

Effects of Xanthine Derivatives on Phosphatidylcholine Secretion in Rat Type II Pneumocytes in the Presence of Activated Eosinophils

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ABSTRACT—We have previously reported that activated eosinophils enhanced the phosphatidylcholine (PC) secretion in type II pneumocytes. In this study, we have determined whether xanthine derivatives affect the PC secretion increased by activated eosinophils. Theophylline enhanced the increased PC secretion at 10^{-5} M. 8-Phenyltheophylline dose-dependently enhanced the PC secretion. The enhanced secretion by either theophylline at 10^{-5} M or 8-phenyltheophylline was suppressed by superoxide dismutase in combination with catalase. Pentoxifylline did not enhance the PC secretion increased by activated eosinophils, although it increased the PC secretion by itself. The PC secretion increased by theophylline at 10^{-3} M or pentoxifylline was not suppressed by superoxide dismutase in combination with catalase. The present results suggest that xanthine derivatives increased the PC secretion in the co-culture of type II pneumocytes and activated eosinophils possibly through the inhibition of phosphodiesterases or the antagonism of adenosine receptors of the eosinophils.

Keywords: Pneumocyte (type II), Phosphatidylcholine secretion, Eosinophil, Superoxide anion, Xanthine derivative

Xanthine derivatives have been widely used in the treatment of asthma and inhibit the late response to an inhaled allergen, suggesting that the drugs have effects on the inflammatory response (1, 2). However, it has been reported that theophylline had no effect on bronchial hyperresponsiveness in asthmatic subjects, which may be related to eosinophil recruitment and its activation (3). The bronchial hyperresponsiveness is suppressed by glucocorticoid, cyclooxygenase inhibitor and thromboxane inhibitor (4, 5), which suggests that eicosanoids such as prostaglandins, leukotriene and thromboxane A_2 play important roles to induce the pathogenesis. We have speculated that the reason for the ineffectiveness of theophylline may be that theophylline at clinical concentrations has a potential to increase the level of oxygen free radicals. The speculation is supported by the observation that theophylline increased opsonized zymosan-stimulated oxygen-free radical release from guinea pig and human eosinophilia (6). Eosinophils infiltrated into the lung release many cytotoxic proteins, leukotrienes, platelet-activating factor, and various oxygen-derived

toxic metabolites such as superoxide anions, H_2O_2 , and hydroxy radicals (7, 8). The release of cytotoxic proteins and other mediators from eosinophils causes desquamation and damage to respiratory epithelial cells (9).

The type II pneumocytes produce pulmonary surfactant to maintain normal lung functions (10). Pulmonary surfactant, which is composed of phospholipids and apoproteins, lowers the surface tension at the air-liquid interface in the lung and provides for alveolar stability. In addition to the vital role, pulmonary surfactant also plays an important role to facilitate and maintain airway mucociliary clearance (11–18). We have shown that activated eosinophils increase the secretion of phosphatidylcholine (PC), the predominant component of pulmonary surfactant, in a primary culture of type II pneumocytes (19). The increase of PC secretion was mediated by superoxide anions released from activated eosinophils. In addition, we have reported that xanthine derivatives also increased PC secretion possibly through cAMP-dependent and cAMP-independent pathways in type II pneumocytes (20). From pathophysiological and clinical points of view, it seems important to investigate how xanthine derivatives affect PC secretion in type II pneumocytes

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in the presence of activated eosinophils. Therefore, we have examined whether theophylline, 8-phenyltheophylline and pentoxifylline affect the PC secretion in the co-culture of type II pneumocytes and activated eosinophils. We used these drugs because their properties are advantageous for the present studies. Theophylline has multiple effects including an inhibition of phosphodiesterases (cAMP-phosphodiesterase (21) or cGMP-phosphodiesterase (22)), an antagonism of adenosine receptors (23), and an alteration of intracellular calcium mobilization (2). 8-Phenyltheophylline is an adenosine receptor antagonist that does not inhibit phosphodiesterase (24), and pentoxifylline is a phosphodiesterase inhibitor without an adenosine receptor antagonistic activity (25, 26).

MATERIALS AND METHODS

Primary rat type II pneumocyte culture

Type II pneumocytes were isolated from the lungs of adult specific-pathogen-free male Wistar rats (180–200 g) according to the method of Dobbs et al. (27). This method routinely yielded 10^7 cells per rat. The cells were suspended at 10^6 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 74 kBq/ml [methyl- ^3H]choline (specific activity, 3.0 TBq/mmol), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, plated on 24-well tissue culture plate (Falcon 3047), and then cultured at 37°C in 5% CO_2 -air for 18 hr. Non-adherent cells were removed from the wells by washing before the assay. For cellular identification, the sample was stained with a tannic acid and polychrome stain (28) and alkaline phosphatase stain (29). The purity of the type II pneumocytes monolayer was $95 \pm 3\%$ (mean \pm S.E., $n=8$). The viability of type II pneumocytes was $98 \pm 2\%$ (mean \pm S.E., $n=8$) as judged by the trypan blue exclusion test.

Eosinophil isolation and opsonized zymosan preparation

Eosinophils were isolated from peritoneal exudates of Hartley guinea pig by a modification of the method described by Pincus (30). Briefly, peritoneal eosinophil-rich exudates were produced by a weekly intraperitoneal injection of 7,500 units/ml of polymyxin B sulfate for at least 8 weeks. Fifty milliliters of Hank's balanced salt solution containing 20 units/ml sodium heparin was injected intraperitoneally the next day after the last injection of polymyxin B sulfate. The abdomen was massaged gently, and the fluid was collected. The fluid was fractionated by centrifugation through solutions of Nycodenz. The purified fraction of eosinophils was collected from the interface between 1.088 and 1.098 g/ml in the gradient. The eosinophils were 95% pure as determined in Litt's stained smears. To prepare opsonized zymosan, zymosan A in Hank's

balanced salt solution was boiled, washed, and then incubated with fresh rat serum for 30 min at 37°C . After centrifugation and washing 4 times, opsonized zymosan was suspended in Hank's balanced salt solution at a concentration of 10 mg/ml and stored at -70°C until the use.

Metabolic labeling of PC and treatment of cultures

Secretion of PC by cultured type II pneumocytes was determined as follows: The cells were rinsed with fresh serum- and antibiotics-free medium to remove [methyl- ^3H]choline and unattached cells. Eosinophils, opsonized zymosan and various agents (theophylline, 8-phenyltheophylline, pentoxifylline) were added 30 min after the rinse and then the incubation was performed for 90 min. At the end of the incubation period, the medium was aspirated off, the cells were lysed with 2 ml ice-cold 0.05% Triton X-100 solution and lipids were extracted from both the cells and the medium with chloroform and methanol (2 : 1, v/v). PC was separated from the other phospholipids by thin-layer chromatography (31), and its radioactivity was measured with a liquid scintillation counter after an addition of 5 ml Aquasol II to each sample. Secretion was expressed as the amount of [^3H]PC in the medium after the 90-min incubation, as a percentage of that in cells plus medium. [^3H]PC secretion for 90 min was $0.69 \pm 0.06\%$ (mean \pm S.E., $n=20$) in control culture.

Measurement of superoxide anions generation

The generation of superoxide anions was measured by the reduction of horse heart ferricytochrome c (32). Isolated eosinophils (10^5 cells/ml) were preincubated with a drug (theophylline, 8-phenyltheophylline or pentoxifylline) at 37°C for 15 min, and 10 μM cytochrome c and 100 μM opsonized zymosan were added. The eosinophils were incubated for 90 min. The medium was collected and centrifuged at $400 \times g$ for 5 min at 4°C , and the supernatant was removed. Light absorbance at 550 nm was determined in a spectrophotometer. The generation was expressed as a percentage of the absorbance against that of control.

Detection of cell membrane damage and chemicals

The presence or absence of cytoplasmic leakage due to cell membrane damage following the treatment was determined by measuring lactate dehydrogenase (LDH) activity in the culture medium with a commercial LDH assay kit (Nippon Shoji Co., Ltd., Osaka). The LDH activity released into the medium did not exceed 1% of the total cell content in all experiments (data not shown).

The rats and guinea pigs were purchased from Kyudo Farm (Fukuoka), tissue culture medium from Nissui Pharmaceutical Co., Ltd. (Tokyo) and fetal bovine serum from JRH Bioscience (Lenexa, KS, USA). [Methyl-

^3H]choline and Aquasol II were obtained from NEN Research Products (Boston, MA, USA). Other reagents and biochemicals were from Sigma Chemicals Co. (St. Louis, MO, USA).

Statistics

PC secretion values and superoxide anions generation for each group were expressed as means \pm S.E. The difference among groups was assessed by Duncan's multiple range test (a non-parametric test). $P > 0.05$ was considered not significantly different.

RESULTS

Generation of superoxide anions from activated eosinophils

The generation of superoxide anions from eosinophils was increased by opsonized zymosan, and the increase was dependent on the number of eosinophils (10^4 to 10^6 cells/ml). The sufficient number of eosinophils to increase the ^3H]PC secretion in rat type II pneumocytes was 10^5 cells/ml, and the increase was similar to the result in our previous report (19). Addition of superoxide dismutase in combination with catalase completely suppressed the increased generation of superoxide anions (Fig. 1). These results indicated that isolated eosinophils were intact in their functions.

Effects of xanthine derivatives on superoxide anions generation from eosinophils stimulated by opsonized zymosan

Theophylline showed a biphasic action on superoxide

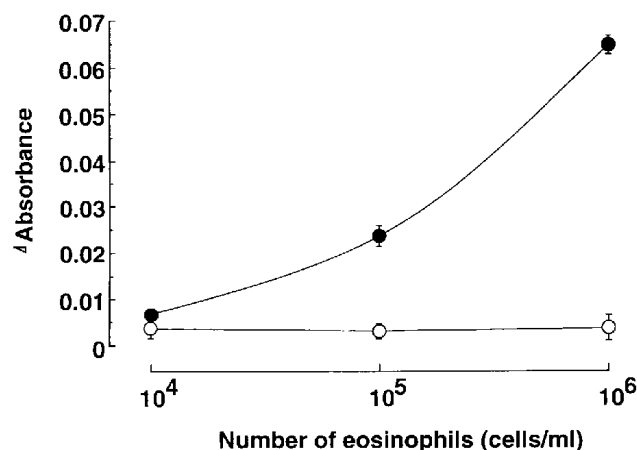


Fig. 1. Generation of superoxide anions from opsonized zymosan activated eosinophils alone (●) or with superoxide dismutase (2 units/ml) and catalase (1000 units/ml) (○). The values of superoxide anion generation were expressed as absorbance at 550 nm of reduced cytochrome c formed from ferricytochrome c. Data are each the mean \pm S.E. of 8 experiments.

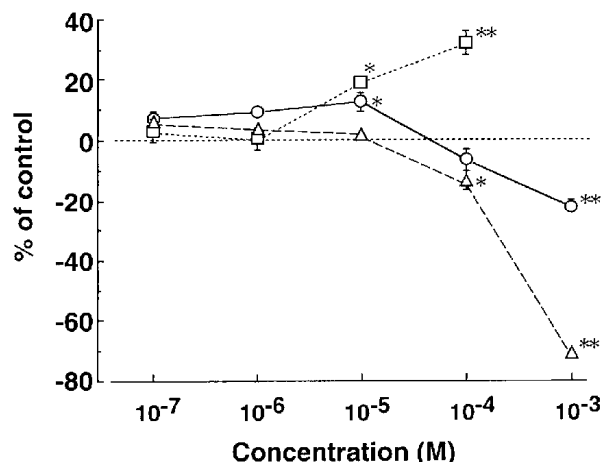


Fig. 2. Effects of xanthine derivatives on superoxide anions generation in activated eosinophils. Eosinophils were preincubated with the indicated concentration of theophylline (○), 8-phenyltheophylline (□) and pentoxifylline (△) before an addition of opsonized zymosan. Data are each the mean \pm S.E. of 6 experiments. * and **: Significant at $P < 0.05$ and $P < 0.01$ vs control, respectively.

anions generation from eosinophils stimulated by opsonized zymosan (Fig. 2). Theophylline increased the superoxide anions generation at concentrations of 10^{-6} and 10^{-5} M, whereas it inhibited the generation at concentrations of 10^{-4} and 10^{-3} M. In contrast, 8-phenyltheophylline increased the superoxide anions generation, whereas pentoxifylline inhibited it dose-dependently. A significant potentiation of the generation by 8-phenyltheophylline occurred at concentrations of 10^{-5} to 10^{-4} M.

Effects of xanthine derivatives on the PC secretion increased by activated eosinophils in primary culture of rat type II pneumocytes

Theophylline increased the PC secretion in rat type II pneumocytes dose-dependently with the doses higher than 10^{-4} M and the result was similar to that in our previous report (20). Theophylline at 10^{-5} M or lower concentrations did not affect the secretion. However, theophylline at 10^{-5} M enhanced the PC secretion increased by activated eosinophils (Fig. 3). The enhanced secretion was suppressed by superoxide dismutase in combination with catalase. However, superoxide dismutase and catalase had no effect on the enhanced secretion by theophylline at 10^{-3} M in the presence of activated eosinophils.

Whereas 8-phenyltheophylline had no effect on the PC secretion in type II pneumocytes, 8-phenyltheophylline dose-dependently enhanced the PC secretion increased by activated eosinophils (Fig. 4). The enhanced secretion by 8-phenyltheophylline was suppressed by superoxide dismutase in combination with catalase, and it was completely suppressed by further addition of adenosine

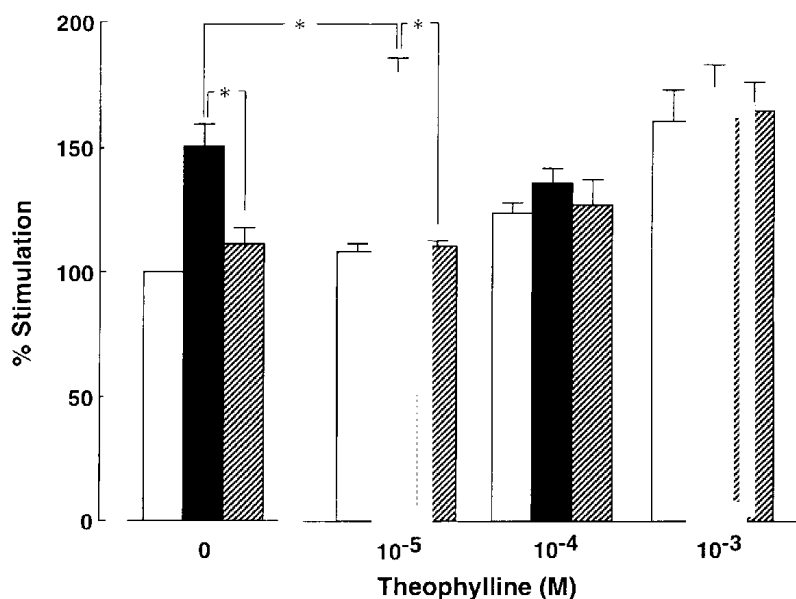


Fig. 3. Effect of theophylline on the PC secretion increased by activated eosinophils in type II pneumocytes. Type II pneumocytes were incubated with the indicated concentrations of theophylline alone (open bars), theophylline with activated eosinophils (solid bars), or theophylline with activated eosinophils in the presence of superoxide dismutase (2 units/ml) and catalase (1000 units/ml) (hatched bars). Data are each the mean \pm S.E. of 5 experiments. *: Significant at $P < 0.01$.

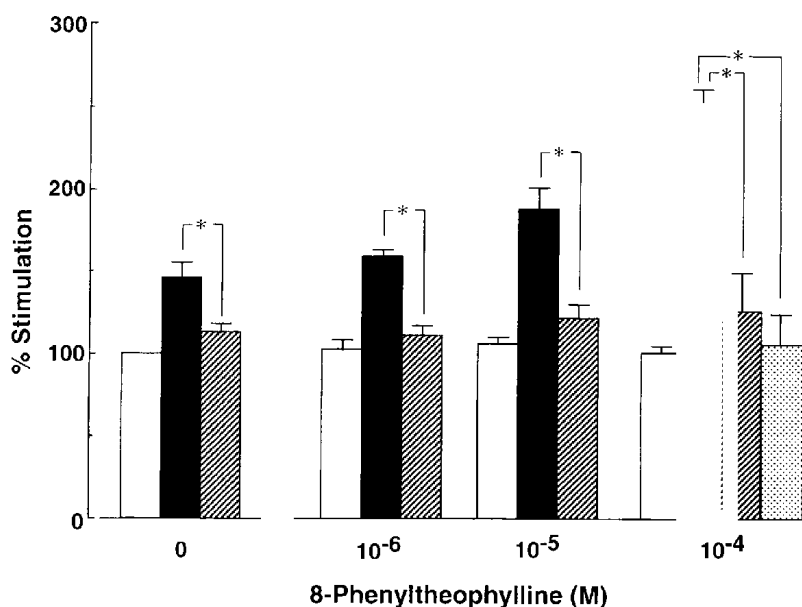


Fig. 4. Effect of 8-phenyltheophylline on the PC secretion increased by activated eosinophils in type II pneumocytes. Type II pneumocytes were incubated with the indicated concentrations of 8-phenyltheophylline alone (open bars), 8-phenyltheophylline with activated eosinophils (solid bars), or 8-phenyltheophylline with activated eosinophils in the presence of superoxide dismutase (2 units/ml) and catalase (1000 units/ml) (hatched bars), and further addition of adenosine deaminase (1 units/ml) (stippled bar). Data are each the mean \pm S.E. of 5 experiments. *: Significant at $P < 0.01$.

deaminase.

Pentoxifylline increased the PC secretion in type II pneumocytes. However, pentoxifylline did not enhance

the PC secretion increased by activated eosinophils (Fig. 5). The PC secretion increased by pentoxifylline was not suppressed by superoxide dismutase in combination with

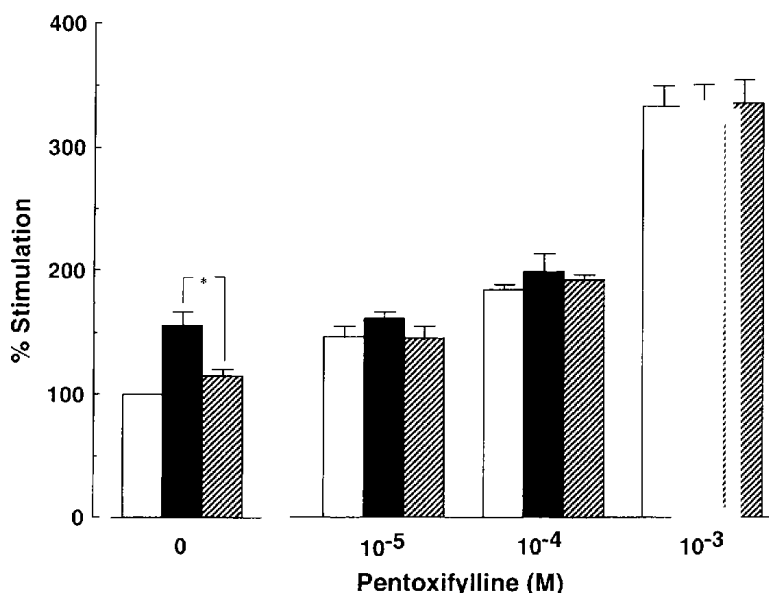


Fig. 5. Effect of pentoxifylline on the PC secretion increased by activated eosinophils in type II pneumocytes. Type II pneumocytes were incubated with the indicated concentrations of pentoxifylline alone (open bars), pentoxifylline with activated eosinophils (solid bars), or pentoxifylline with activated eosinophils in the presence of superoxide dismutase (2 units/ml) and catalase (1000 units/ml) (hatched bars). Data are each the mean \pm S.E. of 5 experiments. *: Significant at $P < 0.01$.

catalase.

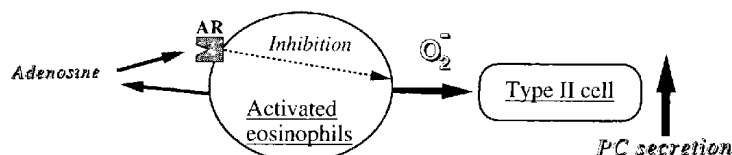
DISCUSSION

Eosinophils infiltrated into the lung are activated in subjects with asthma. The infiltration and activation are induced by chemical mediators, including PAF and histamine released from mast cells stimulated by allergens inhalation. Therefore, it seems important to obtain information on the influence of eosinophils on the secretion of pulmonary surfactant. We previously reported that the activated eosinophils increased the PC secretion in type II pneumocytes, and the increase was suppressed by superoxide dismutase and catalase (19). The results suggested that activated eosinophils stimulated the secretion of pulmonary surfactant, at least in part, through superoxide anions. The pulmonary surfactant secretion increased by activated eosinophils may be a kind of defense mechanism to attenuate oxidative cellular damages caused by oxidant radicals released from activated eosinophils, because pulmonary surfactant is known to be an inert material against oxidative reaction (33). The increased pulmonary surfactant also may play important roles to facilitate and maintain airway mucociliary clearance (11–18). Hence, the study of the effect of drugs on the PC secretion in the presence of eosinophils is important because eosinophils are regarded as potent proinflammatory cells with considerable tissue-injuring capacity, possibly casually involved in the development of lung dis-

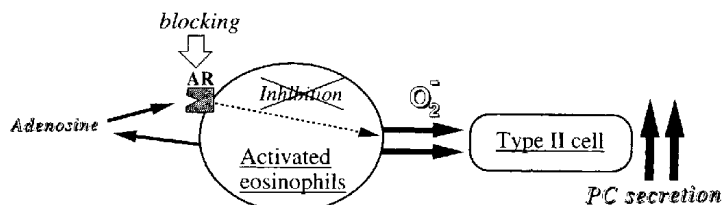
eases such as asthma (34–36).

In the present study, we found that xanthine derivatives affected the PC secretion increased by activated eosinophils in rat type II pneumocytes. Theophylline showed a biphasic action on superoxide anions generation from activated eosinophils. There was a biphasic action of theophylline in the effect on the PC secretion in the co-culture of type II pneumocytes and activated eosinophils. We have proposed a hypothesis whereby theophylline enhanced the PC secretion increased by activated eosinophils possibly through two distinct mechanisms (Fig. 6); one is the antagonism of adenosine receptors on the eosinophils which increase the release of superoxide anions, because exogenously applied adenosine suppressed the superoxide anions generation from activated eosinophils (6). Thereby, the superoxide anions induce PC secretion in the type II pneumocytes. The other mechanism is an inhibition of phosphodiesterase in the eosinophils as well as the pneumocytes. The inhibition of phosphodiesterase in eosinophils decreases the release of superoxide anions through the increase of intracellular cAMP. Thus, the PC secretion increased by superoxide anions is suppressed by theophylline. At the same time, however, the increase of intracellular cAMP in the type II pneumocytes induces the PC secretion. The ineffectiveness of theophylline at 10^{-4} M may be due to a balanced situation between the extent of the suppression of the PC secretion increased by activated eosinophils and the extent of the increase of PC secretion through intracellular

A) No xanthines



B) Theophylline (low conc.) & 8-Phenyltheophylline



C) Theophylline (high conc.) & Pentoxifylline

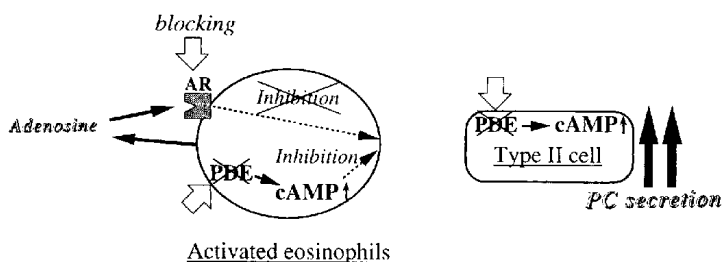


Fig. 6. Possible mechanisms of action of xanthine derivatives on the PC secretion in type II pneumocytes in the presence of activated eosinophils. A: Activated eosinophils generate superoxide anions (O_2^-) to induce PC secretion in type II pneumocytes. Endogenous adenosine may be released from eosinophils by itself to suppress superoxide anions generation through its own adenosine receptor (AR) in eosinophils. B: Theophylline at 10^{-5} M and 8-phenyltheophylline antagonize adenosine receptors on the eosinophils, which increase the release of superoxide anions, because exogenously applied adenosine suppressed the superoxide anion generation from activated eosinophils (6). Thereby, the superoxide anions induce PC secretion in the pneumocytes. An open arrow indicates the site of action of xanthine derivatives. C: Theophylline at 10^{-3} M and pentoxifylline inhibit phosphodiesterases (PDE) in the eosinophils as well as the pneumocytes. The inhibition of phosphodiesterases in eosinophils decrease the release of superoxide anions through the increase of intracellular cAMP. Thus, the PC secretion increased by superoxide anions is suppressed by theophylline. At the same time, however, the increase of intracellular cAMP in the type II pneumocytes induces the PC secretion. Open arrows indicate the sites of action of xanthine derivatives.

cAMP in type II pneumocytes. The balance seems important to determine the effect of theophylline on PC secretion by type II pneumocytes in the presence of activated eosinophils. The hypothesis we proposed here is supported by our findings on 8-phenyltheophylline and pentoxifylline.

8-Phenyltheophylline, which is an adenosine receptor antagonist and lacks a phosphodiesterase inhibitory action (24), had no direct effect on the PC secretion in rat type II pneumocytes, but it dose-dependently increased PC secretion in the presence of activated eosinophils. The increase of the PC secretion was reduced by superoxide

dismutase in combination with catalase, and it was completely suppressed by the further addition of adenosine deaminase. These results indicated that 8-phenyltheophylline increased PC secretion through activated eosinophils. Therefore, the stimulatory effect of theophylline on superoxide anion generation at the low concentration may participate in the increase of PC secretion in rat type II pneumocytes in the presence of activated eosinophils, because theophylline has an antagonistic effect on adenosine receptors (37, 38). However, the source of endogenous adenosine has to be considered. It seems that endogenous adenosine is released from eosinophils by

themselves to control superoxide anions generation through its own adenosine receptors in eosinophils, although it remains unclear to date.

It is known that pentoxifylline increases intracellular cAMP concentrations and the PC secretion in the type II pneumocytes (20) and that pentoxifylline decreases oxygen radicals release in polymorphonuclear leukocytes (39–41). The PC secretion increased by theophylline at the high concentration (10^{-3} M) as well as pentoxifylline was not suppressed by superoxide dismutase and catalase. These results suggested that the theophylline at the high concentration obviously increased the PC secretion in rat type II pneumocytes through the inhibition of phosphodiesterase of the pneumocytes, although it suppressed the PC secretion increased by superoxide anions from activated eosinophils. The effect of theophylline at 10^{-3} M used here, however, may be out of consideration in a clinical situation, since the concentration seems to be higher than the plasma concentration of theophylline in clinical use.

In conclusion, we found that xanthine derivatives increased the PC secretion through two distinct mechanisms in the co-culture of type II pneumocytes and activated eosinophils.

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