but the effects of isoflurane have not been previously demonstrated. Some studies /11/ reported that isoflurane augments expression of proinflammatory cytokines (such as TNF-α) in rat alveolar macrophages, and TNF-α can inhibit the synthesis of SP-A. It is not yet clear whether Iso affects SP-A mRNA. It was reported that halothane anesthesia decreased the lung SP-C mRNA content /2/.

H_2O_2 markedly decreased the SP-A content of the culture medium and AT II cells, obviously the result of lipid peroxidation. Lipid peroxidation can destroy SP-A, and decrease SP-A content /5/. It is interesting that our results showed that isoflurane aggravated the decrease of SP-A induced by H_2O_2. This may be due to isoflurane

involvement in lipid peroxidation which facilitates this process. It has been reported that halothane and isoflurane increases pulmonary artery endothelial cell sensitivity to oxidant-mediated injury and aggravates lung injury /12/. Nielsen *et al.* /13/ found that desflurane decreased the content of vitamin C in lung tissue. Sato *et al.* /14/ reported that sevoflurane induced lipid peroxidation in guinea-pig liver microsomes. This is supported by an observation showing that exposure to halothane increased inflammatory lung injury *in vivo* as well as oxidant-induced cell injury i*n vitro* /15/. Recently, it has been reported that Iso /3/ and sevoflurane /4/ induced organisms to generate ROS. Iso, halothane, desflurane and sevoflurane are all halogenated anesthetics. However, the effects of isoflurane remain to be investigated.

In conclusion, this study shows that Iso (at clinical or superclinical concentrations) decreased the SP-A content of the culture medium and AT II cells, especially under peroxidation conditions. SP-A plays a major role in the regulation of alveolar surface tension. In clinical practice, halogenated anesthetics, including isoflurane, may be not the best choice for patients with existing lung injury, especially under peroxidation conditions, such as ischemia and reperfusion, trauma, shock, intoxication or severe systemic infection, etc.

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