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Synthetic biotinylated peptide compounds derived from Asp-hemolysin: Novel potent inhibitors of platelet-activating factor

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ABSTRACT

Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent inflammatory mediator, is implicated in many inflammatory diseases and may possibly serve as a direct target for anti-inflammatory drugs. We have previously reported that Asp-hemolysin-related synthetic peptides (P4—P29) inhibit the bioactivities of oxidized low-density lipoprotein (ox-LDL) containing PAF-like lipids by direct binding to ox-LDL, which plays a key role in the atherosclerotic inflammatory process. In this study, we investigated whether these peptides inhibit the bioactivities of PAF by binding to PAF and its metabolite/precursor lyso-PAF. In *in vitro* experiments, P21, one of the peptides, bound to both PAF and lyso-PAF in a dose-dependent manner and markedly inhibited PAF-induced apoptosis in human umbilical vein endothelial cells. Moreover, in *in vivo* experiments, P4 and P21, particularly their N-terminally biotinylated peptide compounds (BP4 and BP21), inhibited PAF-induced rat paw oedema dose dependently and markedly, and showed sufficient inhibition of the oedema even at doses 150–300 times less than the doses of PAF antagonists. These results provide evidence that direct binding of N-terminally biotinylated peptide compounds derived from Asp-hemolysin to PAF and lyso-PAF leads to a dramatic inhibition of the bioactivities of PAF, both *in vitro* and *in vivo*, and strongly suggesting that these compounds may be useful as a novel type of anti-inflammatory drug for the treatment of several inflammatory diseases caused by PAF.

1. Introduction

Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent lipid mediator that plays an important role in several physiological events (Ishii et al., 1998) and, potentially, in many inflammatory diseases, such as asthma, anaphylaxis, and atherosclerosis (Evangelou, 1994; Ishii and Shimizu, 2000). PAF is produced and released by various tissues and cell types, including platelets, neutrophils, macrophages and endothelial cells in response to stimuli (Hanahan et al., 1980; McIntyre et al., 1985; Chao and Olson, 1993). The bioactivities of PAF are mediated via its interaction with a specific PAF receptor (Chao and Olson, 1993; Ishii and Shimizu, 2000). PAF is inactivated by PAF acetylhydrolase, yielding lyso-PAF (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) (Arai et al., 2002; Prescott et al., 1990). Conversely, lyso-PAF is catalyzed by lyso-PAF acetyltransferase, yielding PAF (Prescott et al., 1990; Shindou and Shimizu, 2009).

Presently, several compounds that attenuate the bioactivities of PAF are known. PAF antagonists, such as CV-3988, *rac*-3-(*N*-*n*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate (Terashita et al., 1983) and WEB-2086, 3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3α][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone (Casal-Stenzel et al., 1987), which inhibit PAF binding to PAF receptor, are currently used in clinical research. Unfortunately, however, PAF antagonists do not provide a viable therapeutic option against PAF-induced inflammatory diseases because PAF can also function via a PAF receptor-independent mechanism (Dyer et al., 2010). Therefore, there are no anti-inflammatory drugs targeting PAF in clinical use today.

Asp-hemolysin is a hemolytic and toxic protein from *Aspergillus fumigatus*, consists of 131 amino acid residues and has a molecular mass of 14,275 Da (Ebina et al., 1994; Yokota et al., 1977). We have previously reported that oxidized low-density lipoprotein (ox-LDL), an atherogenic lipoprotein that exists in atherosclerotic arteries (Rosenfeld, 1991; Steinberg et al., 1989; Witztum, 1993), inhibits the hemolytic activity of Asp-hemolysin, and that Asp-hemolysin binds to ox-LDL in a concentration-dependent manner (Fukuchi et al., 1998; Kudo et al., 1999). We have shown that Asp-hemolysin binds specifically to ox-LDL with high affinity (the dissociation constant, $K_d = 0.63$ μg/ml). Its binding specificity is distinct from any receptor of ox-LDL (Kudo et al., 2001). Moreover, we have shown that lysophosphatidylcholine (LPC), one of the major phospholipid

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components of ox-LDL, inhibits the binding of Asp-hemolysin to ox-LDL (Kudo et al., 2002; Kumagai et al., 2006). Also, we have reported that Asp-hemolysin-related synthetic peptides (P4–P29, Fig. 1) inhibit ox-LDL-induced macrophage proliferation and LPC-induced apoptosis in human umbilical vein endothelial cells (HUVECs) (Kumagai et al., 2005; Tsutsumi et al., 2006), and that the Tyr-Lys-Asp-Gly (YKDG) sequence in the peptides (Fig. 1) is important for binding to ox-LDL, and the binding of ox-LDL to the peptide is attributable to LPC (Kumagai et al., 2006).

In terms of structure and bioactivity, PAF bears a marked resemblance to LPC and phospholipid oxidation products (PAF-like lipids) contained in ox-LDL particles (Tokumura et al., 2000). In this study, we investigated the influence of Asp-hemolysin-related synthetic peptides on PAF bioactivities. The results in this paper indicate that direct binding of the peptides, particularly the N-terminally biotinylated peptide compounds, to PAF and lyso-PAF leads to a dramatic inhibition of PAF bioactivities, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, C-16) was purchased from Enzo Life Sciences Inc. (Plymouth Meeting, PA, U.S.A.). Lyso-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, C-16) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). PAF antagonists CV-3988 and alprazolam, (8-chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3α][1,4]benzodiazepine, were both purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA, Fraction V RIA grade, A-7888) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise noted.

2.2. Synthetic peptides

Asp-hemolysin-related P21, N-terminally biotinylated peptide compounds (BP21 and BP4), and N-terminally biotinylated RGDS (Arg-Gly-Asp-Ser) tetrapeptide derived from fibronectin were synthesized by Bio Synthesis Inc. (Lewisville, Texas, U.S.A.). Asp-hemolysin-related P4 was synthesized by GL Biochem (Shanghai, China). These peptides were purified by reverse high-pressure liquid chromatography and subsequently analyzed by laser desorption mass spectrometry. The amino acid sequences and structures of Asp-hemolysin-related peptides are shown in Figs. 1 and 2.

Peptide name	*Sequence*
P29	IKNASLSWGKW**YKDG**DKDAEITSEDVQQK
P24	LSWGKW**YKDG**DKDAEITSEDVQQK
P21	IKNASLSWGKW**YKDG**DKDAEI
P16	LSWGKW**YKDG**DKDAEI
P15	IKNASLSWGKW**YKDG**
P11	WGKW**YKDG**DKD
P9	WGKW**YKDG**D
P7	**YKDG**DKD
P6	W**YKDG**D
P4	**YKDG**

Fig. 1. Sequences of the synthetic peptides (P4–P29) derived from Asp-hemolysin. Each amino acid is represented as a single letter.

2.3. Time-resolved fluorometric assay

Assay of binding of the synthetic peptides to PAF and lyso-PAF was performed according to the dissociation-enhanced lanthanide fluorometric immunoassay (DELFIA) protocol with minor modifications, utilizing lanthanide chelates and time-resolved fluorometry (Soini and Kojola, 1983). BP21 was used as the peptide probe in this binding assay. One hundred microliters of PAF or lyso-PAF (0–30 μM) was plated in wells of a 96-well Nunc-Immuno Maxisorp microtiter plate (C96, Thermo Scientific Inc.) and then was incubated overnight at 4 °C. After removing the lipids, the wells were blocked with Super-Block® Blocking Buffer (Thermo Scientific Inc.). After washing the wells three times with PBS (10 mM phosphate and 150 mM sodium chloride, pH 7.4), 100 μL of 1 μM BP21 was added to each well. After incubation for 30 min at 37 °C, the plate was washed with PBS, followed by incubation with 100 μL of Europium (Eu^{3+})-labeled streptavidin (PerkinElmer, Inc.) (diluted 1:1000 in PBS) for 30 min at room temperature. The plate was washed eight times and the bound Eu^{3+} was dissociated and fluorescence was enhanced by incubation with 100 μL of DELFIA enhancement solution (PerkinElmer, Inc.). After shaking for 5 min, we determined fluorescence using a 1420 $ARVO_{sx}$ DELFIA Research Fluorometer (PerkinElmer, Inc.) with the standard setting for Eu^{3+} (an excitation wavelength of 340 nm and an emission wavelength of 615 nm).

2.4. Cell cultures

HUVECs were purchased from Clonetics (San Diego, CA, U.S.A.). HUVECs were cultured at 37 °C in a humidified 5% CO_2 incubator in EGM-2 medium (Clonetics) containing 2% fetal bovine serum, 0.04% hydrocortisone, 0.1% human epidermal growth factor, 0.4% basic fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% ascorbic acid, 0.1% insulin-like growth factor-1 with the substitution of arginine for glutamic acid at position 3, 0.1% heparin, and 0.1% GA-1000 (Gentamicin/Amphotericin-B). At confluence, cells were trypsinized, counted and diluted to 3×10^5 cells/ml for flow cytometric analysis and cell viability assays. For flow cytometry analysis, 1 ml of cell suspension was added per 35-mm culture dish. In both experiments, HUVECs were used from the second to fifth passages. For the cell viability assay, 0.1 ml of cell suspension was added per 96-well tissue culture plate.

2.5. Flow cytometry

Cell apoptosis was detected using a MEBCYTO® Apoptosis Kit (Medical and Biological Laboratories Co., Ltd.). HUVECs were incubated with PAF (0 or 25 μM) in the absence or presence of P21

BP21 **Btn-**IKNASLSWGKW**YKDG**DKDAEI
BP4 **Btn-YKDG**

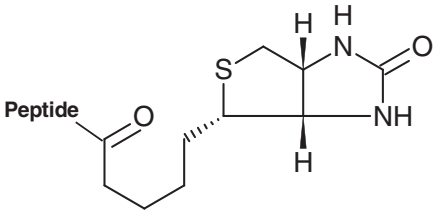

Fig. 2. Amino acid sequences and structures of N-terminally biotinylated peptides derived from Asp-hemolysin. Sequences and structures of N-terminally biotinylated peptides (BP21 and BP4) used in this study are shown here. Each amino acid is represented as a single letter and "Biotinylated" is abbreviated as "Btn".