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Full Length Research Paper

Structural changes and effect of denopamine on alveolar fluid clearance in hypoxic rat lungs

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Terbutaline (β_2-adrenergic agonist) can increase alveolar fluid clearance (AFC) under physiologic and pathologic conditions. It is unknown whether β_1-adrenergic agonists also increase AFC under pathologic conditions. The aim of this study was to investigate the effect of denopamine (β_1- adrenergic agonist) on AFC in hypoxic lung injury and the possible mechanisms involved. Hypoxic rats were exposed to 10% oxygen. 10^{-5} mol/L denopamine alone or combined with β receptor antagonists, Na^+ channel blocker, or Na^+/K^+-ATPase blocker were perfused into the alveolar space of rats exposed to 10% oxygen for 48 h. AFC and total lung water content (TLW) were measured. AFC did not change for the first 24 h but then decreased after 48h exposure to 10% oxygen. The perfusion of denopamine significantly increased AFC in normoxic and hypoxic rats. The AFC- stimulating effect of denopamine lowered with amiloride (a Na^+ channel blocker) and ouabain (a Na^+/K^+-ATPase inhibitor) by 35 and 53%, respectively. Colchicine significantly inhibited the effect of denopamine. Denopamine can increase the AFC during hypoxic lung injury in rats and accelerate the absorption of pulmonary edema.

Key words: β_1-Adrenergic agonist, hypoxia, pulmonary edema, alveolar epithelium.

INTRODUCTION

Alveolar epithelium is currently considered to be not only a limited permeable barrier but also the most likely site for absorption of excess alveolar fluid (Sartori et al., 2001; Hastings et al., 2003; Heberlein et al., 2000). Na^+ enters the apical membranes of alveolar type II cells through amiloride-sensitive ion channels and is actively transported across the basolateral membranes of these cells by the ouabain-sensitive Na^+/K^+-ATPase (Mehta et al., 2004; Cheng et al., 2003). The stimulation of alveolar fluid clearance (AFC) accelerated the resolution of pulmonary edema and facilitated gas exchange across the alveolar epithelium.

It has been reported that β_2-adrenergic agonists increase AFC under physiologic and pathologic conditions (Sartori and Matthay, 2002; Matthay et al., 2000, 2002). However, it is unknown whether β_1-adrenergic agonists increase AFC under pathologic conditions. Hypoxia induced a down regulation of the expression and activity of Na^+ channels and Na^+/K^+-ATPase (Vivona et al., 2001; Wodopia et al., 2000). The purpose of this study was to investigate the effect of β_1-adrenergic agonist on AFC in rats with hypoxic lung injury, and the mechanisms involved.

MATERIALS AND METHODS

All rats received human care, and this study was approved by the institutional ethics committee. The 14 ± 2 (mean ± SD) week old, specific-pathogen-free male Wistar rats weighing 340 ± 40 g (mean ± SD) were provided by animal center, Shengjing Affiliated Hospital, China Medical University, China. Denopamine were purchased from Tanabe Pharmaceutical Co., Ltd., Tokyo. Atenolol, terbutaline, ICI-118551, amiloride, ouabain, colchicine, β-lumicolchicine and Evans blue were purchased from Sigma, St. Louis, MO, USA. Albumin bovine serum, chloral hydrate, phosphoric acid, osmium teroxide,

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propylene oxide, uranyl acetate were purchased from Beijing Superior Chemicals and Instruments CO., LTD, China.

General protocol

Hypoxic exposure (Feng et al., 2005)

For the hypoxia experiments, rats were exposed to hypoxia for up to 72 h in a specially designed chamber (80 × 60 × 50 cm) with 10 small separate chambers in it. It was continuously flooded with nitrogen at 10 L/min. When the concentration reached 10%, the gas flow was maintained at 1 - 2 L/min. The concentrations were continuously monitored with an O_2 analyzer. Carbon dioxide was trapped by soda lime granules in the box. Animals were allowed free access to food and water. The procedure was the same for control normoxic rats, except that the chamber was ventilated with 21% O_2.

Measurement of AFC (Sakuma et al., 2004)

Rats were anesthetized by intraperitoneal administration of chloral hydrate (0.03 ml/10 g) and an endotracheal tube was inserted through a tracheotomy. The rats were exsanguinated through the abdominal aorta. The trachea, lungs, and heart were excised and placed in a humidified incubator at 37°C. The lungs were ventilated with 100% nitrogen. Physiological saline solution (5 ml/kg) containing 5% albumin and Evans blue dye (0.15 mg/ml) was injected into the alveolar spaces through the endotracheal tube. After injection, the lungs were inflated with 100% nitrogen at an airway pressure of 7 cm H_2O. Alveolar fluid was aspirated 1 h after injection. The concentrations of Evans blue-labeled albumin in the injected and aspirated solutions were measured by a spectrophotometer at 621 nm. AFC was estimated by the progressive increase in the concentration of alveolar Evans blue-labeled albumin and calculated as follows (Sakuma et al., 2004): $AFC=[(V_i - V_f)/ V_i]\times100$, and $V_f = V_i \times P_i / P_f$. V_i is the volume of injected albumin solution, and V_f is the volume of final alveolar fluid. P_i is the concentration of Evans blue in the injected albumin solution and P_f is the concentration of Evans blue in the final alveolar fluid.

Morphology change

Tissue samples for electron microscopy and light microscopy were taken from left lung of rats. Samples for electron microscopy were washed in 1% phosphoric acid buffer, fixed with 4% osmium teroxide, and then dehydrated in a graded series of alcohol, transferred to propylene oxide, and embedded in Epon 618. Thin sections were cut using a diamond knife, and then stained with uranyl acetate and lead citrate for electron microscopic studies.

The methods for evaluating the number of endothelial cells and alveolar epitheiai cells were described previously by James et al., (1980) in detail. The numerical density of lung cells (Nv) was determined using an equation $Nv=N_A/\overline{D}$, where N_A = the frequency of occur-rence of nuclear profiles per unit area of a random sectioning plane, and $\overline{D}$ = the mean caliper diameter of pulmonary cell nuclei. The frequency of occurrence of nuclear profiles per unit area of a random sectioning plane (N_A) was determined using the electron microscope. Using previously described techniques (James et al., 1980), we determined the mean caliper diameter of lung nuclei ($\overline{D}$) for control normoxic rats and for rats exposed to hypoxia for 24, 48 and 72 h.

Tissue samples for light microscopy were embedded in paraffin and cut to 10 µm sections, all of these sections were stained with hematoxylin and eosin using standard procedures for light microscopic studies.

Measurement of total lung water content (TLW)

The TLW of the lung was measured by drying the lungs to a constant weight at 60°C for 72 h. The TLW was measured using the Noble method (Sakuma et al., 2001; Wang et al., 2007): TLW = (wet lung weight-dry lung weight)/ dry lung weight.

Specific protocols

Rats were randomly allocated into 19 groups with 10 animals in every group using random number tables.

Effects of hypoxia on lung morphology change and TLW (n=40)

Tissue samples for electron microscopy and light microscopy were taken from left lung of rats exposed to hypoxia for 24 h (n=10), 48 h (n = 10), 72 h (n = 10) and control normoxic rats (n = 10). We measured the TLW of the right lung of rats exposed to hypoxia for 24, 48, 72 h and control normoxic rats.

Effects of hypoxia on AFC (n=40)

Isomolar albumin solutions were injected into the alveolar spaces in rats exposed to hypoxia for 24 h (n=10), 48 h (n=10), 72 h (n=10) and control normoxic rats (n = 10).

Effects of denopamine on AFC (n=40)

10^{-6} - 10^{-3} mol/L Denopamine (selective β_1-adrenergic agonist) increase alveolar fluid clearance in a dose-dependent manner in normoxic rat lungs (Sakuma et al., 2001). Isomolar albumin solutions in the presence of 10^{-5} mol/L denopamine were injected into the alveolar spaces in rats exposed to hypoxia for 24 h (n=10), 48 h (n=10), 72 h (n = 10) and control normoxic rats (n=10).

Effects of terbutaline on AFC (n=10)

10^{-5} and 10^{-4} mol/L terbutaline (selective β_2-adrenergic agonist) increase alveolar fluid clearance in isolated rat lungs (Sakuma et al., 2004). To investigate the effect of terbutaline on AFC in rats exposed to hypoxia, 10^{-5} mol/L terbutaline were injected into the alveolar spaces in rats exposed to hypoxia for 48 h rats.

Effects of β-adrenergic antagonists on AFC modulation by denopamine (n = 20)

Atenolol and ICI- 118551 inhibit the effect of β-adrenergic agonists in a dose-dependent manner (Dana et al., 2001; Gu et al., 2001). To determine whether denopamine-stimulated AFC was mediated by the stimulation of β-adrenoceptors, atenolol (10^{-4} mol/L) (n = 10) or ICI- 118551 (10^{-7} mol/L) (n = 10) was added to the albumin solutions containing denopamine (10^{-5} mol/L) and injected into the alveolar spaces in rats exposed to hypoxia for 48 h rats.

Effects of denopamine on amiloride-sensitive and amiloride-insensitive AFC (n=20)

5 × 10^{-4} mol/L Amiloride or 5 × 10^{-4} mol/L ouabain inhibit the

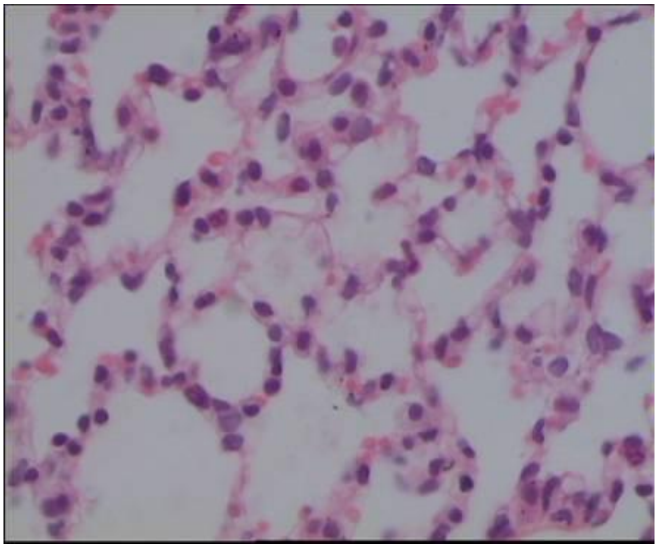

Figure 1a. Control rat (HE ×400). The alveolar space was basically dry, the lung interstice had no edema and the peri-alveolar blood vessels showed no dilations or hyperemia.

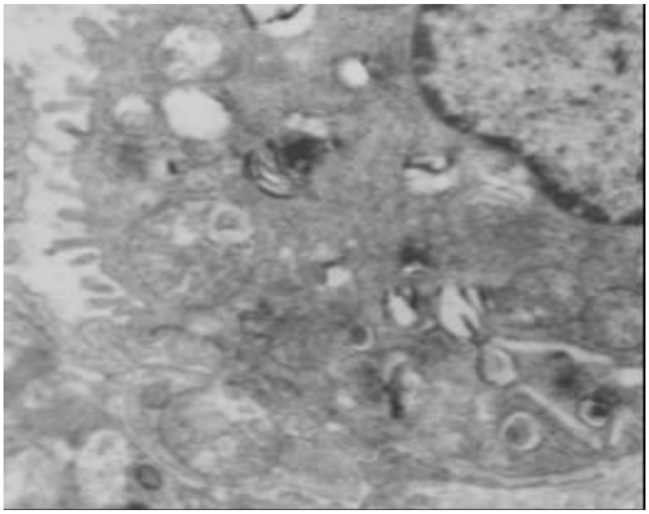

Figure 1b. The epithelial cellular structure of control rat (×12000).

stimulatory effects of terbutaline on AFC (Gu et al., 2001). To determine whether hypoxia altered the effects of denopamine on amiloride-sensitive and amiloride- insensitive sodium channels, an isomolar albumin solution in the presence of 10^{-5} mol/L denopamine plus 5 × 10^{-4} mol/L amiloride (n = 10) or 5 × 10^{-4} mol/L ouabain (n=10) was injected into the alveolar spaces in rats exposed to hypoxia for 48 h.

Effects of the cellular microtubular system on AFC modulation by denopamine (n = 20)

An isomolar albumin solution in the presence of 10^{-5} mol/L denopamine was injected into the alveolar spaces in rats exposed to hypoxia for 48 h and treated with colchicine (0.25 mg/100 g body weight injected intraperitoneally approximately 15 h before the isolated-perfused rat lung experiments) (n = 10) (Saldias et al., 2002). As controls, the effects of β-lumicolchicine (0.25 mg/100 g) body weight injected intraperitoneally approximately 15 h before the isolated-perfused rat lung experiments) on denopamine stimulation (n = 10). β-lumicolchicine is an isomer of colchicine that does not bind tubulin and does not depolymerize microtubules (Saldias et al., 2002). However, it shares other properties of colchicine, such as inhibition of protein synthesis, and it is therefore an appropriate control to demonstrate that the observed effects of colchicine are caused by microtubular disruption. All alveolar solutions contained 5% albumin and Evans blue dye (0.15 mg/ml).

Statistical analysis

The data are presented as means ± SD. Kolmogorov- Smirnov test was used for normality of variables. Bartlett's method and F test were used for homogeneity of variances. All data had confirmed normality of the population and equal variances among different experiment groups. Statistical significance was evaluated by t test between two groups and analysis of variance (ANOVA) post hoc Student-Newman-Keuls method among multiple groups (Prism 4, GraphPad Software, Inc., San Diego, Calif., USA). The level of statistical significance was set at $p < 0.05$.

RESULTS

Morphometric studies

In the control group, the alveolar space was basically dry, the lung interstitium had no edema and the peri-alveolar blood vessels showed no dilations or hyperemia (Figure 1a); the lung epithelial cells (Figure 1b) and capillary endothelial cells (Figure 1c) were intact. At hypoxic for 24 h, the peri-alveolar blood vessels were slightly dilated and had blood stasis (Figure 2a); the Type II epithelial cells had no marked structural changes but the capillary endothelial cell membrane had become thickened, and the cytoplasm was edematous (Figure 2b). At hypoxic for 48 h, the peri-alveolar blood vessels were markedly dilated and had blood stasis. A small amount of edema fluid formed (Figure 3a); other than microvillus being inverted and lying-down and in irregular arrays, the alveolar Type II epithelial cells had no marked structural changes (Figure 3b). At hypoxic for 72 h, the lung interstice was clearly broadened and petechic with hemorrhagic and edematous changes (Figure 4a); the alveolar Type II epithelial cells showed structural damage of lamellar bodies, the mitochondria showed vacuole-like changes, the cytoplasm was edematous, the nucleus became condensed and the microvillus inversion which was lying-down was detached (Figure 4b).

The number of endothelial cells and alveolar type II epithelial cells are given in Figure 5. The total number of alveolar type II epithelial cells remained normal for 72 h. At hypoxic for 72 h, the number of endothelial cells decreased significantly (Figure 5).

The TLW significantly increased to 3.58 ± 0.19, 5.84 ± 0.17 and 6.89 ± 0.23 g/g in rats exposed to hypoxia for

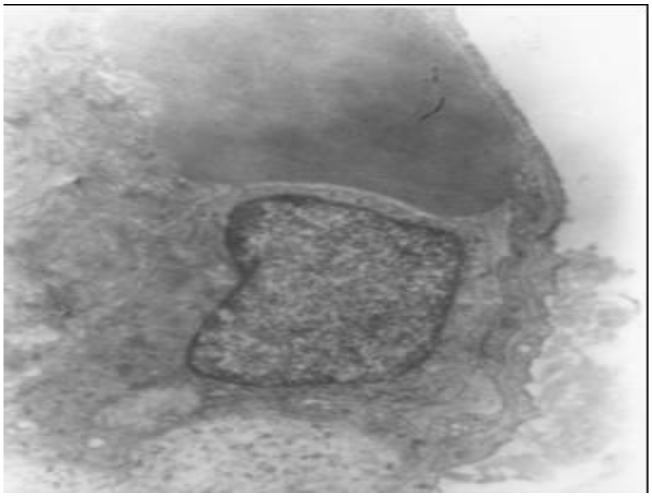

Figure 1c. The capillary endothelial cell of control rat (×10000).

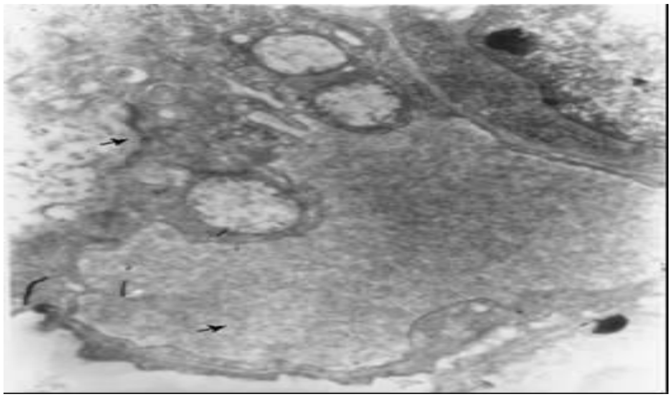

Figure 2b. The capillary endothelial cell of hypoxia rat for 24 h (×1000).The cell membrane became thickened, the cytoplasm was edematous.

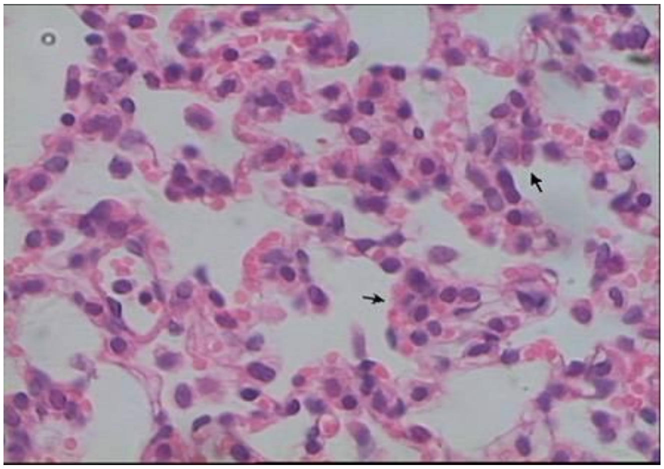

Figure 2a. Hypoxia rat for 24 h (HE ×400) the peri-alveolar blood vessels in rats were slightly dilated and had blood stasis.

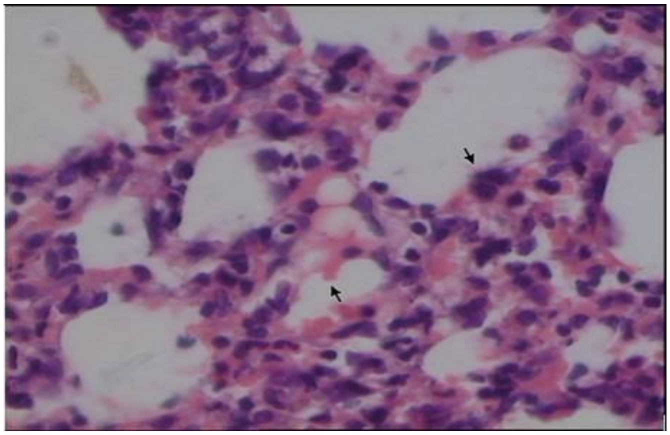

Figure 3a. Hypoxia rat for 48h (HE ×400) the peri-alveolar blood vessels in rats were markedly dilated and had blood stasis. A small amount of edema fluid formed.

24, 48 and 72 h, respectively (Figure 6). However, AFC decreased significantly in rats exposed to hypoxia for 48h (Figure 7).

Effects of β-adrenergic agonists on AFC

10^{-5} mol/L Denopamine significantly increased AFC in rats exposed to hypoxia for 24, 48 and 72 h, and in rats not exposed to hypoxia (Figure 7). There was no significant difference between the basal AFC in control rats without denopamine stimulation and in rats exposed to hypoxia for 48 h stimulated by denopamine (Figure 7). There was a significant difference between the basal AFC in control rats and AFC in rats exposed to hypoxia for 72 h in the presence of denopamine (Figure 7). AFC in rats exposed to hypoxia for 48 h stimulated by terbutaline was 19.0 ±

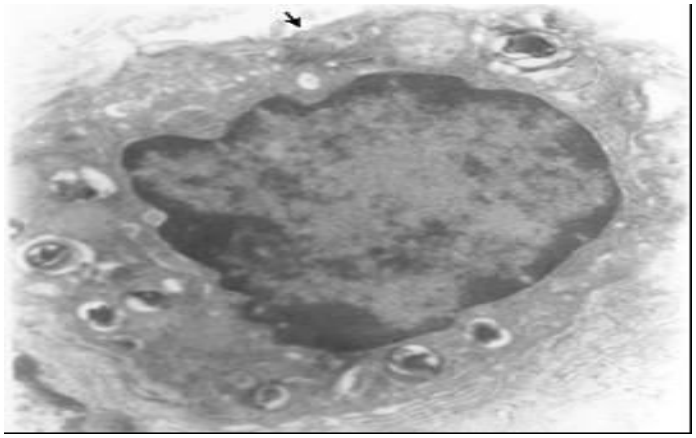

Figure 3b. The epithelial cellular structure of hypoxia rat for 48 h (×10000). The cell had no marked structural change except microvillus inversion lying-down.

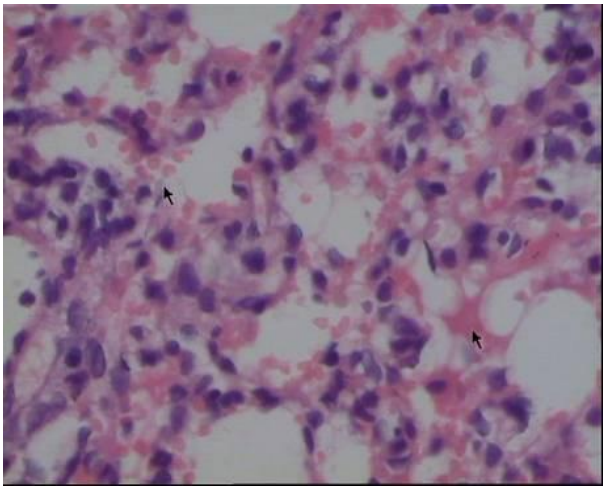

Figure 4a. Hypoxia rat for 72 h (HE ×400) the lung interstice were markedly broadened and petechic with appearance of lung hemorrhage and edema.

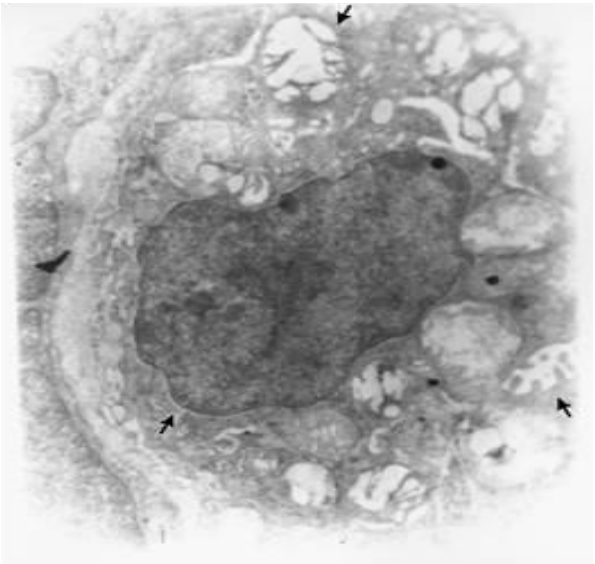

Figure 4b. The epithelial cellular structure of hypoxia rat for 72 h (×8000). Damage of osmiophilic multilamellar body, the mito-chondria showed vacuole-like changes, the nucleus became condensed .

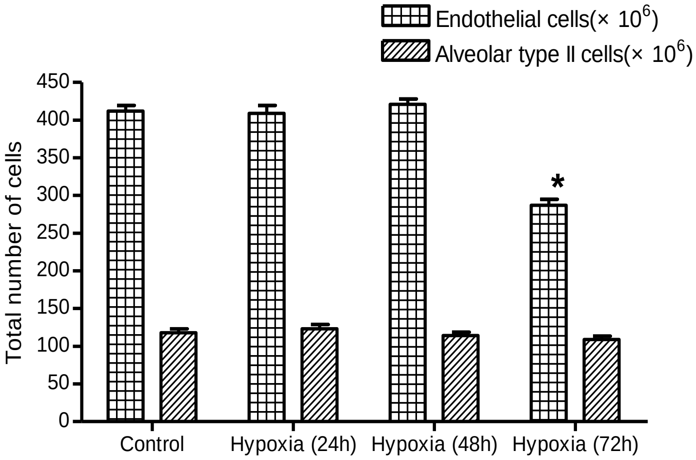

Figure 5. Total number of cells in the alveolar region of rat lungs. Values are means ± SD. n=10 in every group *$p < 0.05$ compared with control endothelial cells (ANOVA: F = 191.9, $p < 0.05$; Student-Newman- Keuls post hoc test). The total number of alveolar type II epithelial cells remained normal for 72 h.

2.03%, and it had no significant difference compared to the effect of denopamine on rats exposed to hypoxia (Figure 8).

Effects of β-adrenergic antagonist on AFC modulation by denopamine

Atenolol (β_1-adrenergic antagonist) inhibited the increase in AFC by denopamine. However, ICI-118551 (β_2-adrenergic antagonist) did not inhibit the increase in AFC by denopamine (Figure 8).

Effects of denopamine on amiloride-sensitive and amiloride- insensitive AFC

Amiloride and ouabain significantly inhibited AFC stimulated by denopamine in rats exposed to hypoxia for 48 h. Amiloride inhibited AFC by 35% and ouabain inhi-bited AFC by 53% (Figure 8).

Effects of colchicine on AFC modulation by denopamine

Colchicine inhibited the stimulatory effect of denopamine on AFC in rats exposed to hypoxia for 48 h, whereas, the isomer β-lumicolchicine did not modify the effect of denopamine on AFC (Figure 8).

DISCUSSION

It is well accepted that β_2-adrenergic agonists can stimulate AFC. But it is unknown whether β_1-adrenergic agonist can stimulate AFC in lung injury. The results of these studies in rats with hypoxic lung injury demonstrated a marked upregulation in alveolar epithelial fluid transport capacity by β_1-adrenergic agonist.

At 24h exposure to 10% oxygen, TLW increased, but AFC did not change significantly. It suggests that AFC is not synchronous with TLW. Meanwhile, the capillary endothelial cells showed structural changes while the alveolar epithelial cells had no marked change. The total

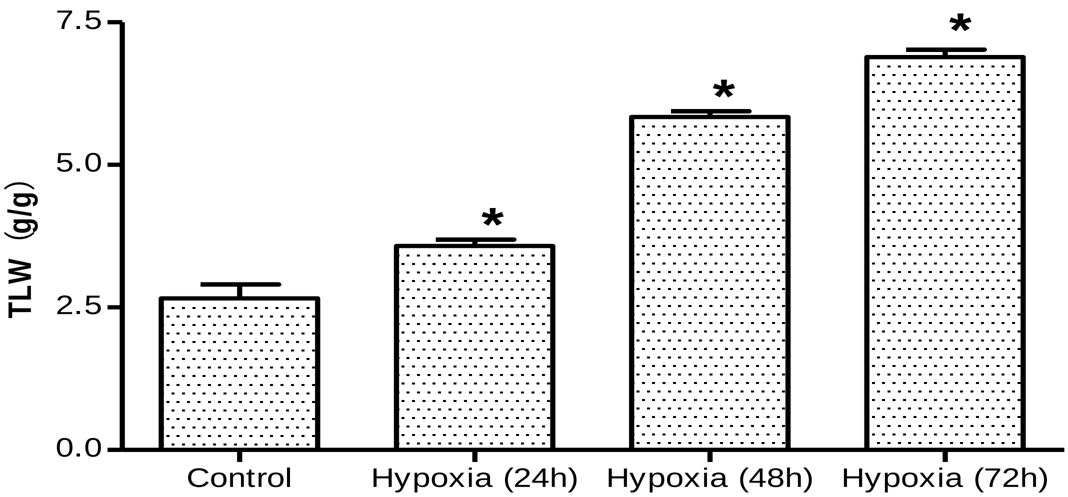

Figure 6. Effects of hypoxia on lung water volume in rats exposed to hypoxia. Values are means ± SD. n=10 in every group * $p < 0.05$ compared with TLW in control rats (ANOVA: F=860.1, P<0.05; Student-Newman- Keuls post hoc test).

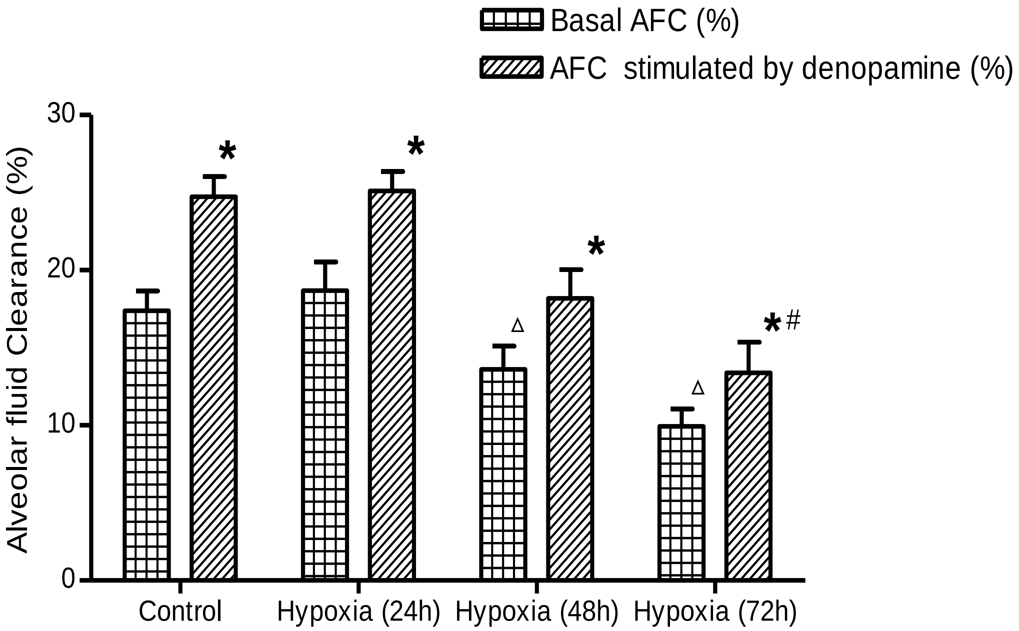

Figure 7. Effects of hypoxia on alveolar fluid clearance. Values are means ± SD. n = 10 in every group. Δ p< 0.05 compared with AFC in control rats (ANOVA: F = 24.34, p<0.05; Student-Newman-Keuls post hoc test), * p < 0.05 compared with basal AFC (unpaired Student's *t*-test), # p< 0.05 compared with basal AFC in control rats (unpaired Student's t-test).

number of alveolar type II epithelial cells remained normal for 72 h. At hypoxic for 72 h, the number of endothelial cells decreased significantly. These suggest that, as compared with the endothelial cell, the alveolar epithelial cell has a stronger anti-injury capacity (Berthiaume et al., 2002). In our study, after rats were hypoxic for 48 h, AFC decreased markedly compared to the control group. But the alveolar epithelial cells still showed no structural change. This is consistent with the previous study by Marie et al., (2001). This can be explained by the necessity of normal structural morphology of alveolar epithelium for maintaining the normal alveolar clearance capacity. The decrease of AFC will not necessarily be accompanied by marked changes to the morphological structures of epithelial cells. When AFC is slightly decreased, the decrease of sodium channel and Na^+/K^+- ATase

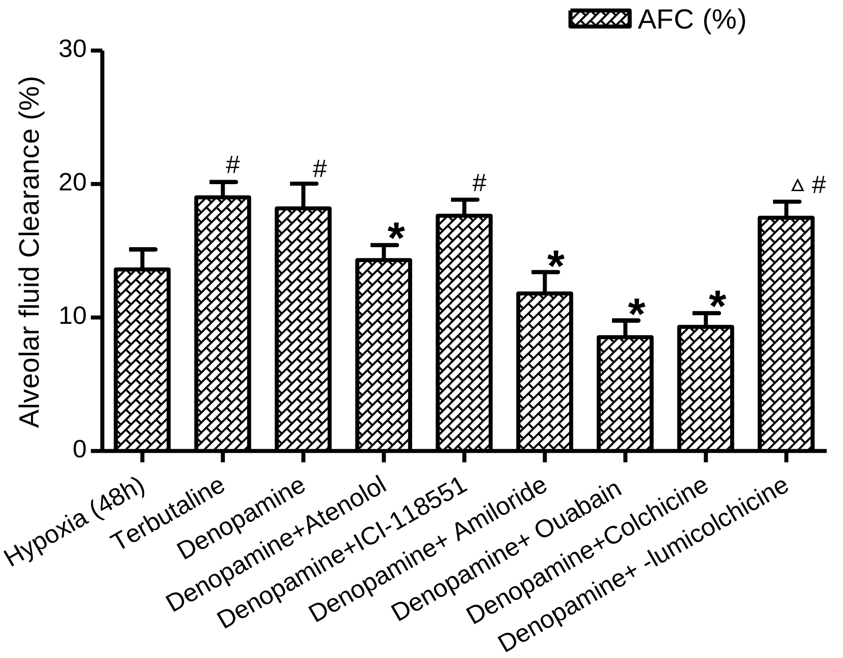

Figure 8. Effects of drugs on AFC modulation by denopamine. Values are means ± SD. n=10 in every group. *p < 0.05 compared with denopamine group (ANOVA), Δ $p < 0.05$ compared with Colchicine +denopamine group (unpaired Student's *t*-test: $t = 9.495$, $P<0.05$), # $p < 0.05$ compared with hypoxia (48 h) group (ANOVA: $F = 8.97$, $p < 0.05$).

activity appeared first before any marked morphological change (Dana et al., 2001).

The main regulating function of alveolar epithelial fluid transport is a catecholamine-dependent regulatory mechanism (Groshaus et al., 2004; Perkins et al., 2004). Both endogenous and exogenous catecholamine can up-regulate the alveolar epithelial fluid clearance. As demonstrated by the present experiment, β_1-adrenergic antagonist atenolol could significantly inhibit the increase in AFC stimulated by denopamine. β_2 Receptor antagonist ICI-118551 had no effect on AFC. It suggests that denopamine acts by stimulating the β_1 adrenergic receptor. Although the percentage of β_1 binding sites were lower than that of β_2 binding sites in the rat lung tissue, there was no difference between the magnitude of denopamine-stimulated AFC and that of terbutaline-stimulated AFC in hypoxic lung injury. Denopamine can remove AFC with hypoxic injury for 48 h back to normal level. However, in 72h hypoxic rats, despite under the action of denopamine, the level of AFC remained lower than that of control rats without denopamine. It suggested that denopamine has a certain limitation in regulating the post-injury alveolar epithelial fluid transport. It means that the exposure of 10% oxygen for 48 h did not cause serious damage to β adrenergic receptor and the injury of alveolar epithelial cell is reversible under a not serious situation.

Neither amiloride nor ouabain inhibited fluid clearance in hypoxic rats, but they did in the normoxic rats. This indicates that hypoxia may down-regulate both the Na^+ channel and Na^+/K^+-ATPase (Suzuki et al., 1996). As demonstrated by the present experiment, the Na^+ channel blocker amiloride and the Na^+/K^+-ATPase inhibitor ouabain inhibited the stimulatory effects of denopamine on AFC, suggesting that denopamine upregulated the Na^+/K^+-ATPase and Na^+ channel function in alveolar epithelium. Amiloride reduced 35% of the effect of denopamine on the AFC rate in hypoxic rats while ouabain removed 53% of the effect of denopamine in hypoxic rat. This suggests that denopamine acts mainly through stimulating the Na^+/K^+-ATPase, and terbutaline acts mainly through stimulating the amiloride-sensitive sodium channel (Modelska et al., 1999). The ratio of amiloride-sensitive sodium channel and Na^+/K^+-ATPase is different among different animals. The amiloride-sensitive sodium channel is 40-50% in human and rat and more than 80% in rabbit and mouse (Dada and Sznajder, 2003). The biological function of Na^+/K^+-ATPase in human is not at all negligible.

Saldias et al. (2002) reported that Na^+/K^+-ATPase exists in intracellular pools and in response to specific signals can be rapidly recruited via cell microtubular transport into the plasma membrane. Therefore we studied whether inhibition of cell microtubular transport of Na^+/K^+-ATPase from intracellular pools to the plasma membrane by colchicine could inhibit the stimulatory effects of denopamine on active Na^+ transport. The results suggest that denopamine stimulation of AFC is probably mediated by recruitment of ion-transporting proteins from inner pools to the plasma membrane of the alveolar epithelium. This need to be proved by further studies.

It has been shown that the rate of short-term AFC was not adversely affected by the absence of perfusion or ventilation to the lung (Matthay et al., 2005). AFC is maintained for 1 h in isolated rat lung, and for more than 4 h in *ex vivo* human lung (Saldias et al., 1998). Here we studied AFC under the *ex vivo* conditions. Because of lack of participation of pulmonary circulation, the *ex vivo* model has some advantages. Since there is no blood perfusion and a reduced inflow of a large quantity of protein fluid during the lung injury, this model is more appropriate for short-term studies of AFC (Fukuda et al., 2000).

Denopamine regulates the AFC capacity in hypoxic injury lung mainly through promoting the intracellular transport of Na^+/K^+-ATPase. This drug can accelerate the resolution of pulmonary edema and thus provide more options for treating pulmonary edema in clinical practice.

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