EFFECT OF ISOFLURANE ON PROLIFERATION AND Na^+,K^+-ATPase ACTIVITY OF ALVEOLAR TYPE II CELLS INJURED BY HYDROGEN PEROXIDE

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SUMMARY

The influence of isoflurane (Iso) on proliferation and Na^+,K^+-ATPase activity of alveolar type II cells (ATII cells) injured by hydrogen peroxide (H_2O_2) was investigated. ATII cells isolated and purified from adult Sprague-Dawley rats were randomly divided into six groups: control group, 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 primary culture for 32 hours, the proliferation of ATII cells was detected by MTT assay, and after culture for 24 hours the activity of Na^+,K^+-ATPase and lactate dehydrogenase (LDH) in the cells, and malonaldehyde (MDA) content of the culture medium, were measured by colorimetry. It was found that 0.28 mM and 2.8 mM Iso had no effect on the proliferation of ATII cells ($p > 0.05$), but 75 μM H_2O_2 inhibited their proliferation ($p < 0.05$) compared with untreated controls; 0.28 mM and 2.8 mM Iso significantly decreased Na^+,K^+-ATPase activity of ATII cells compared with untreated control cells ($p < 0.05$), and 75 μM H_2O_2 markedly decreased Na^+,K^+-ATPase activity of ATII cells ($p < 0.01$)

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with untreated control cells. 0.28 mM and 2.8 mM Iso aggravated the decrease of Na^+,K^+-ATPase activity induced by H_2O_2. Iso had no effect on LDH activity and MDA content of the culture medium of normal ATII cells, but significantly increased LDH activity and MDA content of the culture medium of ATII cells injured by H_2O_2. These findings suggest that Iso itself may decrease the activity of Na^+,K^+-ATPase of ATII cells *in vitro* and further damage the cells' function under peroxidation conditions, but has no effect on the proliferation of ATII cells.

KEY WORDS

isoflurane, alveolar type II cells, proliferation, Na^+,K^+-ATPase, hydrogen peroxide, halogenated anesthetics

INTRDUCTION

Alveolar type II (ATII) cells not only synthesize and secrete surfactant but also perform transepithelial solute transport. ATII cells maintain the alveolar space fluid-free by actively transporting Na^+ from the alveolar space to the interstitium. Sodium,potassium-adenosine triphosphatase (Na^+,K^+-ATPase) is one important protein involved in Na^+ transport in alveolar epithelial cells /1,2/. Sodium enters alveolar cells through apical Na^+ channels, and is extruded at the basolateral membrane by the Na^+,K^+-ATPase pump. Water flows passively into the interstitium, where it is removed by the lymphatic system. The procedure maintains the 'dry' state of the alveolar space and enables normal gas exchange. In a previous study we reported that isoflurane (Iso) itself could inhibit the synthesis of phosphatidycholine of ATII cells *in vitro* and further damage the cells' function under peroxidation conditions /3/. Isoflurane is a widely used volatile anesthetic; its effect on the proliferation and Na^+,K^+-ATPase activity of ATII cells has not been investigated. The aim of the current study was to evaluate the influence of isoflurane on cell proliferation and Na^+,K^+-ATPase activity in ATII cells.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: isoflurane from Abbott, elastase from Difco. DMEM, MTT, DNAse I and all chemicals of the highest grade available were purchased from Sigma. A Lambda Bio-20 ultraviolet spectrophotometer (Perkin-Elmer) was used.

Cell culture

Alveolar type II cells were isolated from the lungs of adult Sprague-Dawley rats (180-220 g) as described by Dobbs *et al.* /4/. Rats were weighed and anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital, and injected intraperitoneally with 4,000 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, at 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 in 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 of 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 resuspended in 10 ml of DMEM (containing 10% of newborn calf serum, 100 U/ml of penicillin, 100 U/ml of 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 in 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 ATII cells were identified with a tannic and polychrome stain /5/.

MTT measurement

ATII cells were cultured for 24 hours and then 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: control group, 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 having eight wells. 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 immediately measured with MTT according to the manufacturer's instructions. MTT is a tetrazolium salt, which is decomposed to formazan in mitochondria. After dissociation, formazan is easy to quantify with ELISA. The higher the production of formazan in the mitochondria, the stronger the cell viability; in addition, the mitochondrial production of formazan is a good marker of cell proliferation activity. The details of MTT measurement are as follows: 20 μl of MTT solution (5 mg/ml) was added to each well, and incubated for 3 hours. The solution was then removed from the incubation medium, 100 μl DMSO was added, and the mixture shaken for 10 min, and them measured with an ELISA counter at 490 nm. The formazan content was expressed as an OD value.

Na^+,K^+-ATPase activity, lactate dehydrogenase (LDH) and malonaldehyde (MDA) measurement

ATII cells were cultured for 24 hours in primary medium and digested with elastase. The cells were gently plated at a density of 10^7/ml in 30 mm culture dishes and randomly divided into six groups as above, each group having eight wells. After adding the relevant experimental factors, cells were incubated at 37°C in a 5% CO_2 incubator for 3 hours. After incubation, the culture medium was collected for LDH and MDA assay. Cells were washed gently with DMEM, digested with trypsogen, transferred to 5 ml centrifuge tubes, and centrifuged at 1,000 rpm for 10 min at 4°C. The cell pellet was mixed gently with 5 ml of 0.9% NaCl and centrifuged three times at 1,000 rpm for 10 min at 4°C. The cell pellet was mixed with 30 ml

10 mmol/l Tris-HCl buffer (10 mmol/l Tris-HCl, EDTA 1 mmol/l, pH 7.4), and samples were incubated for 20 min at 4°C, then centrifuged at 17,500 *g*, and the supernatant discarded. This was repeated three times and the precipitate was then mixed with 200 μl to measure Na^+,K^+-ATPase activity by colorimetry. Na^+,K^+-ATPase activity was expressed as units of 1 μmol of inorganic phosphorus produced by ATPase decomposing ATP per mg protein per hour (μmol Pi/mg protein·h). The cellular protein content was determined by the Lowry reaction.

LDH activity and MDA content in the culture medium were determined with LDH kit (Roche) and MDA kit (Roche), respectively, according to the manufacturer's instructions.

Data analysis

Results are presented as means ± SE. Data were analyzed by Student's t-test, and differences were considered significant if $p < 0.05$.

RESULTS

Effect of isoflurane on MTT

0.28 mM and 2.8mM Iso had no effect on the proliferation of ATII cells ($p > 0.05$), but 75 μM H_2O_2 markedly inhibited the proliferation of ATII cells ($p < 0.05$) compared with untreated control cells (Table 1).

Effect of isoflurane on Na^+,K^+-ATPase

0.28 mM and 2.8 mM Iso significantly decreased Na^+,K^+-ATPase activity of ATII cells compared with untreated control cells ($p < 0.05$), and 75 μM H_2O_2 markedly decreased Na^+,K^+-ATPase activity of ATII cells ($p < 0.01$) compared with untreated control cells. 0.28 mM and 2.8 mM Iso aggravated the decrease of Na^+,K^+-ATPase activity induced by H_2O_2 (Table 2).

TABLE 1

Effect of isoflurane (Iso) on the proliferation of ATII cells

	OD value	
	Untreated cells	**H_2O_2-treated cells**
Control	0.56 ± 0.14	0.31 ± 0.10 [a]
0.28 mM Iso	0.54 ± 0.15	0.30 ± 0.07 [a]
2.8 mM Iso	0.52 ± 0.14	0.29 ± 0.08 [a]

[a] $p < 0.01$ compared with untreated cells.

TABLE 2

Effect of isoflurane (Iso) on Na^+,K^+-ATPase activity of ATII cells

	Na^+,K^+-ATPase activity (μmol Pi/mg protein·h)	
	Untreated cells	**H_2O_2-treated cells**
Control	5.43 ± 0.75	3.29 ± 0.47 [a]
0.28 mM Iso	4.30 ± 0.33 [b]	2.21 ± 0.73 [ab]
2.8 mM Iso	4.16 ± 0.41 [b]	2.01 ± 0.22 [ab]

[a] $p < 0.01$ compared with untreated control cells.
[b] $p < 0.05$ compared with control cells (untreated or H_2O_2-treated).

Effects of isoflurane on LDH activity and MDA content of culture medium of ATII cells

Iso had no effect on LDH activity and MDA content of the culture medium of normal ATII cells, but significantly increased LDH activity and MDA content of culture medium of ATII cells injured by H_2O_2. Details are shown in Table 3.

TABLE 3

Effects of isoflurane (Iso) on LDH activity and MDA content of culture medium of ATII cells

	Untreated cells		H_2O_2-treated cells	
	LDH (μmol/l)	MDA (nmol/l)	LDH (μmol/l)	MDA (nmol/l)
Control	138 ± 6	1.39 ± 0.09	215 ± 19 [a]	2.55 ± 0.19[a]
0.28 mM Iso	142 ± 9 [b]	1.42 ± 0.18 [b]	219 ± 22[ab]	2.60 ± 0.15 [ab]
2.8 mM Iso	144 ± 10 [b]	1.45 ± 0.13 [b]	241 ± 10[ab]	2.83 ± 0.20 [ab]

[a] $p < 0.01$ compared with untreated control cells.
[b] $p < 0.05$ compared with control cells (normal or H_2O_2-treated).

DISCUSSION

Na^+,K^+-ATPase is the one of major proteins involved in Na^+ transport in alveolar epithelial cells. In the present study, we found that 0.28 mM and 2.8mM Iso had no effect on the proliferation of ATII cells, but 75 μM H_2O_2 markedly inhibited the proliferation of ATII cells; 0.28 mM and 2.8 mM Iso significantly decreased Na^+,K^+-ATPase activity of ATII cells, and 75 μM H_2O_2 markedly decreased Na^+,K^+-ATPase activity of ATII cells. 0.28 mM and 2.8 mM Iso aggravated the decrease of Na^+,K^+-ATPase activity induced by H_2O_2. It is reported that 0.28 mM and 2.8 mM isoflurane is equal to a blood concentration of 1 MAC (minimal alveolar concentration) and 10 MAC, respectively, in clinical practice (1 MAC is the minimum alveolar concentration of inhaled anesthetic at 1 atmosphere that prevents movement in 50% of subjects in response to a painful stimulus) /6/. We found no difference in inhibition of Na^+,K^+-ATPase activity between 0.28 mM and 2.8 mM Iso.

The mechanism by which Iso decreases Na^+,K^+-ATPase activity remains unknown. The decrease in the activity of Na^+,K^+-ATPase may result from a direct action of Iso on the pump protein itself, or indirect modifications of intracellular mediators, such as ATP or cytosolic free calcium, or by modifying the lipid environment of the protein. Halothane was previously reported to decrease Na^+,K^+-ATPase activity and reduce ATP content in ATII cells /1/. In addition,

halothane and isoflurane increased pulmonary artery endothelial cell sensitivity to oxidant-mediated injury, and aggravated lung injury /7/. Nielsen *et al.* /8/ found that desflurane decreased the content of vitamin C in lung tissue. Sato *et al.* /9/ reported sevoflurane induced lipid peroxidation in guinea-pig liver microsomes. Isoflurane is a halogenated anesthetic similar to halothane, desflurane and sevoflurane, but the effects of isoflurane have not been demonstrated. We found Iso had no effect on the MDA (an end product of lipid peroxidation) content of the culture medium of normal ATII cells. Iso may modify membrane fluidity. A certain degree of fluidity has been shown to be essential for Na^+,K^+-ATPase activity. Halogenated anesthetics could induce modification in membrane fluidity, an effect that might affect the activity of numerous membrane proteins, among which are transport systems /1,10/.

The decrease in the activity of Na^+,K^+-ATPase may result from cytotoxic effects. It was reported that halothane has cytotoxic effects /11-13/, but we found Iso had no effect on LDH activity in the culture medium of normal ATII cells. In addition, we did not find any effect of Iso on the proliferation of ATII cells, so the decrease of Na^+,K^+-ATPase activity did not result from the change of cell number.

H_2O_2 markedly decreased Na^+,K^+-ATPase activity and inhibited the proliferation of ATII cells, which is obviously the result of lipid peroxidation /14/. However, Iso aggravated the decrease of Na^+,K^+-ATPase activity induced by H_2O_2 and significantly increased LDH activity and MDA content of culture medium of ATII cells injured by H_2O_2. It is possible that Iso facilitates lipid peroxidation.

In conclusion, this study shows that Iso (at clinical or superclinical concentration) decreased the Na^+,K^+-ATPase activity of ATII cells, especially under peroxidation conditions. Na^+,K^+-ATPase plays a major role in the regulation of alveolar fluid balance. In clinical practice, halogenated anesthetics may be not the best choice for patients with existing lung injury, especially under peroxidation conditions, such as ischemia and reperfusion, trauma, shock, intoxication, severe systemic infection, etc.

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