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Detection and Characterization of 45 kDa Platelet Activating Factor Acetylhydrolase in Cerebrospinal Fluid of Children with Meningitis

Tae Chul Moon, Mi Suk Kim, Su Jeong Lee[1], Tae Yoon Lee[1], Soon Hak Kwon[2], Suk Hwan Baek[3], and Hyeun Wook Chang

College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea, [1]Department of Microbiology, College of Medicine, Yeungnam University, Daegu 705-717, Korea, [2]Department of Pediatrics, Kyungpook National University Hospital, Daegu, 700-721, Korea, and [3]Department of Biochemistry and Molecular Biology, College of Medicine, Yeungnam University, Daegu 705-717, Korea

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Platelet activating factor acetylhydrolase (PAF-AH) activity has been identified in cerebrospinal fluid (CSF) samples taken from children with meningitis. We reported that PAF-AH activity is significantly increased, by about 3 fold, in patients with meningitis compared to control subjects. Because of limited knowledge about this enzyme in CSF, we examined the biochemical properties of CSF PAF-AH. PAF-AH of CSF was calcium independent, showed a broad pH spectrum and was relatively heat stable. In addition, this enzyme activity was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF), partially inhibited by *p*-bromophenacylbromide (*p*-BPB), uninhibited by iodoacetamide, and moderately stimulated by dithiothreitol (DTT). PAF-AH of CSF did not degrade phospholipid with a long chain fatty acyl group at *sn*-2 position. This enzyme hydrolyzed PAF and oxidatively modified phosphatidylcholine. Furthermore, we identified a monomeric polypeptide with a molecular weight of approximately 45 kDa by Western blot using human plasma PAF-AH antibody. These results suggested that plasma type PAF-AH activity exist in CSF taken from children with meningitis.

Key words: Platelet activating factor, Cerebrospinal fluid, 45 kDa PAF-acetylhydrolase, Meningitis, Oxidative phospholipid

INTRODUCTION

Platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is one of the most potent lipid mediators that is involved in a variety of physiological events. PAF-acetylhydrolase (PAF-AH) catalyzes the inactivation of PAF, is an enzyme that hydrolyzes the acetyl ester at the *sn*-2 position of PAF and converts it to the inactive metabolite, lyso PAF. This enzyme is widely distributed in mammalian tissues and plasma and is thought to be a defense mechanism to protect the host against the toxic effects of PAF and other biologically active oxidized phospholipids. Higher levels of PAF-AH have been found in plasma and other body fluids in a variety of different diseases (Hirashima *et al.*, 1994; Narahara *et al.*, 1996; Triggiani *et al.*, 1997; Tsuji *et al.*, 1998; Morgan *et al.*, 1999). Arditi *et al.* reported that cerebrospinal fluid (CSF) PAF concentrations are significantly higher in children with *H. influenzae* meningitis than in controls (Arditi *et al.*, 1990). Therefore, regulation of CSF PAF concentration in bacterial meningitis may be important for patient recovery from bacterial infections.

We have recently reported the PAF-AH activity in CSF samples taken from children with a variety of neurological conditions (85 patients; mean age 3.8 years) to determine if it is involved in the defense mechanism against the toxic effect of inflammatory mediators in the central nervous system (Chang *et al.*, 2002). We suggested that a new PAF-AH isozyme exists in the CSF according to its biochemical characteristics. However, our description in this on the protein level of CSF PAF-AH in meningitis was not comprehensive. Therefore, in the present study, we

Correspondence to: Hyeun Wook Chang, College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea
Tel: 82-53-810-2811, Fax: 82-53-811-3871
E-mail: hwchang@yu.ac.kr

studied the biochemical properties of PAF-AH activity and identified a monomeric polypeptide with a molecular weight of approximately 45 kDa by Western blotting using human plasma PAF-AH antibody. This molecular weight is the same that of previously described human plasma acetylhydrolases.

MATERIALS AND METHODS

We evaluated 12 patients, aged newborn to 14 years (mean age 3.8 years), who had a spinal tap for a variety of reasons at the Department of Pediatrics, Kyungpook National University Hospital, Daegu, Korea. 1-*O*-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine ([^{3}H]acetyl-PAF) was purchased from NEN (Boston, MA, USA). Bovine serum albumin, phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, and dithiothreitol (DTT) were purchased from Sigma Chem. Co. (St. Louis, MO, USA)

Assaying of PAF-AH activity

The standard incubation system to assay PAF-AH comprised 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 20 nmole [^{3}H-acetyl] PAF (3,000 cpm/nmole) and 40 μL CSF in a total volume of 250 μL. After 30 min at 37°C the reaction was stopped by adding 2.5 mL of chloroform/methanol (4:1) and 250 μL of water. Aliquots (600 μL) of the upper phase were used for radioactivity measurement to determine the amount of acetate liberated.

Preparation of 1-acyl-2-[1-^{14}C]-linoleoyl-glycerophosphocholine (2-linol-PC)

Acyl-2-[1-^{14}C]-linoleoyl-*sn*-glycero-3-phosphocholine was synthesized as described previously (Arai *et al.*, 1985).

Preparation of oxidized phosphatidylcholine

Oxidized phospholipid was prepared as described previously (Shimojo *et al.*, 1974). Briefly, labeled [2-^{14}C]-linoleoylphosphatidylcholine and unlabeled 2-linoleoyl-phosphatidylcholine were mixed to 3,000 cpm/nmole. Phosphatidylcholine in 1 mL of 90% acetate was mixed with 2 mL of an oxidation solution (24 mM $KMnO_4$, 20 mM $NaIO_4$), and then the mixture was stirred for 2 h at room temperature. The oxidized phosphatidylcholine was separated with a silica gel plate, and the band corresponding to oxidized phosphatidylcholine was scrapped off and extracted with chloroform.

SDS-PAGE/immunoblot analysis

CSF samples (40 μg) were separated by 12% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred in 20% methanol, 25 mM Tris, and 192 mM glycine to a nitrocellulose membrane (Schleicher and Schull, Dassel, Germany). The nitrocellulose membrane was then blocked by incubation in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% nonfat milk. Subsequently, the membrane was incubated with anti-human plasma antiserum (Cayman Chemicals, Ann Arbor, MI, USA) or intracellular PAF-AH α1, α2 and type II antibody (a gift from Professor H. Arai, University of Tokyo, Japan) for 4 h, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham Corp., Newark, NJ, USA).

RESULTS

Factors affecting PAF-AF activity

PAF-AH exhibited pH optima in the weak acid to alkaline region. At values below pH 4 the enzyme was inactivated but at higher pH values, up to 11, the enzyme activity remained (Fig. 1A). In addition, PAF-AH is relatively heat stable (Fig. 1B). It has been reported that the optimal pH of PAF-AH purified from human plasma and rat adipocyte was pH 7.8 (Stafforini *et al.*, 1987; Son *et al.*,

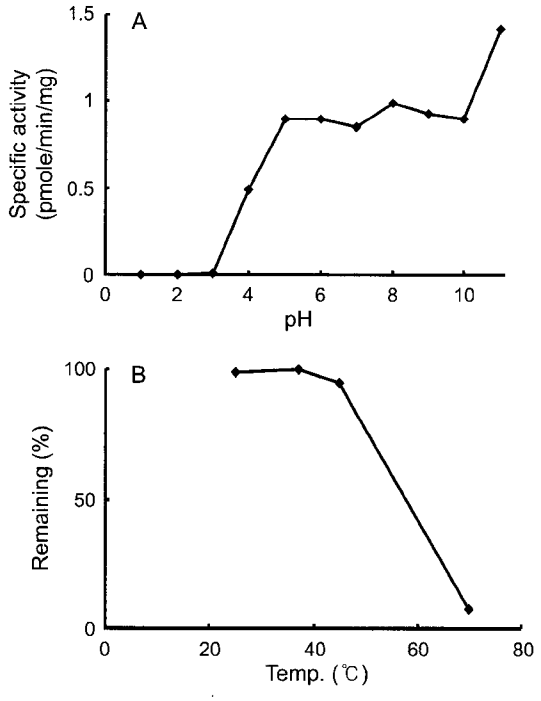

Fig. 1. Effects of pH (A) and temperature (B) on PAF-AH activity. The crude CSF (40 μL) and 20 nmole of [^{3}H-acetyl]PAF were incubated for 30 min at 37°C in the presence of 5 mM EDTA. The buffers used were 100 mM sodium acetate-acetic acid with a range from pH 4 to 5, 100 mM Tris-maleic acid buffer from pH 5 to 6, 100 mM Tris-HCl buffer from pH 6 to 9, and 100 mM glycine-NaOH buffer from pH 9 to 11. The crude CSF (40 μL) was preincubated for 10 min prior to the addition of substrate, after which incubation mixtures were incubated for 30 min. PAF-AH activity was measured as described in MATERIALS AND METHODS.

1997). Next, we examined the heat stability of PAF-AH by preincubating the protein at various temperatures for 15 min and performing assay in the enzyme activity. The activity of this enzyme relatively stable below 40, but over 40 the enzyme activity is gradually decreased (Fig. 1B).

Effects of various chemicals on CSF PAF-AH activity

PMSF inhibited the PAF-AH activity in a dose dependent manner, as shown in Table I. Various compounds were tested for their effects on the activity of PAF-AH (Table I). Both cofactors of phospholipase A_2, Ca^{2+}, and a chelating agent, EDTA had no effect upon the enzyme activity. *p*-BPB, which has been shown to block various phospholipase A_2 activities by derivatizing the histidine residue(s) at their active sites, inhibited the activity of the enzyme by about 60% at 5 mM. Furthermore, PMSF which has been shown to block by derivatizing the serine residue(s) at their active site (Karasawa *et al.*, 1994; Son *et al.*, 1997) inhibited the activity of the enzyme by about 80%. On the other hand, NaF, acting as a phosphatase inhibitor, and DTT, which hydrolyze the disulfide bond and changed the conformation of protein, did not affect enzyme activity. Iodoacetamide, an inhibitor of enzymes that needs free sulfhydryl groups for their activities, could not inhibit the activity of the enzyme. These results suggested that the active site of PAF-AH in might be serine residue(s) and histidine residue(s).

Table I. Effect of various compounds on the activity of CSF and human plasma acetylhydrolase. Each compound was incubated with PAF-AH for 15 min at 37°C and then the enzyme reaction was started by adding the substrate.

Compounds (mM)		CSF PAF-AH (Remaining activity, %)[b]	Human plasma (Remaining activity, %)[b]
$CaCl_2$	10	96.9	97.6
$MgCl_2$	10	94.5	96.4
EDTA	10	98.5	98.7
NaF	10	98.7	98.5
p-BPB			
	1	90	80
	2.5	65	50
	5	40	20
Iodoacetamide			
	1	95	91
	2.5	94	87.2
	5	83.2	83.8
PMSF			
	1	73	57
	5	21	20
	10	0	10
DTT			
	1	96	105
	5	118	110
	10	130	120

[a]Each compound was dissolved in ethanol so that the final concentration of ethanol was 5% (v/v). [b]This value represents the average of duplicated determinations.

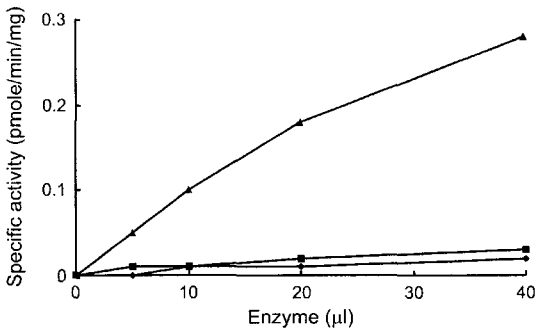

Fig. 2. Hydrolyzing activities of PAF-AH in CSF toward various phospholipids. The crude enzyme was incubated with radio-labeled substrate. The enzyme had little or no activity against phosphatidylcholine (◆) or phosphatidylethanolamine (■) with two long acyl chains. In contrast, PAF-AH could hydrolyze artificially oxidized phosphatidylcholine (▲).

Substrate specificity

The ability of the crude PAF-AH to attack phospholipids other than PAF was investigated (Fig. 2). The enzyme had little or no activity against phosphatidylcholine or phosphatidylethanolamine with two long acyl chains. In

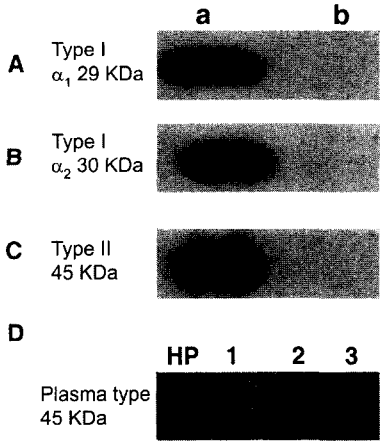

Fig. 3. Identification of PAF-AH protein in CSF. CSF samples (40 μg) were separated by 12% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The immunoblot was then probed with anti-human plasma PAF-AH antibody (1:500) or intracellular PAF-AH α1, α2 and typeantibody at a dilution of 1:1,000. A: Intracellular type 1 subunit mAb; a, mouse testis; b; CSF PAF-AH. B: Intracellular type 2 subunit mAb; a, mouse testis; b, CSF PAF-AH. C: Intracellular type Ab; a, mouse liver; b, CSF PAF-AH. D: Human plasma polyclonal antiserum HP; 30% PAF hydrolytic activity of human plasma PAF-AH; 1, 5% PAF hydrolytic activity of CSF PAF-AH; 2, 20% PAF hydrolytic activity of CSF PAF-AH; 3, 50% PAF hydrolytic activity of CSF PAF-AH. The amounts of all protein used were 10 μg except CSF.

contrast, PAF-AH could hydrolyze phosphatidylcholine that had an oxidatively- fragmented acyl chain at the *sn*-2 position to a degree approximately 40% as efficient a substrate as PAF. This result clearly demonstrates that acetylhydrolase is distinct from phospholipase A_2, which utilizes long chain diacyl phospholipids as substrate.

Identification PAF-AH protein by western blot analysis

CSF samples (40 μg) were separated by 12% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The immunoblot was then probed with anti-human plasma PAF-AH antibody (1:500) or intracellular PAF-AH α1, α2 subunit and type II antibody at a dilution of 1:1,000. Fig. 3A-C shows that CSF PAF-AH did not bind any intracellular type PAF-AH. But, CSF PAF-AH was bound with human plasma antiserum in a manner dependent on PAF hydrolytic activity (Fig. 3D). These results suggested that plasma type PAF-AH activity exists in cerebrospinal fluid (CSF) samples taken from children with meningitis.

DISCUSSION

We have recently reported the PAF-AH activity in CSF samples taken from children with a variety of neurological conditions (Chang *et al.*, 2002). In this follow-up study, we therefore examined the biochemical properties of PAF-AH and what kind of PAF-AH isozyme exists in CSF of patients with meningitis. A series of biochemical and enzymological evaluations revealed that at least two types of PAF-AH exist, namely, the intracellular (cytosolic) and extracellular (plasma) types (Yanoshita *et al.*, 1988). Blank *et al.* reported that the plasma type had a higher molecular weight than the intracellular type and was resistant to protease whereas the intracellular activity was sensitive (Blank *et al.*, 1983). PAF-AH purified from guinea pig peritoneal fluid after *E. coli* lipopolysaccharide administration was resistant to *p*-BPB, NaF and trypsin, like human plasma PAF-AH (Karasawa *et al.*, 1994). PAF-AH purified from bovine brain cytosol, including intracellular type, was inhibited by *p*-BPB or DTT, but not by iodoacetamide, and was essential for the optimal activity needed by a reducing agent (Mitsuharu *et al.*, 1993). Table I summarizes the effect of various chemicals on CSF PAF-AH and human plasma PAF-AH activity. CSF PAF-AH did not hydrolyze the ester bond of phospholipids with a long chain fatty acyl group at the *sn*-2 position (data not shown). CSF PAF-AH is distinguished from phospholipase A_2, which requires a divalent cation, usually Ca^{2+}, and which shows a broad range for fatty acids esterified at the *sn*-2 position. Furthermore, CSF PAF-AH was inhibited by PMSF, as were other purified PAF-AH (Karasawa *et al.*, 1994; Son *et al.*, 1997), indicating that the active center of this enzyme is a serine residue.

It has been reported that the intracellular and plasma types hydrolyze oxidatively modified phospholipid (Karasawa *et al.*, 1994; Matsuzawa *et al.*, 1997; Son *et al.*, 1997). Oxidative degradation of phospholipids results in the formation of biologically active products (Itabe *et al.*, 1988), and these phospholipids are involved in some pathological states including disorganization of membrane structure. CSF PAF-AH dose-dependently hydrolyzed artificially oxidized phosphatidylcholine as a substrate. It is suggested that PAF-AH could hydrolyze the oxidative lipids of biomembrane, lyso derivatives with a long chain fatty acyl group that were reacylated and then the properties of biomembrane could be recovered. Recently, McIntyre *et al.* demonstrated the PAF-like bioactivity of a certain type of oxidatively fragmented phosphatidylcholine, suggesting a pro-inflammatory action of these lipids. It has generally been assumed that (per)oxidized fatty acyl chains in phosphoglyceride may be preferentially hydrolyzed *in vivo*. Phospholipase A_2 has been regarded as a good candidate for the hydrolysis of these chains, since in parallel with phospholipid degradation, the accumulation of lysophospholipids was often observed after oxidative tissue damage (Bazen *et al.*, 1970; Chien *et al.*, 1978; Edgar *et al.*, 1982; Glende *et al.*, 1986). However, the biochemical properties of the enzyme(s) that scavenge (per)oxidized phospholipids remain uncertain. Intracellular PAF-AH, which is constitutively active and selectively hydrolyzes oxidized phospholipids, may participate in the removal of such toxic phospholipids.

We identified a monomeric polypeptide with a molecular weight of approximately 45 kDa on 12% reducing SDS-PAGE (Fig. 3D), which is similar that of other known PAF-AH. Stafforini *et al.* purified human plasma PAF-AH and determined its molecular weight to be 43 kDa (Stafforini *et al.*, 1987). This group also purified human erythrocyte PAF-AH with a molecular weight of 25 kDa (Stafforini *et al.*, 1993). Karasawa *et al.* purified acetylhydrolase of guinea pig peritoneal fluid with a molecular weight of 63 kDa (Karasawa *et al.*, 1994). Hattori *et al.* reported the purification of PAF-AH from bovine brain cytosol, and demonstrated that the native enzyme consisted of subunits with molecular weights of 45 (β), 30 (α1) and 29 kDa (α2) (Hattori *et al.*, 1993). Fig. 3 shows that CSF PAF-AH did not bind any intracellular human PAF-AH mAb. In this experiment, we used mouse testis or mouse liver as positive control for intracellular PAF-AH type I α1, α2 subunit and intracellular type II enzyme (40 kDa), respectively. Fig. 3D shows that CSF PAF-AH bound human plasma type polyclonal antiserum in a dependent manner on PAF hydrolytic activity. Considering the fact that the blood-brain barrier is damaged by meningitis, the increase in CSF

PAF-AH activity seems to be due to overflow of the peripheral circulation into the intrathecal space. The results suggested that CSF PAF-AH from children with meningitis may be the plasma type. The function of CSF PAF-AH remains unknown. Further study will be require to elucidate the physiological role of CSF PAF-AH in neurological diseases.

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