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Biotinylated heptapeptides substituted with a D-amino acid as platelet-activating factor inhibitors

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ABSTRACT

Platelet-activating factor (PAF), a potent lipid mediator, is implicated in many inflammatory diseases, and therefore may serve as a direct target for anti-inflammatory drugs. We previously reported that synthetic biotinylated peptides having a Tyr-Lys-Asp-Gly sequence markedly inhibit PAF-induced inflammation by direct binding, and that two synthetic fluorescence-labelled heptapeptides (Lys-Trp-Tyr-Lys-Asp-Gly-Asp and D-Lys-Trp-Tyr-Lys-Asp-Gly-Asp) with high stability in plasma specifically bind to PAF-like lipids (oxidized- and lyso-phosphatidylchoine). In this study, synthetic heptapeptides (Lys-Trp-Tyr-Lys-Asp-Gly-Asp) coupled to a biotin molecule through the N-terminal amino group and ε-amino group of N-terminus Lys, (Btn)KP6 and K(Btn)P6, respectively, and their biotinylated peptides substituted with D-Lys at the N-terminus, (Btn)dKP6 and dK(Btn)P6, respectively, were investigated for their effects on PAF-induced inflammation. In the experiments using a rat model of hind paw oedema, (Btn)KP6, K(Btn)P6, (Btn)dKP6, and dK(Btn)P6 significantly inhibited PAF-induced paw oedema, with the highest inhibitory effect exhibited by dK(Btn)P6. The inhibitory effect of D-Tyr-D-Lys-D-Asp-Gly tetrapeptide on PAF-induced paw oedema was much lower than that of Tyr-Lys-Asp-Gly tetrapeptide. In the experiments using tryptophan fluorescence spectroscopy, (Btn)KP6, K(Btn)P6, (Btn)dKP6, and dK(Btn)P6 bound to PAF dose-dependently, with dK(Btn)P6 showing the strongest binding affinity, indicating that its affinity appears to be closely correlated with its inhibitory effect on PAF-induced inflammation. These results suggest that direct binding of (Btn)KP6, K(Btn)P6, (Btn)dKP6, and dK(Btn)P6 to PAF can lead to marked inhibition of PAF-induced inflammation, and these agents, particularly dK(Btn)P6, may be useful as anti-inflammatory drugs targeting PAF with high stability in plasma.

1. Introduction

Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator released from various types of cells, including endothelial cells, neutrophils, and macrophages (Chao and Olson, 1993). PAF is considered to be involved in several physiological conditions (Ishii et al., 1998) and particularly, in the pathogenesis of many inflammatory diseases such as anaphylaxis, asthma, and atherosclerosis (Evangelou, 1994; Ishii et al., 1998; Ishii and Shimizu, 2000). PAF acts *via* binding to its specific PAF receptor (Chao and Olson, 1993; Ishii and Shimizu, 2000). PAF is degraded to the biologically inactive metabolite lyso-PAF (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) by PAF acetylhydrolases (PAF-AHs), whereas lyso-PAF is catalysed by lyso-PAF acetyltransferases (lyso-PAF ATs), yielding PAF (Prescott et al., 1990; Shindou and Shimizu, 2009).

We have recently reported that N-terminal biotinylated peptides derived from Asp-hemolysin, a hemolytic and toxic protein from *Aspergillus fumigatus* (Yokota et al., 1977; Ebina et al., 1994), inhibit PAF-induced inflammatory responses dose-dependently and markedly by directly binding to PAF, and that these peptides show sufficient inhibition of PAF-induced inflammation even at doses 150- to 300-fold lower than the doses of PAF antagonists *in vivo* (Sato et al., 2012, 2013). Both a biotin molecule bound at the N-terminus and a Tyr-Lys-Asp-Gly region (especially a Tyr-Lys-Asp region) in the peptides were found to be essential for marked PAF inhibition (Sato et al., 2012, 2013). Thus, biotinylated peptides are useful in the treatment of many inflammatory diseases mediated by PAF, but the *in vivo* stability of the peptides remains unknown because peptides are generally degraded by peptidases. Recently, we have reported that both a heptapeptide (Lys-Trp-Tyr-Lys-Asp-Gly-Asp) coupled to fluorescein isothiocyanate (FITC) through ε-amino group of N-terminus Lys, (FITC)KP6, and (FITC)KP6 substituted with D-Lys at the N-terminus, (FITC)dKP6, with a higher plasma stability, specifically bind to oxidized phosphatidylcholine

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(Btn)KP6	Biotin-(N-terminal amino group)-Lys-Trp-Tyr-Lys-Asp-Gly-Asp
(Btn)dKP6	Biotin-(N-terminal amino group)-D-Lys-Trp-Tyr-Lys-Asp-Gly-Asp
K(Btn)P6	Biotin-(ε-amino group)-Lys-Trp-Tyr-Lys-Asp-Gly-Asp
dK(Btn)P6	Biotin-(ε-amino group)-D-Lys-Trp-Tyr-Lys-Asp-Gly-Asp
BdP4	Biotin-D-Tyr-D-Lys-D-Asp-Gly
BP4	Biotin-Tyr-Lys-Asp-Gly

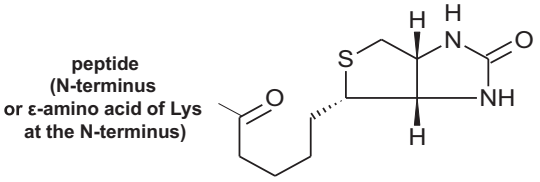

Fig. 1. Amino acid sequences and structures of biotinylated peptides used in this study. Each amino acid is represented by the three-letter code.

and lysophosphatidylcholine (LPC), which are PAF-like lipids, in oxidized low-density lipoprotein (ox-LDL); ox-LDL is known to be a proinflammatory and proatherogenic factor found in atherosclerotic plaques (Sato et al., 2014, 2015; Tokumura et al., 2000).

In this study, biotin-coupled synthetic heptapeptides (Lys-Trp-Tyr-Lys-Asp-Gly-Asp) through the N-terminal amino group and ε-amino group of N-terminus Lys, (Btn)KP6 and K(Btn)P6, respectively, and their biotinylated peptides substituted with D-Lys at the N-terminus, (Btn)dKP6 and dK(Btn)P6, respectively, were investigated for their effects on PAF-induced inflammation (Fig. 1). Results indicate that these biotinylated heptapeptides, especially the peptides substituted with a D-Lys, markedly inhibit PAF-induced inflammation *in vivo* by binding PAF, and that the change in heptapeptide structures induced by the substitution of a D-amino acid and the binding of a biotin molecule in the peptide can affect peptide action on PAF-induced inflammation.

2. Materials 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.). 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 specified.

2.2. Synthetic peptides

(Btn)KP6, K(Btn)P6, (Btn)dKP6, dK(Btn)P6, and biotinylated tetrapeptide BdP4 were all synthesized and purified by GL Biochem Ltd. (Shanghai, China). Biotinylated tetrapeptide BP4 was synthesized and purified by Bio Synthesis Inc. (Lewisville, Texas, U.S.A.). A purity > 95% was assessed by high performance liquid chromatography (HPLC) and mass spectroscopy. The amino acid sequences and structures of biotinylated peptides in this study are shown in Fig. 1.

2.3. Evaluation of PAF-induced rat hind paw oedema

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 (approval no 13-1 and 14-3).

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, 2013). All injections were performed with sterile 1-ml syringes with 27-gauge needles (Terumo Corporation, Tokyo, Japan) under ether anaesthesia. The subplantar injection surface of the hind paw was injected with 50 μl PAF (1 nmol/paw), which was dissolved in a sterile solution (vehicle) containing 0.25% BSA, 150 mM sodium chloride, and 10 mM tris (hydroxymethyl)aminomethane (Tris, pH 7.5), 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 biotinylated peptide (Fig. 1) dissolved in sterile 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 nmol/rat 15 min prior to PAF stimulus.

2.4. Tryptophan fluorescence spectroscopy

Spectra were recorded using a Hitachi F2500 fluorescence spectrophotometer (Tokyo, Japan) at room temperature (22–25 °C) in PBS (10 mM phosphate and 150 mM sodium chloride, pH 7.4). Excitation and emission bandwidths were both adjusted to 5 nm, tryptophan excitation wavelength was set to 295 nm to minimize interference from tyrosine fluorescence, emission was monitored from 320 to 380nm, and the values were read at 348 nm. PAF C16 (0–30 μM) was incubated for 30 min at 37 °C in PBS (pH 7.4) in the absence or presence of (Btn)KP6, K(Btn)P6, (Btn)dKP6, or dK(Btn)P6, all of which contain a tryptophan residue (1 μM). The difference in intensity between the lipid and lipid-free (peptide alone) conditions was calculated.

2.5. Statistical analysis

All results are expressed as the mean ± S.D. (standard deviation). The data were analysed by means of a paired Student's *t*-test and by one-way ANOVA post-hoc test. The difference was considered statistically significant when the *P* value was less than 0.05.