European Journal of Pharmacology 745 (2014) 46–51

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

C-reactive protein specifically enhances platelet-activating factor-induced inflammatory activity *in vivo*

Akira Sato*, Keitaro Oe, Hikaru Yamanaka, Izumi Yokoyama, Keiichi Ebina

Faculty of Pharmacy, Iwaki Meisei University, 5-5-1, Chuodai-Iino, Iwaki, Fukushima 970-8551, Japan

ARTICLE INFO

Article history:
Received 31 July 2014
Received in revised form
16 September 2014
Accepted 6 October 2014
Available online 22 October 2014

Keywords:
C-reactive protein
Platelet-activating factor
Inflammation
Oedema
Inflammatory disease

ABSTRACT

Platelet-activating factor (PAF) is a potent lipid mediator that is implicated in numerous inflammatory diseases. C-reactive protein (CRP) is an acute-phase plasma protein that increases rapidly and dramatically in response to inflammation. In this study, we investigated the effect of the interaction between CRP and PAF on inflammatory responses *in vivo*. From binding analysis using a time-resolved fluorometric assay, CRP bound to PAF and its precursor/metabolite lyso-PAF in a concentration-dependent manner. In addition, CRP bound to several phospholipids containing lysophosphatidylcholine, which bears structural resemblance to PAF and lyso-PAF, sphingosylphosphorylcholine, and lysophosphatidylethanolamine more readily than to lysophosphatidic acid and lysophosphatidylserine. In *in vivo* experiments using a rat model of hind paw oedema, CRP increased PAF-induced rat paw oedema in a dose-dependent manner, without causing the oedema itself, but it did not increase histamine and serotonin-induced paw oedema. Furthermore, the receptor for CRP, lectin-like oxidized low-density lipoprotein receptor 1 was not involved in the increase in PAF-induced inflammatory responses caused by CRP. These results indicate that CRP can specifically enhance PAF-induced inflammatory activity through binding to PAF and lyso-PAF. Therefore, CRP may accelerate the pathogenesis of numerous 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 the pathogenesis of numerous inflammatory diseases such as asthma, anaphylaxis, and atherosclerosis (Evangelou, 1994; Ishii and Shimizu, 2000). PAF is produced and released by various types of tissues and cells, including platelets, macrophages, neutrophils, and endothelial cells, in response to stimuli (Chao and Olson, 1993; Hanahan et al., 1980; McIntyre et al., 1985). 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) (Arai et al., 2002; Prescott et al., 1990), whereas lyso-PAF is catalysed by lyso-PAF acetyltransferases, yielding PAF (Prescott et al., 1990; Shindou and Shimizu, 2009).

C-reactive protein (CRP) is an acute-phase plasma protein that increases rapidly and dramatically, as much as 1000-fold or more, in response to inflammation, infection, and tissue injury (Black et al., 2004). CRP is mainly produced in the liver, but can also be secreted by endothelial cells under certain conditions (Venugopal et al., 2005) and by smooth muscle cells (Calabro et al., 2003). For this reason, CRP has been used as the prototypic inflammatory biomarker (Gabay and Kushner, 1999).

CRP is composed of five identical non-covalently attached subunits. The molecular mass of each subunit is approximately 23 kDa (Shrive et al., 1996). CRP has been shown to bind to oxidized low-density lipoprotein (ox-LDL), which is an atherogenic form of LDL and a constituent of atherosclerotic lesions. CRP has a phosphocholine-binding site, which participates in the binding of CRP to ox-LDL. Moreover, CRP recognizes phosphocholine and other various ligands. Several recent studies have shown that lectin-like ox-LDL receptor 1 (LOX-1 receptor), which was originally found and identified as an endothelial receptor for ox-LDL (Sawamura et al., 1997), is a novel receptor for CRP (Shih et al., 2009), and CRP was found to enhance vascular permeability via LOX-1 receptor *in vivo* (Fujita et al., 2009).

PAF bears a marked resemblance to lysophospholipids, especially lysophosphatidylcholine (LPC), and phospholipid oxidation products (PAF-like lipids) in ox-LDL particles (Tokumura et al., 2000). In this study, we investigated the effect of the interaction of

* Corresponding author. Tel.: +81 246 29 5364; fax: +81 246 29 5414.
E-mail address: a-sato@iwakimu.ac.jp (A. Sato).

http://dx.doi.org/10.1016/j.ejphar.2014.10.020
0014-2999/

CRP with PAF on inflammatory responses *in vivo*. The results indicate that CRP specifically enhances PAF-induced inflammatory responses through direct binding to PAF and its precursor/metabolite lyso-PAF.

2. Materials and methods

2.1. Materials

Recombinant human CRP was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, C16) was purchased from Enzo Life Sciences Inc. (Plymouth, PA, USA.). Lyso-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, C16) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bovine serum albumin (BSA; fraction V RIA grade, A-7888) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Palmitoyl-lysophosphatidylcholine (LPC), 1-palmitoyl-lysophosphatidic acid (LPA), and 1-stearoyl-lysophosphatidylethanolamine (LPE) were all purchased from Avanti Polar Lipids (Alabaster, AL, USA). Lysophosphatidylserine (LysoPS) from the bovine brain was purchased from Doosan Serdary Research Laboratories (Toronto, ON, Canada). Sphingosylphosphorylcholine (SPC) was purchased from BIOMOL Research Laboratories Inc. (Plymouth, PA, USA). Histamine dihydrochloride and serotonin (5-hydroxytryptamine hydrochloride) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Normal mouse immunoglobulin G (IgG) was purchased from Santa Cruz Biotechnologies, Inc. (Dallas, TX, USA), and mouse anti-human LOX-1 receptor monoclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise noted.

2.2. Time-resolved fluorometric assay

A binding assay for CRP binding to PAF, lyso-PAF, and various phospholipids 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), and as previously described (Gang et al., 2012). One hundred microliters of PAF (C16), lyso-PAF (C16), 1-palmitoyl-LPC, 1-palmitoyl-LPA, LysoPS from the bovine brain, 1-stearoyl-LPE, or SPC (each 20 μM), which was dissolved in TBS (10 mM Tris and 150 mM sodium chloride, pH 7.5) containing BSA (2.2 μM) and then sonicated for 5 min, were plated in wells of a 96-well Nunc-Immuno Maxisorp microtiter plate (C96, Thermo Fisher Scientific Inc., Waltham, MS, USA) and incubated overnight at 4 °C. After removing the lipids, the wells were blocked with 0.5% gelatin dissolved in TBS for 45 min at room temperature. After washing the wells three times with TBS, 100 μl of CRP (0.2–20 μg/ml) in TBS containing 5 mM calcium chloride, 0.1% gelatin, and 0.02% Tween-20, was added to each well. After incubation for 2 h at 37 °C, the plate was washed with TBS containing 5 mM calcium chloride, 0.1% gelatin, and 0.02% Tween-20, and then incubated with 100 μl of 1:1000 anti-human CRP mouse monoclonal antibody (clone-8, C1688; Sigma-Aldrich; St. Louis, MO, USA) for 1 h at 37 °C. This was followed by incubation with 100 μl of 1:100 Europium (Eu^{3+})-labelled rabbit anti-mouse IgG (PerkinElmer, Inc., Waltham, MS, USA) for 1 h at 37 °C. The plate was washed six times, 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, the fluorescence was determined 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).

2.3. Measurements of rat hind paw oedema induced by PAF, lyso-PAF, histamine, and serotonin

Animal care and experimental procedures complied 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 (200–250 g body weight) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Measurements of hind paw oedema were conducted as described in previous studies (Henriques et al., 1992; Sato et al., 2012, 2013). Each endpoint was taken as the time as peak response of hind paw oedema induced by PAF and histamine. Since serotonin is known to induce a linear increase in the paw oedema within 1 h, the endpoint was taken as 30 min after the injection (Cole et al., 1995).

PAF (20 μM) or lyso-PAF (20 μM) was dissolved in sterile TBS (pH 7.5) containing 0.25% BSA and 2 mM calcium chloride, sonicated for 5 min, and pre-incubated with CRP (0–50 μg/ml, which corresponds to approximately 0–0.48 μM) at 37 °C for 1 h. All injections were performed with sterile 1-ml syringes with 27-gauge needles under ether anaesthesia. The subplantar surface of the hind paw of the rat was injected with 100 μl of the pre-incubation mixture (PAF and lyso-PAF: 0 or 2 nmol/paw, CRP: 0–5 μg/paw). After 45 min (the time of peak response of PAF injection), the paw oedema was quantified by measuring the increase in paw volume (ml) using a water displacement method.

Histamine (1 mg/ml) or serotonin (100 μg/ml) was dissolved in sterile TBS (pH 7.5) containing 2 mM calcium chloride and pre-incubated with CRP (0–50 μg/ml) at 37 °C for 1 h. The subplantar surface of the hind paw of the rat was injected with 100 μl of the pre-incubation mixture (histamine: 100 μg/paw, serotonin: 10 μg/paw, CRP: 0–5 μg/paw). After 30 min for histamine (the time of peak response of histamine injection), and 30 min for serotonin (which induces a linear increase in the paw oedema within 1 h), the paw oedema was quantified by measuring the increase in paw volume (ml). Moreover, to examine the effect of the receptor for CRP, LOX-1 receptor, on the PAF-induced inflammatory activity, the subplantar surface of the hind paw of the rat was injected with 100 μl of mouse anti-LOX-1 receptor IgG and control (mouse) IgG (3 μg/paw each) in the presence of CRP (5 μg/paw). After 45 min (the time of peak response of PAF injection), the paw oedema was quantified by measuring the increase in paw volume (ml).

2.4. Statistical analysis

All results are expressed as the mean ± S.D. The data obtained by comparing the two groups were statistically analysed by means of a paired Student's *t*-test (Figs. 4 and 5). The other data were statistically analysed using one-way analysis of variance followed by a ANOVA-Tukey's test. The difference was considered statistically significant when the *P* value was less than 0.05.

3. Results

3.1. Binding analysis of CRP to PAF, lyso-PAF, and various phospholipids

We investigated the binding of CRP to PAF, lyso-PAF, and various phospholipids by a time-resolved fluorometric assay. CRP was added to microtitre wells pre-coated with each lipid. After washing, the amounts of the lipid–CRP complex remaining in each well were determined using both a mouse anti-human CRP monoclonal antibody and Eu^{3+}-labelled rabbit anti-mouse IgG. As a shown in Fig. 1, CRP strongly bound to PAF, lyso-PAF, LPC, and SPC, all of which have a phosphorylcholine group. CRP also bound