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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MBP INCREASES PC SECRETION IN TYPE II PNEUMOCYTES

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 ∼10³ cells/rat. Cells were suspended at 10⁶ 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 U/ml 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 (6). The purity of the type II pneumocytes was 95 ± 7% (SE; n = 8 cultures), and their viability was 97 ± 8% (n = 8 cultures) as confirmed by the trypan blue exclusion test.

Preparation of guinea pig MBP. Eosinophils were isolated from peritoneal exudates of Hartley guinea pigs by a modification of the method originally described by Pincus (24). Briefly, peritoneal eosinophil-rich exudates were produced by weekly intraperitoneal injections of 7,500 U/ml of polymyxin B sulfate for at least 8 wk. Furthermore, 50 ml of Hanks’ balanced salt solution containing 20 U/ml of sodium heparin was injected intraperitoneally the day after the last injection of polymyxin B sulfate. The abdomen was massaged gently, and the peritoneal 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. Eosinophils were 96% pure as determined with Litt’s stained smears. MBP was purified from isolated guinea pig eosinophils according to the procedure described by Gleich et al. (11). Briefly, suspensions of eosinophils in 0.34 M sucrose were repeatedly pipetted, then centrifuged for 10 min at 400 g at 4°C to remove unbroken cells. The opalescent supernatants were transferred to another tube and centrifuged at 25,000 g for 20 min at 4°C. The pellet was solubilized in 0.02 M acetate buffer, pH 4.3, in 0.01 M HCl and analyzed by gel filtration on 1.2 × 45-cm columns of Sephadex G-50. The purified MBP was homogeneous as confirmed by the presence of a single electrophoretic protein band on SDS-PAGE gels as described previously (10; data not shown). The concentration of MBP was determined as described previously (10, 12) and the peak secretion was observed at an MBP concentration of 8 × 10⁻³ M.

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

Statistical analysis. The concentration of PC is expressed as mean ± SE. Differences among groups were assessed by Duncan’s multiple range test (a nonparametric test). P < 0.05 denoted the presence of a significant difference.

RESULTS

Isolation of eosinophil granule proteins and their effect on PC secretion. Similar to a previous study on eosinophil granule proteins of guinea pigs (30), suspensions containing eosinophil granule proteins (EPO, EDN, ECP, and MBP) showed three separate peak fractions by gel filtration on columns of Sephadex G-50 when each fraction was monitored for changes in absorbance at 277 nm (Fig. 1, top). The first peak (G2) corresponded to the void volume of the column and contained a variety of bands as observed on SDS-PAGE gels. The second peak (G3) contained two slightly separated bands, whereas the third peak (G4) contained one major band with a molecular weight < 14,000. These results indicated that the third peak contained only MBP.

Next, we examined the effects of various peak fractions, including the fraction preceding the void volume (G1), on PC secretion in primary cultures of rat type II pneumocytes. Each fraction was ultrafiltered, concentrated, and resuspended at a granule concentration corresponding to 1 × 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 without increasing LDH activity in the culture medium. The MBP-induced increase of PC secretion was concentration dependent, and the peak secretion was observed at an MBP concentration of 8 × 10⁻³ M (Fig. 2). Furthermore, examination of the kinetics of PC secretion showed that it commenced within 5 min of the addition of MBP (8 × 10⁻³ M) and then increased progressively throughout the observation period (90 min; Fig. 3). The profile was not different from that of the control culture. In contrast, the addition of terbutaline (1 × 10⁻⁶ M), which was used as protein kinase A-related agent, resulted in a steep increase in the first 30 min, but this was followed by a small increase throughout the remaining 60 min (Fig. 3).

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 $8 \times 10^{-9}$ M MBP. H-7 ($1 \times 10^{-5}$ M), a protein kinase C inhibitor, and BAPTA-AM ($5 \times 10^{-6}$ 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 ($6 \times 10^{-6}$ 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 pneumo-

![Fig. 1. Top: fraction profile of eosinophil granule protein by gel filtration on columns of Sephadex G-50. Each fraction was monitored for changes in absorbance at 277 nm. G1, fraction preceding void volume; G2, eosinophil peroxidase (EPO) fraction; G3, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) fraction; G4, major basic protein (MBP). Bottom: effects of each fraction on PC secretion in primary cultures of rat type II pneumocytes. Each fraction was ultrafiltered, concentrated, and resuspended at a granule concentration corresponding to $1 \times 10^6$ eosinophils/ml culture medium. Values are means ± SE expressed as amount of [3H]phosphatidylcholine (PC) in medium after 90-min incubation as percentage of that in cells plus medium (% of control) from 6 experiments. [3H]PC secretion after 90 min was $0.75 \pm 0.07\%$ in control cultures ($n = 5$). Significant difference from control value: *$P < 0.05$; **$P < 0.01$.

![Fig. 2. MBP concentration-dependent secretion of PC from type II pneumocytes. Isolated pneumocytes were incubated with indicated concentrations of MBP for 90 min. □, Value for terbutaline ($1 \times 10^{-6}$ M; positive control). Values are means ± SE from 6 experiments. [3H]PC secretion after 90 min was $0.51 \pm 0.04\%$ in control cultures (not incubated with MBP; $n = 5$).

![Fig. 3. Kinetics of MBP-stimulated PC secretion in type II pneumocytes. ○, Control culture; ●, MBP ($8 \times 10^{-9}$ M); ▲, terbutaline ($1 \times 10^{-6}$ M). Values are means ± SE from 6 experiments. [3H]PC secretion after 90 min was $0.89 \pm 0.03\%$ in control cultures ($n = 5$).]
Fig. 4. Effects of several inhibitors on MBP-induced PC secretion in type II pneumocytes. Each inhibitor was added 10 min before addition of MBP (8 × 10⁻⁹ M), followed by further incubation for 90 min. H-89 (6 × 10⁻⁶ M), HA-1004 (1 × 10⁻⁵ M), H-7 (1 × 10⁻⁵ M), and 1,2-bis(2-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-AM (5 × 10⁻⁶ M) were used as inhibitors of PC secretion pathways. Values are means ± SE from 6 experiments. [³H]PC secretion after 90 min was 0.88 ± 0.06% in control cultures (n = 5). *Significant difference from MBP alone, P < 0.05.

cytos and that such increases were MBP concentration dependent (Fig. 2). Furthermore, we also demonstrated that MBP-stimulated PC secretion was significantly inhibited by H-7, a protein kinase inhibitor, and BAPTA-AM, an intracellular Ca²⁺ chelator (Fig. 4).

Eosinophils infiltrate the airways and lungs of asthmatic patients and release many granule proteins, leukotrienes, platelet-activating factor, and various oxygen-derived toxic metabolites. The direct involvement of these substances on lung surfactant has been reported (8, 25). However, the effect of the eosinophil itself on the secretion of surfactant is poorly understood. In a previous study by Okumura et al. (22), activated eosinophils were found to increase PC secretion in primary cultures of rat type II pneumocytes, which was partly mediated by superoxide anion released from these cells. However, we speculated that the granule proteins, in addition to superoxide anions, were 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 cAMP-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 partly inhibited the MBP-increased PC secretion. H-7 is a relatively selective protein kinase C inhibitor, but it also exerts a slight inhibitory
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-glycero-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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