cDNA Cloning and Expression of Biologically Active Platelet Activating Factor-Acetylhydrolase (PAF-AH) from Bovine Mammary Gland

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Platelet activating factor (PAF)-acetylhydrolase (PAF-AH) is an enzyme that hydrolyzes the acetyl ester at the sn-2 position of PAF, and converts it to the inactive metabolite, lypo-PAF. This enzyme is distributed widely in the intracellular as well as the extracellular matrix and is believed to be a defense mechanism that protects the host against the toxic effects of PAF and other biologically active oxidized phospholipids. Purification and expression of cDNA cloning of the intracellular and extracellular types of PAF-AH from several sources from different species have been reported. In this study, the cDNA for PAF-AH was cloned by reverse transcription (RT)-PCR from total RNA of bovine mammary gland. The complete amino acid sequences from the cDNA contains 444 amino acids and was identical to that of the PAF-AH isolated from the bovine spleen cDNA library except for two mismatches of amino acid residues (Thr-247 to Met and Ile-431 to Thr). Recombinant PAF-AH was expressed in HEK 293 cells, which exhibited enzyme activity in the in vitro assay system. Furthermore, recombinant bovine PAF-AH was identified by western blot using human plasma PAF-AH antibody as a monomeric polypeptide with a molecular weight of approximately 43 kDa. This protein can be applied to in vivo models to test its protective role against the deleterious PAF actions.

Key words platelet activating factor (PAF)-acetylhydrolase; cloning; bovine mammary gland; HEK 293 cell; expression

Platelet activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is one of the most potent lipid mediator involved in a variety physiological events. PAF, which is synthesized by a variety of stimulated cells, acts through the cell surface receptors although intracellular PAF accumulation might also influence the cell function. The synthesis and degradation of PAF is tightly regulated to prevent high concentrations of PAF observed in many diseases. For example, high levels of PAF were detected in the serum of septic patients. The plasma PAF levels also increase in the human plasma during acute asthma attacks. The pathologic events of PAF have also been demonstrated in PAF receptor-knockout and -transgenic mice, suggesting that PAF is a critical mediator of inflammation. Recombinant plasma PAF-AH had a marked effect in blocking the late-phase pulmonary inflammation. Recombinant plasma PAF-AH also decreased the mortality in an active anaphylactic shock model.

Previously, we found that bovine Colostrums contained high levels of plasma type PAF-AH activity (Chang et al. personal communication). Therefore, bovine mammary gland was used as a source to produce PAF-AH found in the Colostrums. In this study, the cDNA of the PAF-AH from the bovine mammary gland was cloned and expressed in HEK293 cells. The recombinant PAF-AH expressed was identified by western blot using human plasma PAF-AH antibody as a monomeric polypeptide with a molecular weight of approximately 43 kDa and this enzyme strongly hydrolyzed against PAF in vitro assay system. These results suggest that plasma type PAF-AH activity may exist in bovine mammary gland.

MATERIALS AND METHODS

Reverse Transcription (RT)-PCR Bovine mammary gland was obtained from a local slaughterhouse and pulverized in liquid nitrogen. One gram of frozen tissue was homogenized in TRIZol (Invitrogen, Carlsbad, CA, USA.) to isolate total RNA according to the manufacturer’s instructions. First strand cDNA was produced by reverse transcription using RNA PCR kit (Takara, Kyoto, Japan). In brief, one microgram of total RNA was added to the PCR mixture containing MgCl2 (5 mM), Taq DNA polymerase (Takara) and primers in the RNA PCR buffer. The primers were derived from previously deposited nucleic acid sequences.

Reverse Transcription (RT)-PCR was carried out at 42 °C for 30 min. The PCR reaction was carried out using the first strand cDNA, Taq DNA polymerase (Takara) and two intracellular forms, PAF-AH Ib and PAF-AH II. Plasma type PAF-AH is a monomeric polypeptide with a molecular weight of approximately 45 kDa, which is usually associated with the plasma lipoproteins. The intracellular PAF-AH type Ib is a heterotrimer complex composed of three different subunits whereas PAF-AH type II is a monomeric protein that exhibits broad substrate specificity than PAF-AH Ib. These enzymes show different biochemical properties and molecular structures. According to the specificity and the chemical nature of the substrate molecules, PAF-AH has been classified as a group VII phospholipase A2.

There are a number of studies in which an abnormal PAF-AH activity is associated with diseases including asthma, sepsis, and ischemic stroke. Recombinant PAF-AHs have been used in various in vitro and in vivo models to test for their clinical benefits of inflammation. Pretreatment of the animals with recombinant plasma PAF-AH has been shown to block the PAF-induced acute inflammation. In a murine model of allergen-induced asthma, the administration of recombinant plasma PAF-AH had a marked effect in blocking the late-phase pulmonary inflammation. Recombinant plasma PAF-AH also decreased the mortality in an active anaphylactic shock model.

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acid sequence (Accession number NM_174578; 5’-ACG-GTACCCAGCTCAGCTGGAGATG-3’ (KpnI site underlined) and 5’-GCCAGCTGCTTATTAATCTAAATTG-GTC-3’ (XbaI site underlined). A 30-cycle program with each cycle comprising 94 °C (1 min), 58 °C (1 min), and 72 °C (2 min) was carried out in a thermocycler (GeneAmp PCR System 2400, Perkin-Elmer-Cetus, Norwalk, CT). Obtained PCR product was separated by agarose gel electrophoresis.

Cloning and Sequence Determination of Bovine Plasma Type PAF-Acetylhydrolase (PAF-AH) Gene The PCR product was digested with KpnI and XbaI and subcloned into pCR3.1 vector (Invitrogen). After cloning, both strands of nucleotide sequence were determined using Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 (Applied Biosystems, Foster City, CA, U.S.A.). The PAF-AH gene cloned into pCR3.1 was named pCR3.1/PAF-AH.

Stable Transfection of HEK293 Cells with the Recombinant PAF-AH HEK293 cells were cultured grown at 37 °C, 5% CO₂ in RPMI 1640 (Invitrogen) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 0.1 mM nonessential amino acids, and 10% heat-inactivated fetal calf serum (HyClone, Logan, UT, U.S.A.). The pCR3.1/PAF-AH or pCR3.1, the vector alone as a control, was transfected to HEK293 cells by the LipofectAMINE Reagent (Invitrogen) according to the manufacturer’s instructions. To establish the stable cell line expressing PAF-AH protein, HEK293 cells were transfected with recombinant plasmid, pCR3.1/PAF-AH and moved 48 h later into 96-well plates by limiting dilution of cells in selection medium containing 500 µg/ml G418 (Invitrogen). Selection media were changed every 2–3 d to form the colony. After 3 weeks, wells containing a single colony were chosen and expanded up to T75-flask. The established stable cell line was named HEK293/PAF-AH.

Assay of PAF-AH Activity HEK293/PAF-AH cells were seeded (0.7×10⁶ cells/ml) and cultured for 3 d in protein-free medium (JBI, Daegu, Korea). The activity of PAF-AH from culture supernatants was measured as described previously.22) In brief, the standard incubation system for the assaying of PAF acetylhydrolase comprised 50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 µM of [3H-acetyl] PAF (Du Pont-New England Nuclear.), and the sample 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.

SDS-PAGE and Western for PAF-AH Expression Analysis Cells were treated with a lysis buffer consisting of 1% Triton X-100, 1 mM EDTA, 350 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulfonylfluoride (PMSF). The lysate was centrifuged at 15000 rpm for 20 min to sediment insoluble fractions. Protein concentration was quantified by use of the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL, U.S.A.) using bovine serum albumin as standard. After measuring the protein concentration, 20 µg of protein was separated by 12% reducing SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). The membrane was blocked in Tris–buffered saline (pH 7.4) containing 5% non-fat dried milk powder for 2 h at room temperature and probed with anti-human plasma PAF-AH polyclonal antibody (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) at 4 °C overnight with gentle shaking. After washed with TBST, the membrane was incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (Bio-Rad, Hercules, CA, U.S.A.) for 1 h. Finally, the membranes were washed and developed using an ECL system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

RESULTS

Cloning of cDNA Encoding PAF-AH from Bovine Mammary Gland In order to obtain the PAF-AH cDNA from the bovine mammary gland, the total RNA was isolated and PCR was performed using the cDNA from the bovine mammary gland total RNA as a template. Gene specific primers were designed from the mature bovine plasma type PAF-AH gene. Approximately 1.3 kb of the amplified PCR products were identified (Fig. 1). The PAF-AH cDNA gene obtained by RT-PCR was cloned into pCR3.1. Figure 2 shows the cDNA sequence and predicted amino acid sequences of the bovine PAF-AH. The cDNA contains an open reading frame of 1332 bp and encodes for 444 amino acids with an expected molecular mass of approximately 43 kDa. This nucleotide sequence had four mismatched sequences when compared with those previously deposited in the GenBank/EMBL databank with accession number NM_174578, of which two amino acid sequences (Thr-247 to Met and Ile-431 to Thr) had been changed. Otherwise, the remainders of the nucleotide and amino acid sequences were identical to those of the bovine PFA-AH including a typical signal sequence and a serine esterase consensus motif GXSGXG.

The Expression of the Biological Active Recombinant PAF-AH in the HEK293 Cells In order to examine the functions of bovine PAF-AH, purified cDNA was cloned into a mammalian expression vector, pCR3.1. The HEK293 cells transfected with either pCR3.1/PAF-AH or pCR3.1 only were cultured in a protein-free medium. Because plasma PAF-AH is a secretory protein, the culture supernatant was used to measure the enzyme activity. No PAF-AH activity was detected in the HEK293 cells only and HEK293 cells transfected with pCR3.1 alone, as shown in Fig. 3. However, 20% of PAF-AH activity was observed in the supernatant of the HEK293 cells transfected with pCR3.1/PAF-AH (Fig. 3). Western blotting was performed to examine the PAF-AH protein expression level in the culture supernatant of the PAF-AH/HEK293 cells using an anti-human PAF-AH polyclonal antisera.
antibody. As shown in Fig. 4, a single protein band with a molecular mass of 43 kDa was detected in the culture supernatants of the P AF-AH/HEK293 cells. No P AF-AH protein was detected in the supernatant of either the HEK293 cell alone or the pCR3.1/HEK293 cell culture as expected. Human plasma P AF-AH was used as the positive control. These results showed that the biological active bovine P AF-AH was successfully expressed in the HEK293 cells.

DISCUSSION

PAF has diverse physiological and pathological functions, as demonstrated by numerous studies in animal models and humans. One approach to block the PAF-induced inflammatory diseases is to use PAF-receptor antagonists. Numerous PAF-receptor antagonists have been developed and showed antibody. As shown in Fig. 4, a single protein band with a molecular mass of 43 kDa was detected in the culture supernatants of the P AF-AH/HEK293 cells. No P AF-AH protein was detected in the supernatant of either the HEK293 cell alone or the pCR3.1/HEK293 cell culture as expected. Human plasma P AF-AH was used as the positive control. These results showed that the biological active bovine PAF-AH was successfully expressed in the HEK293 cells.

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Fig. 2. Nucleotide and Deduced Amino Acid Sequences of the cDNA Encoding Bovine PAF-AH Gene

Different nucleotide and amino acid sequences from those of deposited bovine plasma PAF-AH are underlined and circled, respectively. The start codon (ATG) and stop codon (TAA) are bolded. The predicted amino acid sequence is shown by the single-letter amino acid code under the nucleotide sequence. The amino acid sequences for enzyme active sites are marked by boxes.

Fig. 3. PAF-AH Activity from the Supernatants of HEK293 Cells

Cells transfected with pCR3.1 vector alone (pCR3.1/HEK293) or recombinant vector containing PAF-AH gene (PAF-AH/HEK293) were cultured for 3 d as described in Materials and Methods. The PAF-AH protein activity in the culture supernatant of HEK293 cells, pCR3.1/HEK293 or PAF-AH/HEK293 transfectant was measured.
some effects. However, many of the PAF receptor antagonists showed disappointing results in the clinic because a number of adverse effects were associated with the antagonists. In contrast, the plasma form of PAF-AH has been reported to regulate the circulating PAF levels and is essential in resolving inflammation. Animal studies using recombinant PAF-AH have shown that a pretreatment with the recombinant PAF-AH blocked PAF-induced edema, prevent asthma-related symptoms, and protect animals from septicemia. These data indicate that plasma PAF-AH is expected to lack the detrimental effects of the PAF receptor antagonists.

In the present study, cDNA of bovine plasma type PAF-AH from the mammary gland was cloned and the recombinant protein was expressed in HEK cells. The nucleotide sequences were isolated from other species including bovine, dog, mouse, chicken, and guinea pig. The current study targeted the cDNA from the bovine mammary gland because bovine colostrums contain high levels of plasma type PAF-AH activity. It was reported that human plasma PAF-AH is heavily N-glycosylated. The possible N-glycosylation sites (Asn-274, Asp-297, and His-352, for PAF-AH) were not changed. The recombinant PAF-AH protein produced in the E. coli did not play an important role in the PAF hydrolyzing activity. Because plasma PAF-AH is known to play an important role in the regulation of diverse pathological processes, recombinant plasma PAF-AH can be applied to in vitro and in vivo models to examine the clinical benefits of it in inflammatory diseases. Studies on the biological activity in animal models of septic shock are currently under way.

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REFERENCES