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INACTIVATION OF 1-ALKYL-2-ACETYL-sn-GLYCERO-3-PHOSPHOCHOLINE BY A PLASMA ACETYLHYDROLASE: HIGHER ACTIVITIES IN HYPERTENSIVE RATS

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SUMMARY — We have partially characterized the properties of a specific acetylhydrolase in plasma from spontaneous hypertensive rats. This enzyme inactivates 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (a lipid involved in platelet aggregating, hypotensive, and allergic responses) by removal of the acetate group. The extent of acetate hydrolysis was linear with both time and protein concentration, and the enzyme had an apparent K_m of 2.5 μM and a V_{max} of 2.6 nmol/min/mg protein. As with an intracellular acetylhydrolase previously characterized by us, the plasma activity was not affected by addition of phosphatidylcholine, EDTA, or Ca^{2+}. However, in contrast to the acetylhydrolase activity in the rat kidney soluble fraction, the plasma activity was associated with a higher molecular weight protein resolved on a Sepharose 6B column and the plasma acetylhydrolase was not inhibited by treatment with trypsin, pronase, or subtilisin. We also compared the acetylhydrolase activity in plasma of age-matched spontaneous hypertensive rats and their normotensive controls, and found approximately 20% higher levels of activity in plasma from the hypertensive animals ($P < 0.01$).

The potent biological effects and metabolism of 1-alkyl-2-acetyl-GPC* (PAF) in platelets, neutrophils, basophils, and macrophages, including the cardiovascular activities of this bioactive species of phospholipids have been reviewed (1-3). It is noteworthy that the hypotensive and platelet aggregation responses appear to involve separate mechanisms (2,4,5). Since the initial investigations of 1-alkyl-2-acetyl-GPC (6-8), and the elegant structural proof by Hanahan et al. (9) that 1-alkyl-2-acetyl-GPC occurs naturally in sensitized basophils, it has become clear that acetate or a propionate group at the sn-2 position is crucial for maximum activity [see (10) for comparison of various PAF analogs and additional references]. Enzyme data have also clearly documented that PAF is produced in cells as a native constituent (11-15).

Cellular inactivation of PAF can occur via a specific acetylhydrolase (16) that catalyzes the hydrolysis of the acetate moiety of PAF. This enzyme appears to have properties distinct from intracellular phospholipase A_2 (16); however, phospholipase A_2 from snake venom (12) and undoubtedly tissue sources of A_2 activity can also hydrolyze the acetate group. Properties of the

*Abbreviations: GPC, sn-glycero-3-phosphocholine; PAF, platelet activating factor; SHR, spontaneous hypertensive rats; WKY, normotensive Wistar-Kyoto rats.

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specific intracellular acetylhydrolase for 1-alkyl-2-acetyl-GPC have been described in detail and a similar activity was also detected in rat plasma (16). Farr et al. described an acid-labile factor in human serum that inactivates PAF (17), which they claimed to be associated with lipoproteins and later attributed to an acylhydrolase activity (18). Because of the central role that the plasma acetylhydrolase can play in regulating the levels of circulating PAF in the vascular compartment, we have examined the properties of acetylhydrolase in plasma (and serum) and show that it has similar but not identical properties to that of the intracellular acetylhydrolase described by us earlier. Lability of acetylhydrolase during chromatography has hindered the purification of this enzyme activity (unpublished results).

MATERIALS AND METHODS

Male SHR* and WKY* rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). The SHR rats, 8- to 10-months old, were used in all experiments except when the intracellular form of acetylhydrolase was prepared or when plasma acetylhydrolase activities were compared to rats with normal blood pressures; in the latter experiments the SHR and WKY were 4.5-month-old (age-matched) animals. Blood pressures were measured by the tail-cuff method and monitored with a Model MK IV physiograph from Narco Bio-Systems, Inc. (Houston, TX). Rats were decapitated and their blood collected in heparinized centrifuge tubes. Plasma was prepared and aliquots from above the buffy layer were withdrawn after centrifuging the blood at 450g for 20 min at 4°C. The plasma was then divided into small aliquots and stored frozen in glass vials at -23°C. Only fresh or once-thawed plasma samples were used in all acetylhydrolase assays; the acetylhydrolase activity appeared stable up to at least 10 days after storage at -23°C. The cytosolic fraction from kidney cortex was prepared (16) from young adult male CDF rats from Charles River Breeding Laboratories (Wilmington, MA).

1-Hexadecyl-2-[^{3}H]acetyl-GPC (250 μCi/μmol) and unlabeled 1-hexadecyl-2-acetyl-GPC, 1-palmitoyl-2-acetyl-GPC, and 1-alkyl-2-propionyl-GPC were prepared and purified as previously described (10,16). Phosphatidylcholine (from egg yolk), subtilisin, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Glass-distilled chloroform was from Burdick and Jackson Laboratories (Muskegon, MI), trypsin from Grand Island Biological Co. (Grand Island, NY), and pronase-CB and Azocoll from Calbiochem-Behring Corp. (La Jolla, CA).

In experiments to assess the acid lability of the acetylhydrolase activity, plasma samples were diluted 1:20 with water, placed in an ice bath, adjusted to pH 2.0 or 3.0 by slow addition of 0.1 N HCl at 0° and maintained at these pHs for 5 min, and then readjusted to pH 7.4 with 0.1 N NaOH. The acid treated plasma was then diluted with water to a final 1:40 dilution for the acetylhydrolase assay.

Acetylhydrolase activity was assayed essentially as before (16). Each incubation mixture contained protein (25-50 μl of 1:50 diluted plasma) and 10 μM 1-hexadecyl-2-[^{3}H]acetyl-GPC (spec. act. = 25 μCi/μmol) in a total volume of 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4). Incubations were carried out at 37°C for 5 min and the enzymatic reaction was then terminated by addition of 1 ml chloroform and 0.5 ml of saturated sodium bicarbonate solution. After the initial solvent extraction, the aqueous solution containing the released [^{3}H]acetate was transferred to a separate tube and washed three times with 1 ml of chloroform; radioactivity in the aqueous layer was determined by liquid scintillation spectrometry. All data were corrected by subtracting the radioactivity in the aqueous layer obtained from

corresponding incubations of plasma that had previously been placed in a boiling water bath for 10 min; these boiled enzyme blanks usually contained about 2% of the total substrate radioactivity originally added to the incubations.

Effects of proteases on acetylhydrolase activities in plasma and kidney cortex soluble fraction were studied by pre-incubating the standard assay mixtures, minus 1-hexadecyl-2-[^{3}H]acetyl-GPC, for 15 min at 30°C in the presence or absence (controls) of the proteases. The labeled phospholipid substrate was then added for determination of the acetylhydrolase activity.

Sepharose CL-6B from Pharmacia Fine Chemicals (Piscataway, NJ) was packed into a 1.5 x 30-cm column and the protein fractions from either plasma or the rat kidney cortex soluble fraction were resolved using phosphate buffered saline at a flow rate of 0.2 ml/min. The total amount of protein applied to the column was 75 mg for plasma and 15 mg for kidney cortex soluble fraction. Column fractions were collected in 1-ml increments and assayed for acetylhydrolase activity (16) and protein (O.D. at 280 nm).

RESULTS AND DISCUSSION

Acetylhydrolase activity of rat plasma, with PAF as the substrate at saturating levels, was linear with respect to protein (0-100 μg), plasma volume (up to 1.5 μl), and time (0-8 min). Boiling the plasma for 10 min effectively destroyed the enzyme activity. Investigation of the plasma enzyme activity at various pHs revealed a fairly broad optimum range (6.5-8.0), with a maximum activity occurring at pH 7.4. To rule out the possibility of contaminating platelets as a source of the acetylhydrolase, we compared the acetylhydrolase activity of plasma and found that platelet-poor plasma hydrolyzed 10% more substrate than did the platelet-rich plasma. Furthermore, serum that was completely free of platelets had even a slightly higher acetylhydrolase activity than the plasma from which it was prepared. These data clearly indicate that the acetylhydrolase of rat plasma is not due to a contamination from platelets. Overnight fasting of rats had no effect on the acetylhydrolase activity and storage of frozen plasma samples for as long as 10 days at -23°C demonstrated that the enzyme was relatively stable at this temperature.

As previously noted with the intracellular form of acetylhydrolase (16), the addition of 2 and 10 mM EDTA or calcium ions had essentially no effect on plasma acetylhydrolase activities (Table I). These data and the fact that egg phosphatidylcholine at a level of 9 μM did not affect the enzyme activity indicate that the properties of plasma acetylhydrolase are different from those of a typical cellular phospholipase A_2. Both 1-alkyl-2-propionyl-GPC and 1-palmitoyl-2-acetyl-GPC appeared to serve as substrates as indicated by their ability to compete with the labeled 1-alkyl-2-acetyl-GPC (Table I).

The plasma acetylhydrolase from SHR rats had an apparent K_m of 2.5 μM and a V_{max} of 2.6 nmol/min/mg protein when 1-hexadecyl-2-acetyl-GPC was the substrate. Competitive inhibition kinetics with 1-palmitoyl-2-acetyl-GPC as a substrate (data not shown) gave a calculated K_i of 3.2 $\pm$ 0.8 μM for the acyl

TABLE I

EFFECT OF EDTA, Ca^{2+}, AND PHOSPHOLIPIDS ON 1-ALKYL-2-ACETYL-GPC ACETYLHYDROLASE ACTIVITIES IN RAT PLASMA

Addition	Acetylhydrolase Activity
	(% of Control)
EDTA*	
(2 mM)	94, 89
(10 mM)	108, 108
Ca^{2+}*	
(2 mM)	108, 108
10 mM)	114, 116
Egg phosphatidylcholine**	
(9 μM)	116, 117
(19 μM)	71, 66
1-Palmitoyl-2-acetyl-GPC**	
(9 μM)	15, 23
1-Alkyl-2-propionyl-GPC**	
(9 μM)	15, 16

* Incubated 5 min with 10 μM 1-hexadecyl-2-[^{3}H]acetyl-GPC as substrate; the controls hydrolyzed 0.17 nmol/μl plasma/min.

** Incubated 5 min with 1.0 μM 1-hexadecyl-2-[^{3}H]acetyl-GPC as substrate; the controls hydrolyzed 0.06 nmol/μl plasma/min.

analog. The similarity of the K_m with 1-hexadecyl-2-acetyl-GPC as the substrate and the K_i of the acyl analog suggests that the enzyme effectively removes the sn-2 acetate with substrates having either an alkyl or acyl moiety at the sn-1 position. It is also of interest that the K_m for the PAF acetylhydrolase of rat plasma is very close to that of the K_m previously reported (16) for the intracellular enzyme (K_m = 3.1 μM) in the cytosolic fraction of the kidney cortex from rats. Loss of the acetylhydrolase activity after exposure of plasma to acidic conditions (see Methods) indicates that the enzyme is acid sensitive. After incubating rat plasma at pH 3.0 for 5 min, approximately one-third of the acetylhydrolase activity was destroyed. However, when the enzyme was maintained at a lower pH (2.0) for 5 min, about 60% of the acetylhydrolase activity was lost. These data and those presented above

TABLE II

EFFECT OF PROTEASES ON ACETYLHYDROLASE ACTIVITY IN RAT PLASMA AND THE SOLUBLE FRACTION FROM KIDNEY CORTEX*

Treatment	% of Control Acetylhydrolase Activity	
	Plasma	Kidney Soluble Fraction
Trypsin (1.25 mg)	111;116	0.0;0.0
Pronase (1.0 mg)	117;118	0.0;0.0
Subtilisin (1.0 mg)	118;114	12;12

*Plasma and the soluble fraction from kidney cortex were incubated with the proteases for 15 min at 30°C before addition of 1-hexadecyl-2-[^{3}H]acetyl-GPC for the acetylhydrolase assay. Control activities were 1.5 and 4.3 nmol/min/mg protein for plasma and kidney soluble fraction, respectively.

indicate that the inactivation of PAF by the acid-labile factor previously described by Farr et al. (17) is due, at least in part, to an acetylhydrolase activity specific for short chain acyl moieties at the sn-2 position.

Acetylhydrolase activities of plasma and the soluble fraction of the kidney cortex were eluted from the Sepharose 6B column with a K_{av} of 0.4 and 0.6, respectively; this indicates that the intracellular enzyme has a lower molecular weight than the activity associated with the plasma protein. The K_{av} of the plasma activity peak was similar to that calculated by us from the data others have published for the Sepharose 6B separation of the acid-labile factor in human serum (17). Another distinct difference between the acetylhydrolase activities of plasma and kidney was their susceptibility to inactivation by proteases. As shown in Table II, the acetylhydrolase activity in plasma is not inactivated even by relatively high amounts of three different proteases, whereas the activity in the kidney cortex soluble fraction was completely destroyed by trypsin and pronase and greatly inhibited by subtilisin. The resistance of the plasma acetylhydrolase activity to protease treatment could be due to the presence of a protease inhibitor in plasma. However, this possibility appears unlikely since plasma had no effect on hydrolysis of Azocoll by the same levels of either trypsin or pronase that were added to the incubation mixtures in our experiments.

The potential importance of plasma acetylhydrolase in controlling circulating levels of 1-alkyl-2-acetyl-GPC could be another factor to consider in the regulation of blood pressure. Therefore, we examined plasma acetylhydrolase activities in genetic hypertensive SHR rats and in age-paired (similar

weights) normotensive WKY rats and found the acetylhydrolase activities in the SHR rats averaged 20% higher than in the WKY controls. SHR rats (n = 5) with a mean systolic blood pressure of 205 ± 22 mm Hg had plasma acetylhydrolase activites of 1.25 ± 0.13 nmol/min/10 µl plasma, whereas the control WKY rats (n = 5) with a mean systolic pressure of 126 ± 10 mm Hg had plasma acetylhydrolase activities of 1.02 ± 0.06 nmol/min/10 µl plasma. These results demonstrate that the hypertensive SHR rats have a significantly higher plasma acetylhydrolase activity ($P < 0.01$, based on Student's t test) than the normotensive WKY rats. Thus, further studies would appear warranted to assess the role of acetylhydrolase in the pathogenesis of hypertension.

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