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Eicosanoid generation from antigen-primed mast cells by extracellular mammalian 14-kDa group II phospholipase A_2

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The extracellular form of 14-kDa group II phospholipase A_2 has been found to accumulate at various types of inflammatory sites. In the present paper, we have studied the possible role of the extracellular 14-kDa group II phospholipase A_2 in the process of prostaglandin production in activated rat mast cells. When mast cells obtained from the peritoneal cavity of rats were sensitized with IgE, challenged with antigen and then exposed to extracellular 14-kDa group II phospholipase A_2, appreciable release of prostaglandin D_2 was observed. Generation of prostaglandin D_2 was dependent on the concentration of the phospholipase A_2 as well as that of the antigen, while no appreciable prostaglandin D_2 generation was observed with cells in the absence of the antigen. No histamine release was observed under the same conditions. Phosphatidylcholine in mast cell membranes was appreciably hydrolyzed to liberate free arachidonic acid when mast cells were incubated with 14-kDa group II phospholipase A_2 added exogenously in the presence of the antigen. Both the generation of prostaglandin D_2 and the release of arachidonic acid were retarded by inhibitors specific to 14-kDa group II phospholipase A_2. Thus, 14-kDa group II phospholipase A_2 may function in the process of inflammation by acting on IgE-antigen-primed mast cells, which are not fully activated, to generate eicosanoids.

Group II phospholipase A_2; Mast cell; Prostaglandin D_2; Rat

1. INTRODUCTION

Several studies have implicated extracellular phospholipase A_2 in the pathogenesis of disorders of the cardiovascular, gastrointestinal and pulmonary systems, skin and connective tissues [1,2]. Extracellular phospholipase A_2 found in fluid at various sites of inflammation, such as glycogen-induced ascitic fluid in rabbits [3], caseinate-induced ascitic fluid in rats [4], and human synovial fluid in patients with rheumatoid arthritis [5–7], has been purified and identified as a 14-kDa group II phospholipase A_2. Various inflammatory cells such as platelets [7–9] and neutrophils [10] have been shown to contain 14-kDa group II phospholipase A_2. The inflammatory cytokines, such as tumor necrosis factor, interleukin (IL)-1 and IL-6 induced transcription of the 14-kDa group II phospholipase A_2 gene in various cells including rat vascular smooth muscle cells [11], rat mesangial cells [12], rabbit chondrocytes [13], or human hepatocytes [14]. Anti-inflammatory glucocorticoid suppressed the transcription of 14-kDa group II phospholipase A_2 [15]. Injection of endotoxin into rats enhanced the expression of 14-kDa group II phospholipase A_2 in various organs [16]. These observations suggest that extracellular 14-kDa group II phospholipase A_2 may be involved in the process of inflammation.

Mast cells are well known for their central role in allergic and hypersensitivity states and have been implicated in a variety of chronic inflammatory processes [17,18]. Cross-linking of high affinity IgE receptors on mast cell surfaces with IgE and multivalent antigen triggers the release of a variety of chemical mediators stored in granules, for example histamine, and the generation of eicosanoids, such as PGD_2, which has vasodilating and bronchoconstrictive activities [19], or leukotriene C_4 (LTC_4) and LTB_4. As for rodent connective tissue mast cells, lysophosphatidylserine (lysoPS) acts as an essential cofactor for full activation upon various stimuli including IgE-antigen system [20–22]. In the present study, we found that 14-kDa group II phospholipase A_2 located outside mast cells was involved in generation of PGD_2 in IgE- and antigen-challenged rat peritoneal connective tissue mast cells even in the absence of lysoPS.

Abbreviations: PG, prostaglandin; LT, leukotriene; rC3 α, recombinant C3 α; DNP-*Ascaris*, dinitrophenyl-conjugated *Ascaris suum*; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; lysoPS, lysophosphatidylserine; IL, interleukein.

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2. MATERIALS AND METHODS

2.1. *Mast cells*

Mast cells (purity; more than 90%) were isolated from the peritoneal cavity of Wistar rats (Nippon Bio-Supply Center, Tokyo, Japan) as described previously [20].

2.2. *Phospholipase A_2 and its specific inhibitors*

Rat 14-kDa group II phospholipase A_2 was purified from rat platelets using an anti-rat 14-kDa group II phospholipase A_2 monoclonal antibody-conjugated Sepharose column as described previously [23,24]. Preparation of 14-kDa group II phospholipase A_2-specific polyclonal antibody R377 [23] and monoclonal antibody MD7.1 [24] was described previously. Preparation of rat recombinant C3 α (rC3 α; a product of the *E. coli* lacZ-rat C3dg (complement C3 degrading product [25]) gene) and thielocin A1, both of which inhibited group II phospholipase A_2 rather specifically, are described elsewhere (unpublished data).

2.3. *Treatment of mast cells with phospholipase A_2*

Mast cells were suspended in 10 mM Tris-HCl buffer (pH 7.4), which contained 150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 0.1% (w/v) glucose and 0.5% (w/v) gelatin (Sigma, St. Louis, MO) (Tris-gelatin buffer), and adjusted to 1×10^6 cells/ml. The cells were sensitized with 1 μg/ml mouse monoclonal anti-dinitrophenyl (DNP) IgE (Seikagaku Kogyo, Tokyo, Japan) for 30 min at 37°C. The sensitized cells were washed, suspended in Tris-gelatin buffer at 1×10^6 cells/ml, and incubated with 14-kDa group II phospholipase A_2 in the presence or absence of DNP-conjugated *Ascaris suum* (DNP-*Ascaris* (donated by Kissei Pharmaceutical, Matsumoto, Japan). Alternatively, the sensitized cells were stimulated with DNP-*Ascaris* in the presence of 10^{-6} M lysoPS (Funakoshi, Tokyo, Japan). After incubation, the cells were centrifuged at $750 \times g$ for 5 min at 4°C to obtain the supernatant. PGD_2 released into the supernatant was measured using a PGD_2 assay kit (Amersham, Buckinghamshire, UK). Histamine released into the supernatant was determined by a radioenzymatic assay using [^{3}H]methyl-*S*-adenosyl-L-methionine (New England Nuclear, Boston, MA) and a crude preparation of rat kidney histamine methyltransferase [26]. The percentage release of histamine was calculated by dividing the amount in each supernatant by that in a preparation of sonicated cells (Branson Sonifier, 20 pulses, setting 4, 50% pulse cycle).

2.4. *Lipid analysis*

Mast cells (1×10^6 cells/ml) were incubated for 1 h in Tris-gelatin buffer containing 1 μCi/ml [^{3}H]arachidonate (Amersham), washed twice with Tris-gelatin buffer, sensitized with IgE and treated with 10 μg/ml 14-kDa group II phospholipase A_2 and 1 μg/ml DNP-*Ascaris* in the presence or absence of 2 μg/ml thielocin A1 for 15 min at 37°C. The total cellular lipids were extracted by the method of Bligh and Dyer [27] and separated by two-dimensional thin-layer chromatography on silica gel plates (Merck, Darmstadt, Germany), which were developed with a slight modification of the solvent system described by Esko and Raetz [28]. Briefly, the extracted lipids were reconstituted in a small volume of chloroform and applied to silica gel plates (approximately 1×10^5 cells per plate). The plates were developed first with chloroform/methanol/acetic acid (65:25:10, v/v), dried for 60 min under an air stream, and developed in the second dimension with chloroform/methanol/88% formic acid (65:25:10,v/v). The spots which were visible after exposure to I_2 vapor were identified by comparing their positions with those of authentic standard phospholipids. The I_2 was then removed with an air stream, the individual spots were scraped off, and the radioactivity of each was measured.

3. RESULTS

The effect of treatment of rat peritoneal mast cells with purified rat 14-kDa group II phospholipase A_2 on their cellular functions were examined. When unstimulated mast cells were treated with the phospholipase A_2 alone, PGD_2 release into the supernatant was not augmented appreciably (Fig. 1). We then examined the effect of the phospholipase A_2 on antigen-stimulated mast cells. No appreciable PGD_2 generation was observed when mast cells were first sensitized with anti-DNP IgE and then challenged with DNP-conjugated antigen in the absence of lysoPS, which is an essential cofactor for full activation of rat peritoneal mast cells [20–22]. When these IgE-sensitized, antigen-challenged cells were further treated with purified 14-kDa group II phospholipase A_2, concentration-dependent PGD_2 generation was observed (Fig. 1). The concentrations of the enzyme producing an appreciable effect (ranging from 1 to 10 μg/ml) were comparable to those detected at various inflammatory sites [23,29]. PGD_2 generation was also

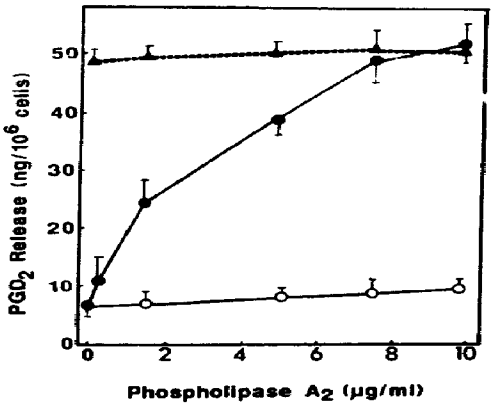

Fig. 1. Effect of extracellular 14-kDa group II phospholipase A_2 on PGD_2 generation by rat peritoneal mast cells. Mast cells sensitized with IgE were incubated with the indicated concentrations of 14-kDa group II phospholipase A_2 in the presence of 1 μg/ml DNP-*Ascaris* (●), DNP-*Ascaris* plus 10^{-6} M lysoPS (▲) or in their absence (○) for 15 min at 37°C, and PGD_2 released into the supernatant was measured as described in section 2. The values indicate averages ± SD (n=3).

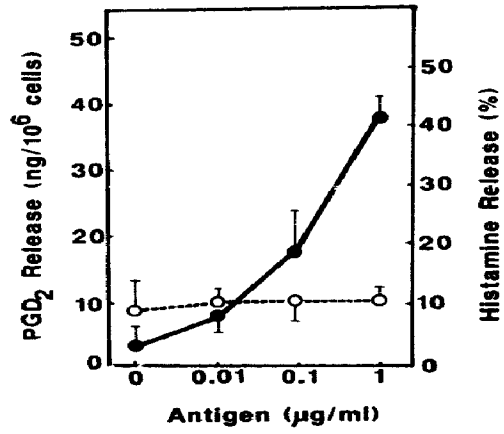

Fig. 2. Effect of antigen concentrations on extracellular 14-kDa group II phospholipase A_2-mediated PGD_2 release from mast cells. The sensitized mast cells were incubated with 14-kDa group II phospholipase A_2 (6 μg/ml) and the indicated concentrations of DNP-*Ascaris* for 15 min at 37°C, and PGD_2 (●) or histamine (○) released into the supernatant was measured as described in section 2. The values indicate averages ± SD (n=3).

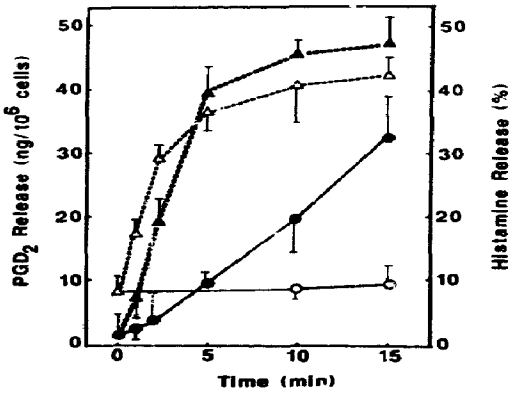

Fig. 3. Time-course of PGD_2 generation by mast cells treated with extracellular 14-kDa group II phospholipase A_2. The sensitized mast cells were treated with 14-kDa group II phospholipase A_2 (6 μg/ml) (circles) or lysoPS (10^{-6} M) (triangles) in the presence of DNP-*Ascaris* (1 μg/ml) for the indicated period at 37°C, and either PGD_2 (closed symbols) or histamine (open symbols) released into the supernatant was measured as described in section 2. The values indicate averages ± SD (n=3).

dependent on the concentration of the antigen (Fig. 2). When the cells were challenged with 1 μg/ml antigen and treated with more than 7.5 μg/ml enzyme, about 50 ng of PGD_2 per 10^6 cells was generated, almost the same amount as that generated by fully activated mast cells; mast cells stimulated with 1 μg/ml antigen in the presence of 10^{-6} M lysoPS generated about 50 ng of PGD_2 per 10^6 cells (Fig. 1). It should be noted here that no further enhancement effect of exogenous 14-kDa group II phospholipase A_2 on PGD_2 generation was observed with cells which were fully activated by treatment with antigen plus lysoPS.

In contrast to PGD_2 generation, release of histamine from mast cells was not affected by exogenous phospholipase A_2 appreciably; mast cells released only about 10% of cellular histamine (the same as the basal level) even in the presence of 14-kDa group II phospholipase A_2 (Fig. 2).

Table I

Effect of 14-kDa group II phospholipase A_2-specific inhibitors on extracellular 14-kDa group II phospholipase A_2-mediated PGD_2 release from mast cells

Treatment	PGD_2 released (ng/10^6 cells)	Inhibition (%)
No treatment	2.1 ± 0.8	
Phospholipase A_2	38.0 ± 1.9	
+ Antibody R377 (40 μg/ml)*	2.0 ± 1.1	100
+ rC3α (2 μg/ml)*	3.6 ± 1.4	90.5
+ Thielocin A1 (2 μg/ml)*	2.6 ± 0.4	94.7
+ Antibody MD7.1 (15 μg/ml)**	37.0 ± 2.5	2.6

The enzyme was preincubated with the indicated amounts of each inhibitor for 30 min at 22°C. Then IgE-antigen-primed mast cells were treated with the inhibitor-treated enzyme (6 μg/ml) and PGD_2 released into the supernatant was quantified as described in Section 2. Values indicate averages ± SD (n=3).

*Sufficient concentration to inhibit phospholipase A_2 activity.

**Sufficient concentration to inhibit the interaction between phospholipase A_2 and heparin.

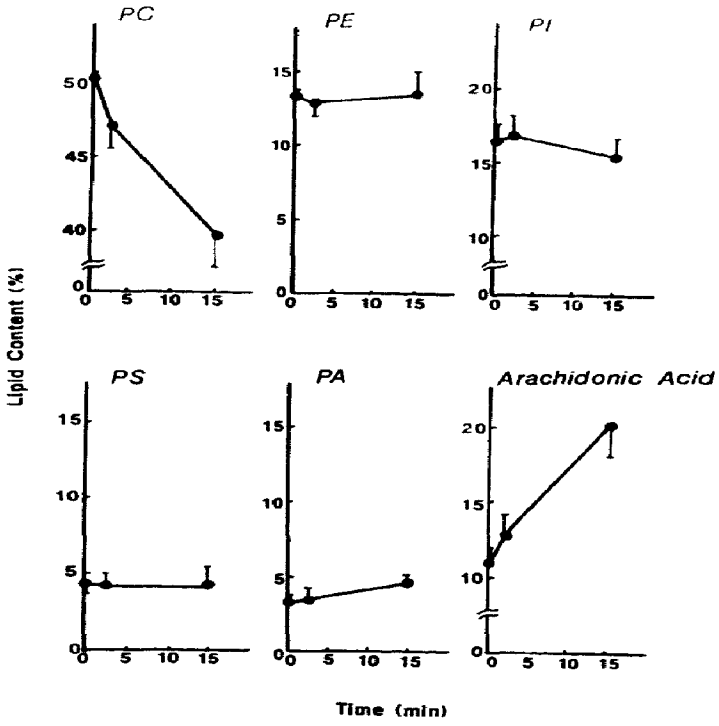

Fig. 4. Time course of phospholipid metabolism of [^{3}H]arachidonate-labeled mast cells after treatment with extracellular 14-kDa group II phospholipase A_2. The procedure is described in section 2. Values indicate phospholipid composition (average (%) ± SD) of each sample (n=3).

The time course of PGD_2 generation induced by antigen plus 14-kDa group II phospholipase A_2 was rather different from the one induced by antigen plus lysoPS. The generation of PGD_2 observed in the presence of 14-kDa group II phospholipase A_2 progressed almost linearly within 15 min after enzyme challenge (Fig. 3). On the other hand, PGD_2 generation by cells supplemented with antigen plus lysoPS reached a plateau approximately 5 min after antigen challenge, paralleling histamine release.

The effect of exogenous 14-kDa group II phospholipase A_2 on PGD_2 generation by mast cells was abolished by pretreatment of the enzyme with 14-kDa group II phospholipase A_2-specific inhibitors, such as antibody R377, thielocin A1 and rC3 α (Table I). A monoclonal antibody MD7.1, which recognizes the heparin-binding domain of rat 14-kDa group II phospholipase A_2 but does not inhibit enzyme activity [24], showed no

Table II

Phospholipid metabolism of [^{3}H]arachidonate-labeled mast cells after treatment with extracellular 14-kDa group II phospholipase A_2

Phospholipids	No treatment	Treatment with phospholipase A_2		
		Intact cells	Antigen-treated cells	
		Thielocin A1 (−)		Thielocin A1 (+)
Sphingomyelin	0.7 ± 0.2	0.8 ± 0.1	1.0 ± 0.3	1.0 ± 0.1
Phosphatidylcholine	51.0 ± 0.2	50.3 ± 1.5	39.9 ± 2.2	50.6 ± 1.2
Phosphatidylinositol	16.5 ± 1.1	15.8 ± 0.3	14.7 ± 1.1	15.6 ± 0.0
Phosphatidylserine	4.3 ± 0.4	4.0 ± 0.5	4.1 ± 1.0	4.0 ± 0.4
Phosphatidylethanolamine	13.1 ± 0.1	13.9 ± 0.8	15.3 ± 0.3	13.7 ± 0.0
Phosphatidic acid	3.1 ± 0.1	3.0 ± 0.5	4.9 ± 0.1	3.7 ± 0.2
Free arachidonate	11.2 ± 0.6	10.8 ± 0.4	20.3 ± 2.0	11.6 ± 1.0

The procedure is described in Section 2. Values indicate phospholipid composition (average (%) ± SD) of each sample (n=3).

inhibitory effect, suggesting that enzymatic activity of group II enzyme may be essential for the generation of PGD_2 in this reaction.

The alteration of the membrane phospholipids in mast cells by treatment with the exogenous 14-kDa group II phospholipase A_2 was next examined. When [^{3}H]arachidonate-labeled cells were exposed to 14-kDa group II phospholipase A_2 in the presence of the antigen, only the radioactivity in phosphatidylcholine (PC) decreased drastically (approximately 50% to 40%) with a concomitant increase in radioactivity of free arachidonate (approximately 11% to 20%) (Fig. 4, Table II). The hydrolysis of PC and thereby the increment of free arachidonate were suppressed almost completely by pretreatment of the enzyme with its specific inhibitor, thielocin A1. No appreciable hydrolysis of PC was observed when intact mast cells were treated with the enzyme under the same conditions (Table II). Therefore, 14-kDa group II phospholipase A_2 appeared to have hydrolyzed PC in the membrane ofantigen-primed mast cells.

4. DISCUSSION

In the present study, we demonstrated that mammalian 14-kDa group II phospholipase A_2 added exogenously to IgE-antigen-primed rat peritoneal mast cells augmented eicosanoid generation in the cells. The concentrations of the enzyme required for the augmentation (1–10 μg/ ml) were within the range detected at various inflammatory sites [23,29], indicating that extracellular 14-kDa group II phospholipase A_2 may contribute to the progression of inflammation, especially allergic reaction, in which mast cells play important roles as effector cells, by hydrolyzing mast cell phospholipids to generate precursors of pro-inflammatory lipid mediators.

14-kDa Group II phospholipase A_2 was able to influence PGD_2 generation only when IgE-receptors on the surfaces of mast cells were cross-linked by multivalent antigens. The activity of several phospholipases A_2 on cell membranes is known to be affected by lipid packing in the outer leaflet of the plasma membrane [30]. Cross-linking of IgE receptors may change the molecular packing of lipids [31], making them susceptible to exogenous 14-kDa group II phospholipase A_2. These observations are in accord with our previous data, which showed that mammalian 14-kDa group II as well as group I phospholipase A_2 added exogenously augmented the generation of PGE_2 by HL-60 granulocytes only in the co-presence of A23187 [32]. We also reported that injection of purified 14-kDa group II phospholipase A_2 into the hind paw of rats with adjuvant arthritis exacerbated the edema, whereas no effect was observed in normal rats [29]. Thus, expression of the pharmacological activity of the exogenous 14-kDa group II phospholipase A_2 may require a certain stage of ongoing inflammation induced by some other factors. A change in the transmembrane distribution of phospholipids may be one of the features of such an activated state. Recently, Bomalaski et al. [33] demonstrated that purified recombinant human 14-kDa group II phospholipase A_2 elicited a dramatic inflammatory, arthritogenic response when injected into the joint space of healthy rabbits, although they also stated that the enzyme was completely inactive in the paw edema inflammation assay using normal rats. Although this discrepancy might be explained by the difference in the species employed, further work must be performed to clarify the molecular mechanisms of the pro-inflammatory effect of exogenous 14-kDa group II phospholipase A_2.

Mammalian 14-kDa group II phospholipase A_2 hydrolyzes phosphatidylethanolamine (PE) or phosphatidylserine (PS) more efficiently than PC in an in vitro assay system [2–10]. The treatment of mast cells with exogenous 14-kDa group II phospholipase A_2 resulted in preferential hydrolysis of membrane PC. It is known that PC is distributed in the outer leaflet of plasma bilayer membrane of mammalian cells, whereas both PE and PS exist almost exclusively in the inner leaflet [34]. Thus, only PC might be available for extracellular 14-kDa group II phospholipase A_2. Alternatively, the

group II enzyme might activate some other cellular phospholipase(s) A_2 which liberate arachidonate from PC. We found that mast cells expressed an arachidonate-preferential cytosolic 85-kDa phospholipase A_2 [35], a cDNA clone of which was recently isolated from U937 cells [36], and showed the possibility that the enzyme might play an essential role in arachidonate metabolism upon application of mast cells with immunochemical stimuli (antigen plus lysoPS) via the IgE receptor [37]. The possibility that exogenous 14-kDa group II phospholipase A_2 activates intracellular phospholipase A_2 such as arachidonate-preferential phospholipase A_2 cannot be ruled out at present.

LysoPS is a potentiator of the degranulation as well as PGD_2 generation by rat peritoneal mast cells [20–22]. One possible mechanism whereby exogenous 14-kDa group II phospholipase A_2 may exert its effect is to generate lysoPS by hydrolysis of membrane PS. However, this possibility can be eliminated for the following reasons: (i) histamine release was not induced by exogenous 14-kDa group II phospholipase A_2. If lysoPS was supplied by this exogenous enzyme, then histamine release might also be induced in the same manner as PGD_2 generation; (ii) no appreciable hydrolysis of PS was observed when cells prelabeled with radiolabeled arachidonate were exposed to the 14-kDa group II phospholipase A_2 in the presence of antigen; (iii) stimulation of mast cells with antigen plus lysoPS is accompanied by a rapid and drastic breakdown of phosphatidylinositol (PI) [38], whereas no appreciable rapid decrease in radioactivity in PI was observed with mast cells treated with antigen and the 14-kDa group II phospholipase A_2 (Fig. 4). It can be concluded that exogenous 14-kDa group II phospholipase A_2 acted on IgE-antigen-primed, but not fully activated mast cells to generate eicosanoids, resulting in the progression of allergic inflammation.

The source of the extracellular 14-kDa group II phospholipase A_2 detected at inflamed sites is still unidentified. It should be noted that activated mast cells also secrete 14-kDa group II phospholipase A_2 (unpublished data). However, the amount of the enzyme released from activated mast cells is too small (less than 1 ng per 10^6 cells) to act on IgE-antigen-primed mast cells to generate a detectable level of eicosanoids. Therefore, 14-kDa group II phospholipase A_2 might be supplied by cells other than mast cells.

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