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PHOSPHOLIPIDS ACTIVATE CATHEPSIN D

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SUMMARY: Total lipids as well as phospholipids extracted from the mitochondrial-lysosomal fraction of porcine adrenal cortex activated the lysosomal cathepsin D of this tissue 30- and 40-fold, respectively, with bovine serum albumin as the substrate. Phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol and cardiolipin were found to activate greatly the cathepsin D. The degree of activation ranged from 6-fold by phosphatidyl ethanolamine to 40-fold by cardiolipin at 1 mM, respectively. These results strongly point to the importance of phospholipids in intracellular protein degradation by lysosomal cathepsin D.

In our study of possible activators of the lysosomal cathepsin D responsible for intracellular protein degradation, we reported previously in these Communications that polyphosphates activate bovine spleen cathepsin D (1). The polyphosphates studied were sodium tripolyphosphate, nucleotides and their analogues, and all were water-soluble in nature.

In the meantime, we found that porcine adrenocortical lysosomes contain a remarkably high activity of cathepsin D which is 6-fold that in porcine liver and 10-fold that in rat liver (2). We recovered this particular enzyme in several multiple forms, and purified the major ones to homogeneity (3).

Because the adrenal cortex is very active in the endocytosis of low-density lipoproteins (4) as well as in the autophagy of mitochondria (5,6), we conceived of the possibility that some of the lipids derived from these materials might serve as activators of the lysosomal cathepsin D. Using one of the major adrenocortical cathepsin D forms, we found first, that its activity is greatly activated by the total lipids and even more so by the phospholipids of the mitochondrial-lysosomal fraction of porcine adrenal cortex, and second, that these effects may be simulated by the authentic phospholipids listed in the Summary.

MATERIALS AND METHODS

Materials: Fresh porcine adrenals were obtained from a local slaughterhouse and processed as described previously (2). The cathepsin D preparation used in

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the present study was porcine adrenocortical "cathepsin D_5" purified to homogeneity as described previously (3). This particular cathepsin D is one of the major forms of the lysosomal cathepsin D in porcine adrenal cortex and has been so termed for the purpose of identification (3). The specific activity was 17.3 U/mg protein as assayed with acid-denatured bovine hemoglobin at pH 3.6 using the fluorescamine method (1).

Bovine serum albumin (Fraction V), ovalbumin, human serum albumin, synthetic DL-α-phosphatidic acid (dipalmitoyl), egg yolk L-α-phosphatidyl choline, synthetic L-α-phosphatidyl ethanolamine (dipalmitoyl), soybean phosphatidyl inositol, synthetic L-α-phosphatidyl glycerol (dipalmitoyl), bovine brain phosphatidyl serine, bovine heart cardiolipin, and bovine brain sphingomyelin were purchased from Sigma Chemical Company, St. Louis, Mo. The molecular weights of the phospholipids of biological origins were calculated by assuming the presence of ester bonds with stearic acid and of the sodium salt.

Subcellular fractions: Differential centrifugation was performed to obtain nuclear, mitochondrial-lysosomal, microsomal and cytosol fractions of the porcine adrenal cortex as described previously (2). The quality of the subcellular fractions was monitored by measuring the distribution profiles of marker enzymes (2). No significant discrepancies were noted in their distribution profiles when compared to the previous results (2).

Extraction of total lipids and phospholipids: The total lipids from the four subcellular fractions of the porcine adrenal cortex were prepared by the method of Bligh and Dyer (7). The phospholipids in the total lipids of the mitochondrial-lysosomal fraction were precipitated by an excess of acetone added to the solution of total lipids in a minimal amount of chloroform. The recovery of the phospholipids from the total lipids was 88% when lipid phosphate was determined.

Lipid suspensions: Phosphatidyl choline was first dissolved in a small amount of methanol-chloroform mixture (2 : 1, v/v), then evaporated down to dryness and suspended in 50 mM sodium acetate buffer, pH 4.6.

To prepare the suspensions of phospholipids other than phosphatidyl choline, it was necessary to add phosphatidyl choline at a molar concentration 9-fold that of the respective phospholipid. The mixture of the desired phospholipid and phosphatidyl choline was made up in methanol-chloroform mixture and processed as described above for preparing the suspension of phosphatidyl choline.

Total lipids and phospholipids from porcine adrenocortical subcellular fractions were suspended in an appropriate volume of 50 mM sodium acetate buffer, pH 4.6, respectively.

Enzyme assays: Samples for the routine cathepsin D assay in the present study contained in a final volume of 100 µl the following components: 50 mM sodium acetate buffer, pH 4.6, 0.5 µg cathepsin D_5, 0.1 mg susbstrate protein and appropriate amounts of the compounds to be tested. The reaction was carried out at 37°C for 20 minutes or less to obtain linear time-courses. The incubation was halted by the addition of 100 µl of 6% trichloroacetic acid. Degradation products were determined by the fluorescamine method (1).

When acid-denatured bovine hemoglobin was used as the substrate to study the effects of lipids, the cathepsin D_5 concentration was reduced to 0.05 µg/tube to obtain a proteolytic rate comparable to that observed with other proteins.

That the total lipids and the phospholipids from porcine adrenocortical subcellular fractions as well as the authentic phospholipids mentioned above were essentially free of proteins was carefully checked by running control incubations in which the lipid suspensions were incubated with cathepsin D_5

but without any other protein substrates. No detectable contamination of these lipids by degradable proteins was found by the fluorescamine method (1).

In other control incubation, the lipid preparations were incubated with bovine serum albumin at various pH values between 2.5 and 6.0 to detect any protease activity. No such activity was detected by the fluorescamine method (1).

Other methods: Protein was determined by the method of Lowry et al.(8) using bovine serum albumin as the standard and lipid phosphate by the method of Fiske and Subbarow (9) after digesting lipid materials with a mixture of sulfuric acid and nitric acid.

RESULTS AND DISCUSSION

Subcellular distribution of cathepsin D activating lipids: We first tested the four preparations of the total lipids from nuclear, mitochondrial-lysosomal, microsomal and cytosol fractions of porcine adrenal cortex for their possible effect on cathepsin D (TABLE I). The total lipids were all effective in activating cathepsin D with bovine serum albumin as the substrate. In terms of the tissue wet weight, the activating effect was found mainly in the mitochondrial-lysosomal fraction.

Kinetic properties of activation by total lipids: The maximal activation by the total lipids from the mitochondrial-lysosomal fraction was found to be about 30

TABLE I. Subcellular distribution of the total lipids and their cathepsin D activating effect.

Fractions	Protein (mg/g)	Total lipids (mg/g)	Cathepsin D activating effect (pmol/min)
Nuclear	48.0 ± 4.3	19.2 ± 1.9	144 ± 18
Mitochondrial-lysosomal	29.5 ± 3.6	22.7 ± 5.4	192 ± 5
Microsomal	16.7 ± 2.0	17.5 ± 2.2	89 ± 23
Cytosol	28.7 ± 1.7	7.9 ± 3.1	12 ± 7

The cathepsin D activating effect of the total lipids from the subcellular fractions was expressed as that enzyme activity which was obtained by subtracting the cathepsin D activity without the total lipids added (25 pmol/min) from the activity with the total lipids added. The enzyme activity was expressed in terms of the initial reaction rate (pmol/min) under the incubation conditions described in the Enzyme assay of the Methods. Amounts of the total lipids from the subcellular fractions were equivalent to 50 μg wet tissue. Values are means ± standard deviation of the mean of 3 experiments. Recoveries of protein, total lipids and cathepsin D activating effect were 101, 102 and 94% , respectively, as compared with those in the whole homogenate.

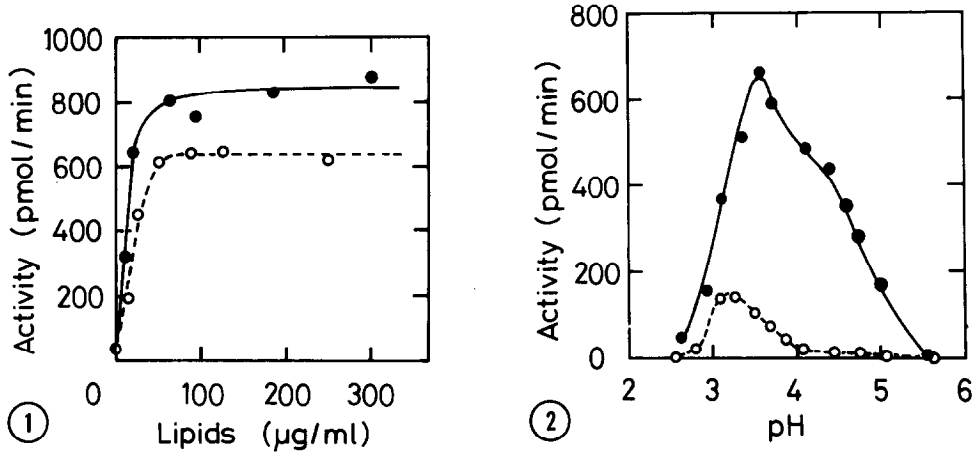

Figure 1. Activation of porcine adrenocortical cathepsin D by varying concentrations of total lipids (o) and phospholipids (•) from the mitochondrial-lysosomal fraction of porcine adrenal cortex. The abscissa indicates the amount of the lipids in terms of the dry weight per milliliter. In terms of the lipid phosphate, 100 μg/ml of the total lipids and of the phospholipids represented a concentration of 0.091 and 0.115 mM, respectively.

Figure 2. pH-Activity profiles of porcine adrenocortical cathepsin D with bovine serum albumin as the substrate in the presence (•) and absence (o) of the total lipids from the mitochondrial-lysosomal fraction of porcine adrenal cortex. To adjust pH-values, 50 mM sodium lactate buffer was used for the pH-range between 2.5 and 4 and 50 mM sodium acetate buffer for that between 4 and 5.5. For this particular experiment, the amount of cathepsin D was reduced to 0.2 μg/tube.

fold higher than the control when 300 μg (dry weight) per milliliter was added to the assay mixture (Figure 1). When bovine serum albumin was used as the substrate, the activation was clearly observed between pH 2.5 and pH 5 (Figure 2). A lesser activation by the total lipids was also observed with ovalbumin, human serum albumin and yeast cytochrome c at pH 4.6, but only 2 to 3 fold the control (data not shown). With acid-denatured bovine hemoglobin, the activation was almost negligible as compared to that seen with bovine serum albumin.

Effects of phospholipids from total lipids: Then we tested whether or not the phospholipids contained in the total lipids of the mitochondrial-lysosomal fraction activate cathepsin D. The phospholipids were in fact very active, and the maximal activating effect was found to be 40-fold at 300 μg (dry weight) per milliliter or at 0.3 mM in terms of the lipid phosphate. The results are plotted in Figure 1 for convenient comparison with those for the total lipids.

TABLE II. Effects of phospholipids on porcine adrenocortical cathepsin D activity against bovine serum albumin.

Compounds tested	Initial reaction rate (pmol/min) with compounds at concentrations shown below	
	0.1 mM	1.0 mM
None	24 (1.0)	
Tristearin	29 (1.2)	45 (1.9)
Phosphatidic acid	206 (8.6)	476 (19.9)
Phosphatidyl ethanolamine	29 (1.2)	132 (5.5)
Phosphatidyl inositol	165 (6.9)	205 (8.6)
Phosphatidyl glycerol	257 (10.7)	299 (12.5)
Phosphatidyl serine	302 (12.6)	515 (21.5)
Cardiolipin	711 (29.7)	983 (41.1)
Sphingomyelin	26 (1.1)	36 (1.5)
Sodium tripolyphosphate	850 (35.5)	

Compounds other than sodium tripolyphosphate were suspended with the aid of phosphatidyl choline added at a molar concentration of 9 : 1 for each concentration of 0.1 and 1.0 mM. Phosphatidyl choline itself did not exert any effect on the cathepsin D activity at all. Values shown in the parentheses are relative activities, the control value without any compound tested being normalized to 1.0. Sodium tripolyphosphate was used at a concentration of 40 mM.

Effects of authentic phospholipids: We then studied whether or not authentic phospholipids can activate cathepsin D. The results are summarized in TABLE II.

Tristearin was not effective at all. Taking our previous results (1) into consideration, this may be due to the complete absence of phosphate in the molecule. Both phosphatidyl choline and sphingomyelin were ineffective. The reason for this might be that the positive charge of the quarternary ammonium base is just enough to quench the influence of an otherwise effective phosphate anion.

If the above explanation holds true, then the amino group might also exert a similar quenching effect on the phosphate group. Indeed, phosphatidyl ethanolamine showed a far lower activation than did phosphatidyl inositol and phosphatidyl glycerol. In the case of phosphatidyl serine, the possible quenching effect of the amino group may be abolished at least partly by the carboxyl group.

Cardiolipin containing two phosphate groups but no amino group exerted the highest degree of activation (40-fold) at 1 mM. That this much activa-

tion is indeed maximal under the experimental conditions specified is shown by the results with sodium tripolyphosphate.

A few words are necessary to explain the difference in the number of effective phosphate groups between the water-soluble polyphosphates reported previously (1) and the hydrophobic activators described here. In the former compounds, three or more phosphate groups were needed for the highest degree of activation. On the other hand, in the latter, cardiolipin with only two phosphate groups produced an even slightly higher degree of activation than did sodium tripolyphosphate. As a possible explanation, we hypothesize that phospholipids constitute a kind of polyphosphate structure by way of micelle formation.

Details of the kinetics of the present findings remain to be elucidated together with the identification of the particular phospholipid in the porcine adrenal cortex that is responsible for the cathepsin D activation. In any case the present study strongly points to the importance of some lipids, especially of some phospholipids, in intracellular protein degradation by lysosomal cathepsin D. In addition, the present results open new aspects of cathepsin D enzymology.

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