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Plasmin converts pro-form of group I phospholipase A_2 into receptor binding, active forms

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Treatment of zymogen of pancreatic-type group I phospholipase A_2 (PLA_2-I) by plasmin, a fibrinolytic enzyme, increases PLA_2 activity as well as receptor binding activity in a dose- and time-dependent manner. Separation of plasmin-treated pro-PLA_2-I by HPLC and amino acid sequence analysis of the products revealed that, in addition to an authentic mature PLA_2-I produced by trypsin, plasmin produced active products which had been modified in the C-terminal region. Thus, PLA_2-I may be involved in physiologic processes which accompany the formation of plasmin.

Phospholipase A_2 (PLA_2), EC 3.1.1.4, constitutes a family of enzymes which hydrolyze the ester bond at the C-2 position of glycerophospholipids (1) Thus, most studies on PLA_2 have been focused on the catalytic activity of PLA_2 and the consequent production of phospholipid-derived signal mediators including prostaglandins, leukotriens, lysophospholipids and platelet-activating factor. However, the recent discovery of a PLA_2 receptor on the membranes of several types of cells raised a new concept that PLA_2 acts not only as an enzyme but also as a ligand for the receptor (2-4).

Two isoforms of mammalian extracellular PLA_2 are well characterized (5). Group I PLA_2 (PLA_2-I) is well known to be produced and released by the pancreas and has been supposed to be important for digestion of phospholipids (6,7). On the other hand, group II PLA_2 (PLA_2-II) is proposed to have some important roles for inflammatory diseases, since elevated levels of PLA_2-II are found in inflammatory lesions and its production is regulated by certain inflammatory cytokines and anti-inflammatory glucocorticoids (8-11).

The PLA_2 receptor exhibits specific binding to only the mature form of mammalian PLA_2-I. It does not bind to the pro-form of PLA_2-I (pro-PLA_2-I), PLA_2-II nor bee or snake

Abbreviations: PLA_2, phospholipase A_2; PLA_2-I, group I phospholipase A_2; PLA_2-II, group II phospholipase A_2.

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venom PLA_2 (3,4). Binding of PLA_2-I to the receptor activates cells and induces cellular responses such as cell growth (2,3), chemotaxis (12), and production of prostaglandin (13), suggesting that PLA_2-I may also be involved in inflammatory diseases accompanied by these cellular responses.

As already mentioned, the major source of PLA_2-I is the pancreas. PLA_2-I is released as a pro-form into pancreatic juice, then pro-PLA_2-I is activated by trypsin in the intestines. However, the pancreas is not the only source of PLA_2-I. PLA_2-I has been found to be produced by other tissues such as lung, stomach and spleen (14-16). However, the mechanism of activation of PLA_2-I in tissues other than the intestines is not known. In this study, we examined the potencies of several mammalian proteolytic enzymes to activate pro-PLA_2-I and found that plasmin, which is also suggested to be important for cellular responses of some diseases, activates pro-PLA_2-I and produces receptor-binding forms of PLA_2-I.

MATERIALS AND METHODS

Preparation of pro-PLA_2-I and mature PLA_2-I

Porcine mature PLA_2-I and pro-PLA_2-I were isolated from the pancreas homogenate according to the method of Nieuwenhuizen et al (17).

Assay of PLA_2 activity

The substrate for the PLA_2 assay was 1-palmitoyl-2-[1-^{14}C]linoleoyl-phosphatidylethanolamine (Amersham). PLA_2 activity was measured by the method described previously (18). PLA_2 activity was expressed as an amount of PLA_2, calculated from a calibration curve using authentic specimens of PLA_2-I.

Binding experiments

Binding of 1 nM ^{125}I-PLA_2-I to rat vascular smooth muscle cells grown in 35-mm dishes in the presence of plasmin-treated PLA_2-I were examined as described by Arita et al. (2).

Treatment of pro-PLA_2-I with plasmin and separation of the products

Porcine pro-PLA_2-I (100 μg/ml) was incubated with several concentrations of plasmin for an indicated period. The reaction buffer was 50 mM Tris-HCl, pH 7.4. The reaction was terminated by addition of the same volume of 2 mM diisopropyl fluorophosphate. The products were separated by a reverse phase-HPLC column (Cosmosil, 5C18, 4.6 x 150 mm, Nacarai Tesque, Japan) with a gradient of acetonitrile in 0.1% trifluoroacetic acid.

Analysis of amino acid sequence

Automated Edman degradation was performed with a Model 477A Applied Biosystems gas phase sequencer equipped with a 120A PTH amino-acid analyzer.

RESULTS AND DISCUSSION

We first examined the effect of several proteolytic enzymes on the activation of pro-PLA_2-I. Porcine pro-PLA_2-I (10 μg/ml) was incubated for 1 hour at 37°C with plasmin (0.1 unit/ml), urokinase (10 μg/ml), kallikrein (10 μg/ml), factor Xa (0.1 unit/ml), thrombin (10 μg/ml), cathepsin G (10 μg/ml), elastase (10 μg/ml) or trypsin (10 μg/ml). Trypsin-treated and plasmin-treated pro-PLA_2-I showed similar PLA_2 activity, and the PLA_2 activity of the thrombin-treated pro-PLA_2-I was approximately 5% of their activity(data not shown). The

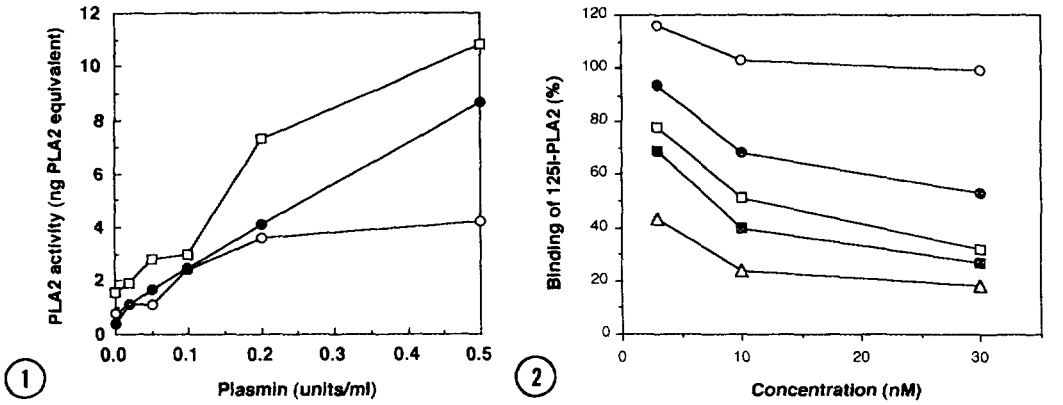

Figure 1. Activation of pro-PLA2-I by plasmin. Porcine pro-PLA2-I was incubated with various concentrations of plasmin for 0.5 hour (open circles), 1 hour (closed circles) or 3 hours (open squares). Plasmin-treated samples (20 ng pro-PLA2-I/assay) were assayed for PLA2 activity.

Figure 2. Inhibition of ^{125}I-PLA2-I binding by plasmin-treated pro-PLA2-I. Porcine pro-PLA2-I was incubated with 0.5 unit/ml plasmin for 0 hour (open circles), 0.5 hour (closed circles), 1 hour (open squares) or 3 hours (closed squares). Binding of ^{125}I-PLA2-I to rat vascular smooth muscle cells were measured in the presence or various concentrations of plasmin-treated pro-PLA2-I or mature PLA2-I (open triangles).

other enzymes did not significantly activate pro-PLA2-I. This finding is supported by the fact that substrate specificity of plasmin and that of trypsin are very similar, and like trypsin and plasmin, thrombin cleaves the carboxyl side of arginine or lysine, although the substrate specificity of thrombin is more strict than that of trypsin or plasmin, which probably depends on the tertiary structure of the substrate proteins. As shown in Fig. 1, activation of pro-PLA2-I was dependent on the plasmin concentration and incubation time.

Figure 2 shows the effects of plasmin-treated pro-PLA2-I on the binding of ^{125}I-PLA2-I to cultured rat smooth muscle cells. Both plasmin-treated pro-PLA2-I and mature PLA2-I inhibited the binding of ^{125}I-PLA2-I, and the potency of inhibition was dependent on the concentration of plasmin.

To analyze the molecular species of plasmin-treated PLA2-I, we separated plasmin-treated pro-PLA2-I with reverse phase HPLC. Peak C in Fig. 3 corresponds to pro-PLA2-I and peak B corresponds to mature PLA2-I, which is a major product of proteolysis of pro-PLA2-I by trypsin. Plasmin digested pro-PLA2-I and produced mature PLA2-I in a dose-dependent manner. However, plasmin-digestion produced another peak designated as peak A. As shown in Fig. 3, peak A had approximately the same PLA2 activity as mature PLA2-I (peak B) while peak C (pro-PLA2-I) did not have PLA2 activity. Figure 4 shows that both peak A and peak B inhibited the specific binding of ^{125}I-PLA2-I to the receptor with the same potency as authentic mature PLA2-I. Peak C did not inhibit the binding (data not shown).

Amino acid sequence analysis confirmed that peak B contained the mature form of PLA2-I (data not shown). Sequence analysis of peak A gave the sequence Asn-Leu-Asp-Thr-

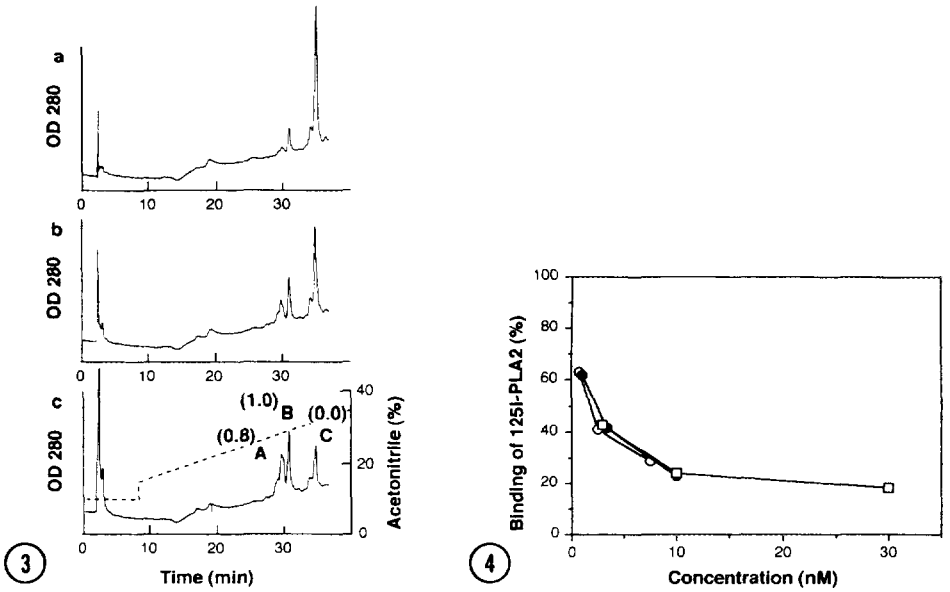

Figure 3. Separation of plasmin-treated pro-PLA2-I by HPLC. Porcine pro-PLA2-I was incubated with 0.02 unit/ml plasmin (a), 0.1 unit/ml plasmin (b) or 0.5 unit/ml plasmin (c) for 3 hours. The samples (50 μg each) were separated by reverse phase HPLC. Peaks A, B and C were collected, dried and dissolved in phosphate buffered saline. Values in parentheses are relative PLA2 activity of each peak.

Figure 4. Inhibition of ^{125}I-PLA2-I binding by the fractions separated by HPLC. Peaks A and B in Fig. 3 were collected and effect of peak A (open circles), peak B (closed circles) or mature PLA2-I (open squares) on ^{125}I-PLA2-I binding to rat vascular smooth muscle cells was analyzed. The PLA2 concentrations in peaks A and B were determined from their PLA2 activity.

Lys-Lys-Tyr in addition to the sequence Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile, which is the N-terminal sequence of mature PLA2-I. The former sequence corresponded to the sequence beginning from Asn^{124}. Since Cys at the C-terminal is linked to Cys^{34} through a disulfide bond (19), we concluded that the major peptide in peak A was Product #1 shown in Fig. 5. Moreover, as a minor component (less than 20% of Product #1), analysis of peak A also gave the sequence Lys-Tyr, which corresponded to the sequence from Lys^{129}. Therefore, peak A was also suggested to contain Product #2 in Fig. 5. As shown in Figs. 3 and 4, the products in peak A, which were modified in the C-terminal region, had enzyme activity and binding activity to the PLA2 receptor, suggesting that the C-terminal region is not important for those activities.

We have shown that plasmin digests pro-PLA2-I and converts pro-PLA2-I into physiologically active, receptor binding forms, which may act as inflammatory mediators. The activity of group II PLA2 in tissues has been suggested to be regulated at the level of synthesis. Inflammatory cytokines up-regulate the synthesis of group II PLA2 and anti-inflammatory glucocorticoids down-regulate it (8-11). On the other hand, as suggested in this report, the activity of PLA2-I may be regulated at the level of processing after secretion. The concentration of pro-PLA2-I in the plasma of healthy volunteers has been reported to vary

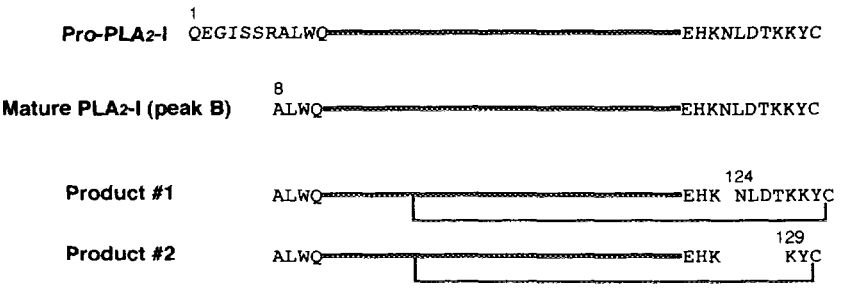

Figure 5. Structures of the products of plasmin digestion of pro-PLA2-I. Solid line represents amino acid sequence between Gln^{11} and Glu^{121}. Thin lines indicate disulfide bonds between Cys^{34} and Cys^{131}.

from 0.1 to 0.3 nM, while the mature form of PLA2-I is not detected in the plasma(20). Therefore, if plasmin is activated in a certain tissue, it may produce the active forms of PLA2-I there and exert enzyme activity and receptor activating activity.

Plasmin is formed by the actions of tissue-type and urokinase-type plasminogen activators which catalyze the conversion of the inactive proenzyme plasminogen to plasmin. The formation of plasmin is negatively regulated by plasminogen activator inhibitor type 1, that binds and inactivates those plasminogen activators (21). Since plasmin degrades extracellular matrix components as well as fibrin, activation of plasmin by plasmin-generating molecules, which assemble on cell surfaces, may promote the processes associated with cell migration (22). These processes include inflammatory response (23), atherosclerosis (24), tumor cell invasion (25), ovulation and implantation (26), and embryogenesis (27). Therefore, our finding suggests that PLA2-I play some role in these processes through its enzyme activity and/or receptor mediated activities, such as cell growth, chemotaxis and prostaglandin formation.

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