J. Biochem. Mol. Biol. Vol. 30, No. 2, pp. 101~105 March 31, 1997

Detection and Characterization of Novel Extracellular Phospholipase A_2 in Urine of Patients with Acute Pyelonephritis

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(Received December 15, 1996)

Abstract : Extracellular phospholipase A_2 activity has been detected in urine of patients with acute pyelonephritis (APN). This enzyme required micromolar Ca^{2+} ion for its maximum activity and showed a broad range of pH (4.5~10) optimum. Urine enzyme hydrolyzed phosphatidylethanolamine (PE) and phosphatidylserine (PS) more effectively than phosphatidylcholine (PC). PLA_2 activity in the urine of patients with APN was about 5-fold higher than that of healthy individuals. When urine was subjected to heparin-Sepharose column chromatography, phospholipase A_2 activity was detected in both heparin-non-binding and binding fractions. Both phospholipase A_2 activities were sensitive less than a micromolar calcium concentration and did not react with anti-human 14-kDa group II phospholipase A_2 monoclonal antibody, HP-1. These findings suggest that two kinds of novel extracellular phospholipase A_2, which may not belong to the 14-kDa group II phospholipase A_2 family, exist in the urine of patients with APN.
Key words : acute pyelonephritis (APN), kidney disease, urine extracellular phospholipase A_2.

Phospholipase A_2 (PLA_2) plays a central role in liberating free fatty acids and lysophospholipids from membrane phospholipids thereby initiating the production of potent proinflammatory lipid mediators, such as eicosanoids, and a platelet activating factor (Waite, 1985). Mammalian cells contain several PLA_2 isozymes including secretory enzymes and a more recently described 85-kDa cytosolic enzyme called $cPLA_2$ (Kim *et al.*, 1991; Kudo *et al.*, 1993; Dennis, 1994). Mammalian extracellular PLA_2s have been classified into two types (Heinrikson *et al.*, 1977), group I and II, based on their primary structures. These traditional group I and II PLA_2s have been isolated as extracellular enzymes, have high disulfide bond content, do not demonstrate preference for fatty acid at the *sn*-2 position of phospholipids, and require mM of Ca^{2+} for catalysis. Group I enzymes are present mainly in digestive organs, such as pancreas and cobra venom (Dijkstra *et al.*, 1981; Fremont *et al.*, 1993), whereas group II enzymes have been found in inflamed sites (Chang *et al.*, 1986; Hara *et al.*, 1989), such as human synovial fluids, and non-pancreatic sources such as platelets (Hayakawa *et al.*, 1988), and spleen (Ono *et al.*, 1988). Unlike the type of traditional low molecular mass secretory PLA_2, $cPLA_2$ exhibits a preference for *sn*-2-arachidonic acid-containing substrate and is optimaly activated under micromolar calcium concentration (Kim *et al.*, 1991; Kudo *et al.*, 1993; Dennis, 1994).

Kidney tissue generates prostaglandins (PGs) in relatively large amounts (Schilondorff *et al.*, 1986). The renal PGs may have an important role in blood pressure regulation. Production of such lipid mediators are induced by PLA_2. Therefore the regulation of production of these lipid mediators is of considerable importance. Recently, Hara *et al.*, reported that at least 4 types of PLA_2 isozymes may contribute to regulation of eicosanoid synthesis in rat kidneys (Hara *et al.*, 1995). Andersen *et al.*, demonstrated that $cPLA_2$ and group II PLA_2 are expressed in human skin and suggested that overexpressed group II PLA_2 could play an important role in psoriatic skin (Andersen *et al.*, 1994). No information was, however, obtained on extracellular PLA_2 activity in the urine of diseased humans, especially kidney disease. Pyelonephritis is one of the most common renal diseases and can be clearly classified, as acute

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and chronic. APN is defined as an acute inflammatory process of the kidney and its adjacent structure, the renal pelvis. In the present study, we describe detection and characterization of novel extracellular PLA_2 activity in the urine of patients with APN.

Materials and Methods

Materials

[1-^{14}C]Linoleic acid and 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerophosphoethanolamine (GPE) were purchased from Amersham (Amersham, UK). Heparin-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of the highest purity available from commercial sources.

Preparation of labeled substrate

1-Acyl-2-[1-^{14}C]linoleoyl-*sn*-GPE was prepared by the method described previously (Arai *et al.*, 1985).

Preparation of enzyme source

Urine was collected from patients with APN who called at Kyungpook National University Hospital. Diagnois of APN was based on clinical and bacteriological studies. Cells and debris were immediately removed from the urine of the patients by centrifugation at 4°C, and then stored at −20°C until used.

Assay of phospholipase A_2

The standard reaction mixture (200 μl), which contained 100 mM Tris-HCl (pH 7.4), 6 mM $CaCl_2$, and 20 nmol substrate (1000 cpm/nmol). The reaction was carried out at 37°C for 20 min, and stopped by adding 1.25 ml of Dole's reagent (Dole *et al.*, 1960). Radioactive free fatty acids released were extracted by the method described previously (Arai *et al.*, 1985).

Analytical procedures

Protein concentration was measured with a Pierce protein assay kit (Kaushal *et al.*, 1986). Bovine serum albumin served as the protein standard.

Results

Cell-free urine of APN was assayed for PLA_2 activity at pH 7.4 using 1-acyl-2-[1-^{14}C]linoleoyl-GPE as a substrate. Evaluation of urine PLA_2 activity in the urine of patients with APN revealed a significantly elevated PLA_2 level. Urine PLA_2 levels varied in patients with APN from 240~5900 cpm/30 μl with a means±S.D of 1880±2280 cpm/30 μl for n=8 patients. The values of PLA_2 in 8 healthy individuals varied from 100~380 cpm/30 μl, with a mean±S.D of 230±105 cpm/30 μl.

To demonstrate the positional specificity of urine phospholipase, the analysis of radioactive products by thin layer chromatography was performed, which revealed that urine phospholipase is of the A_2 type (data not shown). When measured under optimal conditions with various phospholipids differing in their polar head group, PE was the best substrate, followed by PS and PC, the observed specific activites were 14.1, 10.0, 4.2 nmol/min/mg protein, respectively. When 1-acyl-2-[1-^{14}C]linoleoyl-GPE and 1-pal-2-[1-^{14}C]arachidonyl-GPE were used as a substrate, this enzyme did not demonstrate fatty acid preference at the *sn*-2 position of phospholipids (data not shown). Forty percent-$(NH_4)_2SO_4$ supernatant of urine was applied onto a heparin-Sepharose column (2.5×10 cm) preequilibrated with 20 mM Tris-HCl, pH 7.4 containing 1 mM EDTA and 10 mM NaCl. The column was washed and then eluted with the same buffer containing 1 mM EDTA and 1 M NaCl. When individual fractions were examined for PLA_2 activity using 1-acyl-2-[1-^{14}C]linoleoyl-GPE as a substrate, appreciable PLA_2 activity was detected in both heparin-non-binding and binding fractions (Fig. 1) It was ensured that the column was not overloaded by re-applying a part of the pool of the heparin-non-binding fraction to the same column(data not shown).

Factors affecting the PLA_2 activity

The effect of pH on hydrolysis of 1-acyl-2-[1-^{14}C] linoleoyl-GPE by the urine enzyme was determined over a pH range of 3.5~10.5. The activity was detected at a broad pH 4.5~10 range and showed an optimum at pH 7.4 (Fig. 2). When 1-acyl-2-[1-^{14}C] linoleoyl-GPE was used as a substrate, PLA_2 activities

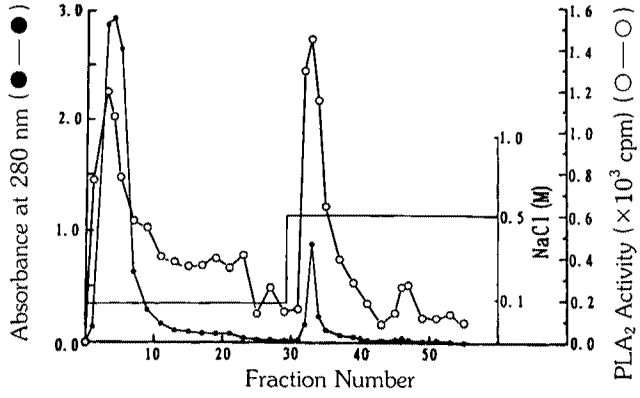

Fig. 1. Heparin-Sepharose chromatography of urine phospholipase A_2. Forty percent-$(NH_4)_2SO_4$ supernatant of urine was applied onto a heparin-Sepharose column (2.5×10 cm) preequilibrated with 20 mM Tris-HCl, pH 7.4 containing 1 mM EDTA and 10 mM NaCl. The column was washed and then eluted with the same buffer containing 1 mM EDTA and 1 M NaCl. The flow rate was 1 ml/10 min per fraction. Protein (●) and phospholipase A_2 activity (○).

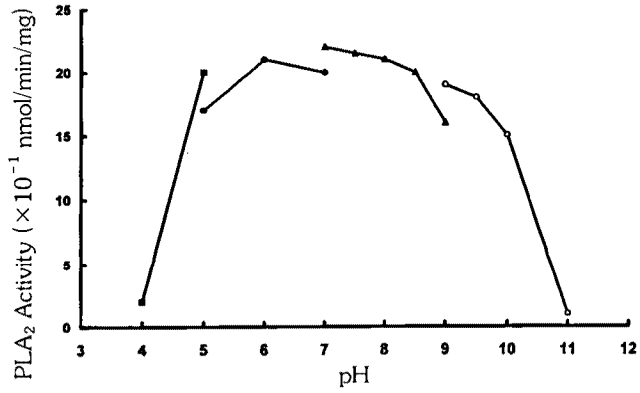

Fig. 2. The pH dependence of urine phospholipase A_2 activity. A reaction mixture containing 40% $(NH_4)_2SO_4$ supernatant fraction (30 μg), 6 mM $CaCl_2$, 10 nmol of 1-acyl-2-[1-^{14}C] linoleoyl-*sn*-glycero-3-phosphoethanolamine was incubated for 20 min at 37°C in a tatal volume of 200 μl. The buffers used were 100 mM sodium acetate buffer (■) within a pH range of 4.0~5.0, 100 mM Tris-malate (●), pH 5.0~7.0, 100 mM Tris-HCl (▲), pH 7.0~9.0 and 100 mM glycin-NaOH (○) pH 9.0~11.0.

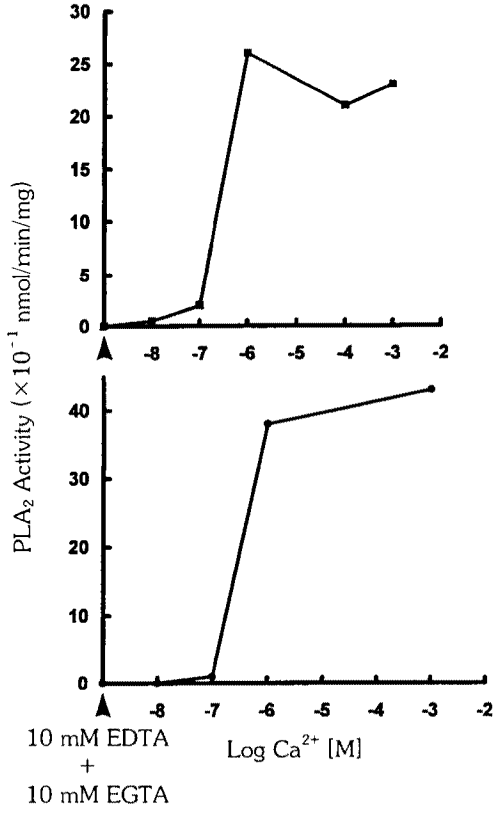

Fig. 3. Effect of calcium ion on the activity of phospholipase A_2. A heparin-non-binding and binding phospholipase A_2 (30 μg) was incubated with 10 nmol of 1-acyl-2-[1-^{14}C] linoleoyl-*sn*-glycero-3-phosphoethanolamine, 100 mM Tris-HCl (pH 7.4) and various concentration of $CaCl_2$ in a total volume of 200 μl for 20 min at 37°C. The absolute concentration of free calcium was calculated using an equation based on the stability constant of the EGTA/$CaCl_2$ system described previously (Duraham *et al.*, 1983). Heparin-non-binding fraction (■), heparin-binding fraction (●).

in a both heparin-non-binding and binding fraction were sensitive to less than a micromolar concentration of calcium ion (Fig. 3); the hydrolyzing activity was not detected in the presence of EDTA or EGTA. Although the negligible PLA_2 activity was observed at 10^{-7} M calcium ion, the activity increased sharply when the free calcium ion concentration increased from 10^{-7} to 10^{-6} M.

The effect of anti-human 14-kDa group II PLA_2 monoclonal antibody, HP-1 (Takayama *et al.*, 1990) on the activity of heparin-non-binding and binding PLA_2s was examined. HP-1 inhibited the human group II PLA_2 in a dose-dependent manner, whereas urine PLA_2 activity did not react with the HP-1, even when added at 10 μg (Fig. 4).

The effect of chemical reagents on the activity of heparin-non-binding and binding PLA_2s is shown in Table 1. Both enzymes were inhibited by mepacrine and *p*-bromophenacyl bromide (*p*-BPB), which is known to be an active site-directed reagent for a large number of secretory PLA_2 (Chang *et al.*, 1986; Hara *et al.*, 1989). No inhibition was observed with dithiothreitol (DTT), which causes spliting and a reduction of disulfide bridges and phenylmethylsulfonyl fluoride (a serine esterase inhibitor).

Discussion

High levels of extracellular PLA_2 associated with an inflammatory process in human and animal models have been extensively reported elsewhere, especially human synovial fluid PLA_2. In the present study, we describe for the first time the presence of extracellular PLA_2 in the urine of patients with APN. One of the most remarkable features of the urine PLA_2 enzymes was its calcium dependence and pH profiles. PLA_2 enzymes generally require calcium ion for their full activity with a few exceptions (Wolf *et al.*, 1985), as is the case for canine myocardium enzymes. Urine PLA_2 was sensitive to micromolar Ca^{2+} concentration, and showed a broad range of pH 4.5~10 optimum but did not show fatty acid preference at the *sn*-2 position of phospholipids. The micromolar Ca^{2+}-dependent PLA_2 is present in the cytosol of a variety of cell types, including human U937 cell, platelet, and macrophage. This $cPLA_2$ translocates from the cytosol to the membrane fraction in a Ca^{2+}-dependent fashion, shows maximal activity at alkaline pH (pH 9.0~10.0), shows no affinity for heparin, and exhibits preference for *sn*-2 arachidonic acid-containing phospholipids. In contrast, group II PLA_2 shows a high affinity for heparin, sensitive to

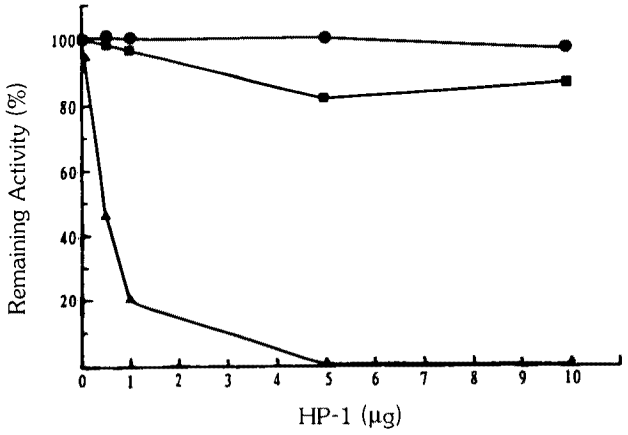

Fig. 4. Effects of anti-human 14 kDa group II phospholipase A_2 monoclonal antibody on the the urine phospholipase A_2 and human 14 KDa group II phospholipase A_2 activities (Hara *et al.*, 1989). Heparin-non-binding and binding enzyme (20 μg) was incubated with the indicated amount of HP-1 in 200 μl of 0.2% BSA-TBS for 1 h at 4°C. After incubation, aliqout was taken and examined for phospholipase A_2 activity. ●, heparin-non-binding fraction; ■, heparin-binding fraction; ▲, human 14 KDa group II phospholipase A_2.

mM Ca^{2+} concentration, and is optimally active at pH 9.0.

Whereas monoclonal antibody raised against human group II PLA_2, HP-1, inhibited human group II PLA_2 in a dose-dependent manner, with an IC_{50} of 0.5 μg, heparin-non-binding and binding PLA_2s were not inhibited by HP-1, even at 10 μg. Anti-rat group II PLA_2 polyclonal IgG also inhibited human group II PLA_2, with an IC_{50} of 10 μg. However, urine PLA_2s was not inhibited by rat group II PLA_2 polyclonal antibody (data not shown). From these results the urine of patients with APN may contain new types of PLA_2 isozyme, not belonging to the group II PLA_2 family.

At present, we don't know why PLA_2 activity in the urine of patients APN was about 5-fold higher than that of healthy individuals. Thus it is of great importantance to understand the pathogenesis of APN and identify the cellular origin of urine PLA_2 and its stimulants. APN is a well-defined clinical syndrome consisting of fever, flank pain with tenderness, often associated constitutional systems, leukocyte and bacteria in the urine. The accompanying anatomical lesions found in the kidney consist of numerous polymorphonuclear leukocytes in the interstitum, sometimes dense enough to be considered as abscesses, and leukocytes in the tubular lumen. Large numbers of bacteria are found in the urine, several hundred thousand per ml in the acute phase, and the most common infecting organism is *Escheria coli*. APN is also an acute inflammatory process of the kidney and renal pelvis. Thus urine PLA_2 in APN may be derived from some inflammatory cells of affected renal tissue, such as granulocytes or renal mesangial cells. The exact source of PLA_2 should be clarifed. Various proinflammatory mediators such as thrombin (Hayakawa *et al.*, 1988), IL-1, IL-6, TNF, and LPS (Oka *et al.*, 1991) are known to induce cellular release of group II PLA_2 . It should be stressed that patients with APN can have endotoxinemia with or without bacterecemia by LPS, which may be an important stimuli of subsequent PLA_2 activation. This means that filtration from the circulatory PLA_2 may be another source of urine PLA_2. Futher study should be performed to clarify this possibility.

Table 1. Effect of Various Agents on the Activity of heparin-non-binding and binding Phospholipase A_2

Treatment	Concentration (mM)	Remaining activity (%)	
		Heparin-non-binding	Binding
No addition		100	100
p-Bromophenacyl-bromide	1.0	94.5	90.5
	3.0	69.8	69.8
Mepacrine	1.0	61.5	39.6
	3.0	22.2	15.9
Dithiothreitol (DTT)	3.0	93.0	90.1
Phenylmethylsulfonyl-fluoride (PMSF)	3.0	96.1	83.1

Heparin-non-binding and binding PLA_2 (30 μg) was preincubated with various agents in 100 mM Tris-HCl (pH 7.4), containing 6 mM $CaCl_2$ in a total volume of 200 μl for 15 min at 37°C. The reaction was started by adding 20 nmol of 1-acyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine as a substrate and was continued for 30 min at 37°C.

Acknowledgements

This work was supported by a grant from the Korea Science and Engineering Foundation(KOSEF 941-0700-040-1). We are grateful to Drs. Ichiro Kudo and Makoto Murakami (Showa University, Japan) for helpful suggestions and providing human group II PLA_2 monoclonal antibody and anti-rat group II PLA_2 polyclonal IgG.

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