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An endothelin-3-related synthetic biotinylated pentapeptide as a novel inhibitor of platelet-activating factor

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ABSTRACT

Platelet-activating factor (PAF), a potent proinflammatory mediator, is involved in many inflammatory diseases. We recently reported that synthetic biotinylated peptides having a Tyr-Lys-Asp-Gly sequence inhibit PAF-induced inflammation by directly binding to PAF. In this study, we investigated the effect of two synthetic biotinylated peptides, both of which have a sequence similar to Tyr-Lys-Asp-Gly—an endothelin-3 (ET-3)-related biotinylated pentapeptide (Tyr-Lys-Asp-Lys-Glu, BPET3) and a scavenger receptor CD36-related biotinylated tetrapeptide (Tyr-Lys-Gly-Lys, BPCD36)—on PAF-induced inflammation by using a rat model of hind paw oedema. BPET3 markedly inhibited PAF-induced oedema in a dose-dependent manner, and the dose that caused 50% inhibition was estimated to be approximately 2.64 nmol/paw. The inhibitory effect of BPCD36 on PAF-induced paw oedema was less than that of BPET3, while a synthetic biotinylated pentapeptide (Lys-Lys-Tyr-Asp-Glu) shuffling amino acid sequence of BPET3, an ET-1-related synthetic biotinylated pentapeptide (Leu-Met-Asp-Lys-Glu), or an ET-2-related synthetic biotinylated pentapeptide (Trp-Leu-Asp-Lys-Glu) did not inhibit PAF-induced paw oedema. Furthermore, intrinsic tryptophan fluorescence studies demonstrated that ET-3 specifically interacted with both PAF and its metabolite/precursor lyso-PAF. These results provide evidence that the Tyr-Lys-Asp region in both ET-3 and BPET3 is essential for marked inhibition of the peptide on PAF-induced inflammation, and strongly suggest that BPET3 may be useful as a novel anti-inflammatory drug targeting PAF.

1. Introduction

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent lipid mediator, plays an important role in several physiological events (Ishii et al., 1998), and may play a role in many inflammatory diseases, such as asthma, anaphylaxis, and atherosclerosis (Evangelou, 1994; Ishii and Shimizu, 2000). PAF is produced 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 binding to a specific PAF receptor (Chao and Olson, 1993; Ishii and Shimizu, 2000). PAF is inactivated by PAF acetylhydrolases, yielding lyso-PAF (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) (Prescott et al., 1990; Arai et al., 2002), whereas lyso-PAF is catalysed by lyso-PAF acetyltransferases, yielding PAF (Prescott et al., 1990; Shindou and Shimizu, 2009).

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 the binding of PAF to the PAF receptor, are currently used in clinical research. However, these PAF antagonists do not provide viable therapeutic options against PAF-induced inflammatory diseases, because PAF can also function via a PAF receptor-independent mechanism (Dyer et al., 2010). Thus, there are no anti-inflammatory drugs targeting PAF in clinical use today.

We recently reported that synthetic peptides derived from Asp-hemolysin, which is a haemolytic and toxic protein (Yokota et al., 1977; Ebina et al., 1994), inhibit the bioactivities of low-density lipoproteins (ox-LDL) containing many phospholipid oxidation products (PAF-like lipids) by directly binding to ox-LDL (Kumagai et al., 2005; Tsutsumi et al., 2006), and that a Tyr-Lys-Asp-Gly (YKDG) region in these peptides is important for binding to ox-LDL (Kumagai et al., 2006). We also reported that these peptides, especially the N-terminally biotinylated peptides, markedly inhibit PAF-induced bioactivities in a dose-dependent manner by direct binding to PAF and its metabolite/precursor lyso-PAF, and that both a YKDG region in the peptides and a biotin bound at the N-terminus of the peptides are necessary for the marked inhibition of PAF bioactivities (Sato et al., 2012). Furthermore, we reported that these biotinylated peptides show sufficient

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BPET3	Btn-YKDKE
A biotinylated peptide shuffling an amino acid sequence of BPET-3	Btn-KKYDE
BPCD36	Btn-YKGK
A biotinylated peptide shuffling an amino acid sequence of BPCD36	Btn-KKYG
BPET1	Btn-LMDKE
BPET2	Btn-WLDKE

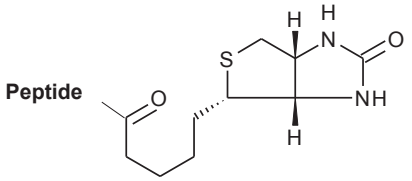

Fig. 1. Amino acid sequences and structures of N-terminally biotinylated peptides used in this study. Each amino acid is represented as a single letter and "Biotinylated" is abbreviated as "Btn.".

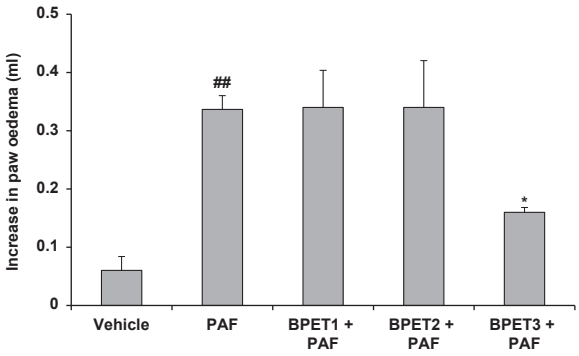

Fig. 2. Effects of ET-related synthetic biotinylated peptides on PAF-induced rat paw oedema. Rats were treated by intraplantar injection with BPET1, BPET2, or BPET3 (10 nmol/paw) 15 min prior to intraplantar injection of PAF solution (1 nmol/paw). One hour after the PAF stimulus, paw oedema was quantified by measuring the increase in paw volume (ml). Each value represents the mean ± S.D. of 3 rats. $^{\#\#}P < 0.05$ compared to rats treated with the vehicle alone. $^{*}P < 0.05$ compared to rats treated with PAF alone.

inhibition of the bioactivities induced by PAF even at doses 150- to 300-fold lower than the doses of PAF antagonists (Sato et al., 2012).

Endothelins (ETs) are a family of 21 amino acid peptides that are produced and secreted by vascular endothelial cells, and are expressed as three biologically active peptides: ET-1, ET-2, and ET-3 [18, 19]. ET-3 has a Tyr-Lys-Asp-Lys-Glu (YKDKE) sequence that is similar to a YKDG sequence in the Asp-hemolysin-related peptides (Fig. 1). CD36, an ox-LDL-specific scavenger receptor, has a Tyr-Lys-Gly-Lys (YKGK) sequence, which is also similar to a YKDG sequence in Asp-hemolysin-related synthetic peptides. In this study, we investigated the effect of ET-3- and CD36-related synthetic biotinylated peptides (BPET3 and BPCD36) on PAF-induced inflammation. The results indicate that BPET3 inhibits PAF-induced inflammation markedly and in a dose-dependent manner *in vivo* and that a YKD region in the peptide is essential for this inhibition.

2. Material and methods

2.1. Materials

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, C16) was purchased from Enzo Life Sciences Inc. (Plymouth Meeting, PA, U.S.A.). Lyso-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, C16) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). 1-Palmitoyl-2-steraroyl-phosphatidylcholine (PC16:0/18:0) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). Bovine serum albumin (BSA, fraction V RIA grade, A-7888) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other regents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise specified.

2.2. Synthetic peptides

ET-3 was purchased from Peptide Institute Inc. (Osaka, Japan). ET-1, ET-2, and ET-3-related N-terminally biotinylated pentapeptides (BPET1, BPET2, and BPET3), CD36-related N-terminally biotinylated tetrapeptide (BPCD36), an N-terminally biotinylated peptide shuffling amino acid sequence of BPET3, and an N-terminally biotinylated tetrapeptide shuffling amino acid sequence of BPCD36 were all synthesised by GL Biochem (Shanghai, China). The amino acid sequences and structures of the biotinylated peptides used in this study are shown in Fig. 1. These peptides were purified by reversed-phase high-pressure liquid chromatography and subsequently analysed by laser desorption mass spectrometry (purity: > 95%).

2.3. Evaluation of PAF-induced paw oedema in rats

Animal care and experimental procedures were in accordance with the principles and guidelines of the Japanese Council on Animal Care and were approved by the Animal Care and Use Committee of Iwaki Meisei University.

Male Wistar rats (weighing 180–220 g) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Measurements of PAF-induced hind paw oedema were conducted as previously described (Henriques et al., 1992; Sato et al., 2012). The subplantar surface of the hind paw was injected with 50 μl of PAF (1 nmol/paw), which was dissolved in a sterile solution (vehicle) containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane (Tris, pH 7.5), and 0.25% BSA, and then sonicated for 5 min. One hour after the PAF stimulus (the time of the peak oedema), the oedema was quantified by measuring the increase in paw volume (ml) by using a water displacement method.

To examine the effect of synthetic peptides on PAF-induced hind paw oedema, each synthetic pentapeptide (BPET1, BPET2, BPET3, BPCD36, or BPET3-related and BPCD36-related shuffled biotinylated peptides) dissolved in phosphate-buffered saline (PBS, pH 7.4) was injected into the subplantar surface of the hind paw (intraplantar) or into the tail vein (intravenous) at a dose of 10 or 20 nmol/rat 15 min prior to PAF stimulus. To examine the dose-dependent effects of BPET3 on PAF-induced rat paw oedema, the peptide dissolved in PBS (pH 7.4) was injected intraplantarly at doses of 0.625, 1.25, 5, 10, or 20 nmol/rat 15 min prior to PAF stimulus. The dose that caused 50% inhibition (ID_{50}) was calculated from the linear-log peptide dose-inhibitory response relationship.

2.4. Tryptophan fluorescence spectroscopy

To examine the interaction of native ET-3 with PAF and with the PAF metabolite/precursor lyso-PAF, we investigated the changes in the intrinsic tryptophan fluorescence of ET-3 in the presence of PAF, lyso-PAF, or PC, which is a phospholipid possessing two fatty acids, unlike PAF or lyso-PAF, each of which has a fatty acid. PAF (C16), lyso-PAF (C16), or PC16:0/18:0 (each 0–30 μM) was incubated for 30 min at 37 °C in PBS (10 mM phosphate and 150 mM sodium chloride, pH 7.4) in the absence or presence of ET-3 containing a tryptophan residue (0.2 μM). The fluorescence