Effects of eosinophil granule major basic protein on phosphatidylcholine secretion in rat type II pneumocytes

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Okumura, Manabu, Hirofumi Kai, Shinya Shinozawa, Yoichiro Isohama, and Takeshi Miyata. Effects of eosinophil granule major basic protein on phosphatidylcholine secretion in rat type II pneumocytes. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L763–L768, 1999.—Eosinophils are involved in inflammatory diseases such as asthma. We previously reported that activated eosinophils increased the phosphatidylcholine (PC) secretion in primary cultures of rat type II pneumocytes. Increased PC secretion was confirmed to be partly mediated by superoxide anions released from activated eosinophils. However, the influence of eosinophil granule proteins on PC secretion is unknown at present. In this study, we determined whether eosinophil major basic protein (MBP) influences PC secretion. MBP dose dependently increased the PC secretion in rat type II pneumocytes without producing any cell damage. The MBP-induced increase in PC secretion was significantly reduced by preadministration of either H-7, a protein kinase inhibitor, or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM, a chelator of intracellular Ca2+, but not by H-89, a protein kinase inhibitor. Our results suggest that the MBP-induced increase in PC secretion may provide mechanical stability and protect against lung atelectasis.

Eosinophils play an important pathological role in allergic diseases such as asthma, and examination of bronchoalveolar lavage fluid of asthmatic patients usually demonstrates a significant increase in eosinophil count (19, 29). Eosinophils release several particles including leukotrienes, platelet-activating factor; various oxygen-derived toxic metabolites such as superoxide anions, H2O2, and hydroxyl radicals (28); and toxic cationic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (9, 11). The cytotoxic proteins and other mediators cause hyperreactivity of respiratory smooth muscles (7) as well as desquamation of and damage to respiratory epithelial cells including type II pneumocytes.

Type II pneumocytes produce lung surfactant to reduce the surface tension of the alveolar air-liquid interface, thereby providing mechanical stability and preventing alveolar atelectasis (2). Hence examination of the influence of cytotoxic proteins and other mediators released from eosinophils on the secretion of lung surfactant is important for our understanding of normal lung physiology as well as of certain pathological pulmonary conditions. Previous studies (2, 15, 21) have shown an increased secretion of phosphatidylcholine (PC), the predominant component of pulmonary surfactant, by a variety of physiological and pharmacological agents. Furthermore, a recent study by Okumura et al. (22) showed that activated eosinophils increase PC secretion in primary cultures of rat type II pneumocytes. Such an increase was not suppressed by ONO-1078, a selective antagonist of peptide leukotrienes, or TCV-309, an antagonist of platelet-activating factor, but was suppressed by a combination of superoxide dismutase and catalase. However, our results also showed that the combined use of both enzymes did not completely inhibit the secretion of PC. These results suggested that increased PC secretion was partly mediated by superoxide anions released from activated eosinophils and might represent one facet of the defense mechanisms aimed at attenuating cellular damage induced by superoxide anions.

Superoxide anions released from eosinophils participate in the early asthmatic reaction, whereas the eosinophil granule proteins participate in the late asthmatic reactions (LAR). Eosinophil granule proteins increase the secretion of histamine by basophils and mast cells (30) and the generation of superoxide anion by lung macrophages (14). However, to our knowledge, the effect of eosinophil granule proteins on the secretion of lung surfactant has not been previously reported.

Based on the above findings and the results of the previous study by Okumura et al. (22) demonstrating failure of the combined use of superoxide dismutase and catalase to completely inhibit the activated eosinophil-induced increase in PC secretion, we hypothesized in the present study that eosinophil granule proteins might increase the secretion of lung surfactant in patients with asthma during LAR. To test this hypothesis, we examined the effects of MBP, a primary constituent of eosinophil granule proteins, on PC secretion in primary cultures of rat type II pneumocytes.

MATERIALS AND METHODS

Animals and chemicals. Rats and guinea pigs were purchased from Kyudo Farm (Fukuoka, Japan), tissue culture medium was from Nissui Pharmaceutical (Tokyo, Japan), and fetal bovine serum was from JRH Bioscience (Lenexa, KS). [Methyl-3H]Choline and Aquasol II were obtained from NEN Research Products (Boston, MA). Sephadex G-50 was ob-
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Primary cultures of rat type II pneumocytes. Type II pneumocytes were isolated from the lungs of adult specific pathogen-free male Wistar rats (body weight 180-200 g) according to the method of Dobbs et al. (4). This method yields ~10^7 cells/rat. Cells were suspended at 10^6 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 74 kBq/ml of [methyl-3H]choline (specific activity 3.0 TBq/mmol), 100 μM of penicillin, and 100 μg/ml of streptomycin; plated on 24-well tissue culture plates (Falcon); and then cultured at 37°C in 5% CO₂-air for 18 h. Nonadherent cells were removed from the wells by washing before the assay. For cellular identification, the sample was stained with tannic acid-polychrome stain (17) and alkaline phosphatase assay. For cellular identification, the sample was stained with Litt's stained smears. MBP was purified from isolated guinea pig eosinophils according to the procedure described by Gleich et al. (24). Briefly, suspensions of eosinophils in 0.34 M sucrose contained only MBP. Each fraction was ultrafiltered, concentrated, and resuspended at a granule concentration corresponding to 1 x 10⁶ eosinophils/ml culture medium. G1 and G3 fractions did not increase PC secretion; in contrast, G2 and G4 fractions significantly increased PC secretion by 21 and 54%, respectively (Fig. 1, bottom).

Effects of MBP on PC secretion. MBP caused an ~1.5-fold increase in PC secretion in primary cultures of rat type II pneumocytes. Each fraction was ultrafiltered, concentrated, and resuspended at a granule concentration corresponding to 1 x 10⁷ eosinophils/ml culture medium. G1 and G3 fractions did not increase PC secretion; in contrast, G2 and G4 fractions significantly increased PC secretion by 21 and 54%, respectively (Fig. 1, bottom).

Effects of several inhibitors on MBP-induced increase in PC secretion. To determine the mechanism involved
in MBP-induced increase in PC secretion, we examined the effects of several inhibitors of intracellular pathways. In these experiments, the inhibitor was added 10 min before the application of 8 \times 10^{-9} \text{ M} \ MBP. H-7 (1 \times 10^{-5} \text{ M}), a protein kinase C inhibitor, and BAPTA-AM (5 \times 10^{-6} \text{ M}), a chelator of intracellular Ca^{2+}, significantly suppressed the MBP-induced increase in PC secretion. However, no synergistic effect was noted when the two inhibitors were added simultaneously. H-89 (6 \times 10^{-6} \text{ M}), a protein kinase A inhibitor, and

**DISCUSSION**

The major finding of the present study was that MBP purified from guinea pig eosinophil granules increased PC secretion in primary cultures of rat type II pneumocytes. Isolated pneumocytes were incubated with indicated concentrations of MBP for 90 min. Terbutaline (1 \times 10^{-6} \text{ M}; positive control). Values are means ± SE from 6 experiments. [\textbf{3H}]PC secretion after 90 min was 0.51 ± 0.04% in control cultures (not incubated with MBP; n = 5).

HA-1004 (1 \times 10^{-5} \text{ M}), a control to H-7, did not influence the MBP-induced increase in PC secretion.
eosinophils during the early stages of asthma and that this increase in pulmonary surfactant secretion by eosinophils in primary cultures of rat type II pneumocytes, which was partly mediated by superoxide anions, was probably involved in the increased PC secretion because such secretion was not completely inhibited by pretreatment with superoxide dismutase combined with catalase. To our knowledge, the possible involvement of eosinophil granule proteins on PC secretion has not been previously investigated.

Previous studies (13, 20) have shown that MBP causes desquamation of and damage to respiratory epithelial cells. Furthermore, MBP has been reported to stimulate histamine release from human basophils and rat mast cells (30). Histamine increases the surfactant secretion by a receptor-mediated process (3). Histamine release is an important pathophysiological reaction in asthma, and the increased surfactant secretion induced by histamine may be one of the protective reactions during the early asthmatic reaction. However, our results showed that the concentration of MBP necessary to increase PC secretion was far less than that of MBP to stimulate histamine release. Our results suggest that secretion of lung surfactant may be increased in asthmatic patients by granule proteins released from eosinophils during the early stages of the LAR. Such an increase in pulmonary surfactant secretion may serve as a protective mechanism against cellular damage caused by cytotoxic granule proteins.

MBP is the primary constituent of eosinophil granules (10, 12) and accounts for >50% of the granule proteins in the guinea pig (11, 16). Furthermore, previous studies have shown high serum concentrations of MBP in patients with eosinophilia (1) and in the sputum and bronchoalveolar lavage fluid of asthmatic patients (5, 29). Based on these early findings, we examined, in the present study, the specific effects of MBP. In addition to MBP, eosinophil granules contain three other major cationic proteins, EPO, EDN, and ECP, that have been purified from peritoneal exudates in the guinea pig. In this study, these granule proteins were separated from each other by Sephadex G-50 as described in a previous study (30). The first protein peak contained EPO, and the second peak contained EDN and ECP. The pooled fraction containing EPO increased PC secretion significantly, but the concentration of EPO was lower than that of MBP. In contrast, the pooled fraction containing EDN and ECP did not increase PC secretion. These results suggested that in addition to MBP, EPO may also participate in increasing PC secretion induced by eosinophils in primary cultures of rat type II pneumocytes. However, taking into consideration the relatively low amount of EPO, our results show that the majority of secreted PC was mediated to a large extent by MBP.

PC secretion from type II pneumocytes is regulated via various intracellular pathways (2, 15, 21, 23). The principal secretion pathway is the activation of a Ca2+ dependent protein kinase, protein kinase C, and high concentrations of intracellular Ca2+. In basophils, MBP stimulates histamine release, which is inhibited by pertussis toxin (27). Also, in mast cells, histamine release is induced by protein kinase C activation by diacylglycerol through a pertussis toxin-sensitive G protein. In type II pneumocytes, H-7, but not HA-1004, significantly but partially inhibited the MBP-increased PC secretion. H-7 is a relatively selective protein kinase C inhibitor, but it also exerts a slight inhibitory
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effect on protein kinase A and protein kinase G as well as on Ca\(^{2+}\)/calmodulin-dependent kinase. In contrast, the HA-1004 dose not have any inhibitory effect on protein kinase C, although it is inhibitory of other kinases, with a potency equivalent to that of H-7. Therefore, MBP may stimulate PC secretion as well as histamine release in basophils and mast cells through protein kinase C activation. In support of this, the kinetics of MBP-stimulated PC secretion (Fig. 3) is similar to that of 1-oleoyl-2-acetyl-sn-glycerol-stimulated PC secretion previously reported (26). BAPTA-AM also significantly inhibited PC secretion increased by MBP, suggesting that intracellular Ca\(^{2+}\) plays an important role in MBP stimulation. The finding that BAPTA-AM in combination with H-7 did not synergistically or additively inhibit MBP-induced PC secretion (Fig. 4) suggests that intracellular Ca\(^{2+}\) and protein kinase C may act on the same signaling pathway of MBP stimulation of PC secretion. Furthermore, incomplete inhibition of PC secretion by BAPTA-AM and H-7 suggests that other pathways may be involved in MBP-induced PC secretion, although further studies with higher concentrations of H-7 and other protein kinase C inhibitors are needed.

In conclusion, our results showed that MBP, the primary constituent of eosinophil granule proteins, increased PC secretion in primary cultures of rat type II pneumocytes. Such an effect of eosinophils on PC, the predominant component of pulmonary surfactant, may provide mechanical stability and prevent lung atelectasis in asthmatic patients during early LAR. Our results also showed that the MBP-induced increase in PC secretion was mediated, at least in part, by protein kinase C and intracellular Ca\(^{2+}\). Further studies are necessary to identify other pathways that regulate PC secretion.

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