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Aspirin induces non-enzymatic formation of platelet-activating factor from lyso platelet-activating factor

Kiyoko Mabuchi-Itoh, Takayuki Sugiura*, Neng-neng Cheng, Keizo Waku

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

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Substantial amounts of platelet-activating factor (PAF) were formed when lysoPAF was mixed with aspirin (e.g. 0.04% of added lysoPAF (200 nmol) was converted to PAF when mixed with aspirin (2 μmol) for 1 h). Non-enzymatic formation of PAF from aspirin and lysoPAF also occurs in the aqueous solution or in organic solvents in time-dependent and dose-dependent manners. Possible meanings of the non-enzymatic formation of PAF are discussed.

Aspirin; Platelet-activating factor; Lysophospholipid; Non-enzymatic acetylation

1. INTRODUCTION

Platelet-activating factor (PAF) is a potent bioactive lipid molecule, the structure of which was finally elucidated as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine in 1979 [1–3]. PAF is known to cause a variety of biological responses, such as the aggregation and degranulation of platelets and polymorphonuclear leukocytes, smooth muscle contraction, increased vascular permeability, hypotension, gastric ulcer and increased bronchohyper-reactivity. It has been postulated that PAF is implicated in the pathogenesis of various allergic and inflammatory diseases, such as endotoxin shock, anaphylactic shock, nephritis and bronchial asthma [4,5]. Several investigators have also suggested that PAF is an important bioactive molecule in several physiological processes, such as pregnancy [4,5].

Recently, we investigated the levels of PAF-like lipid in various multicellular invertebrates [6,7]. We found that PAF-like lipid is widely distributed in various lower order animals, which are usually enriched in alkyl ether phospholipids as well. It seems very likely that PAF plays physiologically important roles even in these lower order animals. In an extended study, we also noticed that a commercial Japanese antifebrile medicine (Mimizu Ippuh San, a mixture of the extract of earthworms, aspirin, acetaminophen and caffeine; Tenshindo Co., Nara, Japan) contains an extremely high level (per lipid phosphorus) of PAF-like material (Sugiura, T., unpublished data). This observation was very curious and unexpected, because either living or dried earthworms themselves contain a lesser amount of PAF-like material. We then tried to explain how a PAF-like material came to be included in this antifebrile medicine. Finally, we reached the conclusion that PAF is formed from lysoPAF through non-enzymatic transfer of the acetyl moiety from aspirin based on the facts that earthworms contain a large amount of lysoPAF (2% total phospholipids; Sugiura, T., unpublished data) and aspirin is known to act as an acetyl donor in the non-enzymatic acetylation of proteins such as cyclooxygenase [8].

In this study, we examined this possibility precisely. We confirmed that a large amount of PAF is actually formed from lysoPAF and aspirin in a non-enzymatic manner. The reaction proceeds either in the presence or absence of aqueous solution. Possible implications of the non-enzymatic formation of PAF or PAF-like materials are also discussed.

2. MATERIALS AND METHODS

2.1. *Chemicals*

LysoPAF (1-*O*-hexadecyl) was purchased from Novabiochem (Läufelfingen, Switzerland). PAF (1-*O*-hexadecyl) was obtained from Bachem (Bubendorf, Switzerland). Aspirin (acetylsalicylic acid) and acetic acid were from Wako Pure Chem. Ind. (Osaka, Japan). Essentially fatty acid-free bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Pre-coated silica gel TLC plates were purchased from Merck (Darmstadt, Germany). The specific PAF antagonists, CV6209 and TCV309, were generous gifts from Takeda Chem. Ind. (Osaka, Japan).

2.2. *Non-enzymatic formation of PAF*

LysoPAF (200 nmol) dissolved in 100 μl of chloroform:methanol (2:1, v/v) and aspirin (2 μmol) dissolved in10 μl of acetone were mixed in a round-bottomed glass tube and organic solvents were quickly removed under a stream of nitrogen. The residue was left at room

*Corresponding author. Fax: (81) (426) 851 345.

Abbreviations: PAF, platelet-activating factor; BSA, bovine serum albumin; PC, phosphatidylcholine.

temperature for 1–24 h. Then 2 ml of 0.25% BSA-containing HEPES–Tyrode's solution was added to the residue and vortexed, and the solution was further diluted with the same buffer. A portion of the diluted solution (25 μl) was taken and added to 225 μl of washed rabbit platelet suspension (in 5 mM HEPES–Tyrode's buffer). The aggregation of washed rabbit platelets by samples and by standard PAF was recorded in a haematracer (Niko PAT 2 M), as described previously [9]. The amount of PAF formed through non-enzymatic acetylation was estimated using a calibration curve with standard PAF. In this case, the final concentration of lysoPAF in each cuvette, either for sample or for standard PAF, was fixed to 1 μM, which is not sufficient per se to induce aggregation, in order to minimize the difference in conditions between tubes. In a separate experiment, the residue was dissolved in a small amount of chloroform:methanol (2:1, v/v) and immediately applied to a TLC plate. The TLC plate was developed with chloroform:methanol:water (65:35:6, v/v) and the silica gel was zonally scraped (2 cm width). Lipids were extracted from the silica gel by the method of Bligh and Dyer [10] and subjected to bioassay. In order to examine whether PAF is formed in aqueous solution, lysoPAF (final 100 μM) and aspirin (1 mM) were incubated in sterile 5 mM HEPES-buffered saline (pH 7.4) at 37°C for 3–24 h. After standing for several hours, a portion of the reaction mixture was taken and added to the rabbit platelet suspension. The amounts of formed PAF were determined as described above.

3. RESULTS AND DISCUSSION

First, we examined whether PAF is formed from lysoPAF and aspirin in tubes in which aqueous solution is absent. As demonstrated in Fig. 1c, a strong platelet-aggregating activity was detected in tubes where lysoPAF and aspirin were mixed and dried together (the molar ratio of lysoPAF to aspirin was 1:10). On the other hand, negligible activity was detected in the cases where either aspirin alone (Fig. 1a) or lysoPAF alone (Fig. 1b) was employed. This observation indicates that

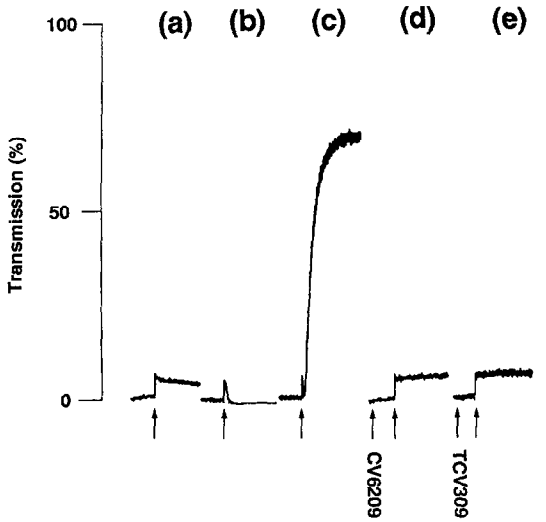

Fig. 1. Aggregation of washed rabbit platelets by PAF-like material formed upon mixing of lysoPAF with aspirin. Platelets were challenged with (a) aspirin alone; (b) lysoPAF alone; (c) the sample obtained after mixing of lysoPAF and aspirin for 1 h; (d) platelets were treated with CV6209 (1 μM) 1 min prior to the addition of the same sample in (c); (e) platelets were treated with TCV309 (1 μM) 1 min prior to the addition of the same sample in c.

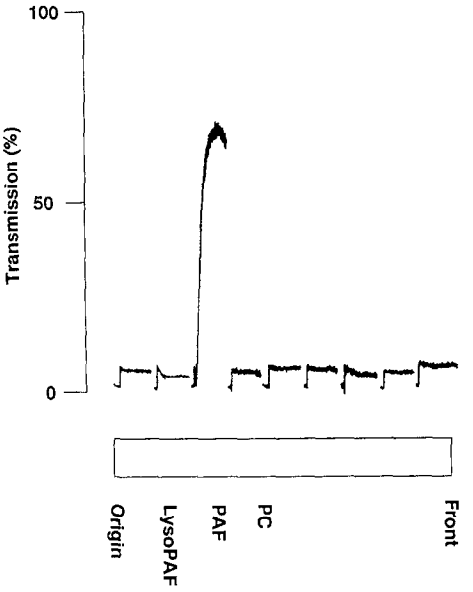

Fig. 2. TLC separation of PAF-like material formed upon mixing of lysoPAF with aspirin. LysoPAF (200 nmol) and aspirin (2 μmol) were mixed and dried for 1 h. Then the residue was dissolved in chloroform:methanol (2:1, v/v) and immediately applied to a TLC plate. The silica gel plate was developed with chloroform:methanol:water (65:35:6, v/v) and zonally scraped (2 cm width). The ability of each fraction to aggregate washed rabbit platelets was examined as described in section 2.

a material with platelet-aggregating activity was formed from lysoPAF and aspirin after mixing.

The effects of PAF antagonists were then examined. Here we used two types of potent and specific PAF antagonists with different chemical structures (CV6209 and TCV309). As depicted in Fig. 1d and e, the pretreatment of platelets with either CV6209 or TCV309 completely blocked the aggregation induced by the sample. This suggests that the bioactive material produced after mixing of lysoPAF with aspirin is PAF itself, which was further confirmed by TLC analysis. The activity co-migrated with authentic PAF and no activity was detected in other fractions (Fig. 2). Hence, it is apparent that PAF is actually formed from lysoPAF and aspirin by a non-enzymatic transacetylation reaction. As shown in Fig. 3, the amounts of PAF increased with time, although the rates of the formation somewhat gradually decreased. About 0.04% and 0.15% of added lysoPAF (total 200 nmol) was converted to PAF after 1 h and 24 h, respectively.

Next we examined whether PAF is formed from lysoPAF and aspirin in the presence of aqueous solution. Fig. 4 demonstrates the formation of PAF in HEPES-buffered saline (pH 7.4) as a function of time. We confirmed that the formation of PAF takes place even in aqueous solution. The amounts of PAF increased almost linearly with time at least up to 24 h. The effect of varying concentrations of aspirin and lysoPAF on the formation of PAF is shown in Fig. 5. The amounts

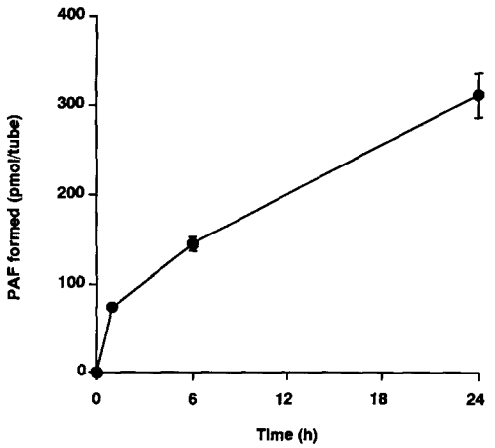

Fig. 3. Formation of PAF upon mixing of lysoPAF with aspirin as a function of time. LysoPAF (200 nmol) and aspirin (2 μmol) were mixed and dried for the indicated periods. Then 2 ml of 0.25% BSA-containing HEPES-Tyrode's solution was added to the tubes. The amounts of PAF were estimated using washed rabbit platelets as described in section 2. Values are the means ± S.D. from three determinations.

of PAF increased with increased concentrations of aspirin (A) and lysoPAF (B). The levels of PAF synthesized during the incubation in the aqueous solution seem to be fairly high. For instance, as up to 17.7 nM (final concentration in tubes) PAF was produced when 100 μM lysoPAF and 10 mM aspirin were incubated together; that is, 0.018% of existing lysoPAF was converted to PAF. We also observed that such non-enzymatic formation of PAF occurs in organic solvents such as ethanol, the rate being somewhat higher than that in the aqueous solution (data not shown). Thus, the contact of lysoPAF with aspirin in either aqueous solution or organic solvents is sufficient to induce the formation of appreciable amounts of PAF. In contrast to the case of aspirin, only a trace amount of PAF was produced when lysoPAF was incubated with 100 mM acetic acid for several days (data not shown), although considerable amounts of PAF were formed when lysoPAF was treated with glacial acetic acid (0.007% of added lysoPAF was converted to PAF during 1 h treatment). In any case, it is apparent that the acetyl moiety is directly transferred from aspirin to lysoPAF through a non-enzymatic process in the case of aspirin-induced PAF formation even in an aqueous solution.

Among various non-steroidal anti-inflammatory drugs, aspirin has a unique characteristic for transferring its acetyl moiety to other molecules. Various types of proteins are known to undergo non-enzymatic acetylation when treated with aspirin [11,12]. The acetylation of cyclooxygenase is of particular importance, because the acetylation of the enzyme protein results in complete loss of the enzyme activity [8]. In the case of cyclooxygenase, the hydroxy group of the serine residue is selectively acetylated by aspirin in a non-enzymatic manner [13]. Such irreversible inactivation of cyclooxygenase is responsible, at least in part, for the anti-inflammatory effects of aspirin observed in vivo.

Here we demonstrated that lysoPAF is also acetylated by aspirin to form PAF. Such a reaction takes place either in the presence or absence of an aqueous solution or organic solvent. To our knowledge, this is the first report on the non-enzymatic formation of PAF from aspirin and lysoPAF. In view of the facts that aspirin is very commonly used in medication and that PAF has powerful and diverse biological activities, it seems important to examine whether such non-enzymatic formation of PAF occurs under various circumstances. We observed that a commercial antifebrile drug, Mimizu Ippuh San, contains a considerable amount of PAF in addition to lysoPAF and aspirin. It seems very likely that most of the PAF present in this drug was formed from lysoPAF and aspirin through the non- enzymatic process described here.

On the other hand, it is not clear at present that non-enzymatic formation of PAF from lysoPAF and aspirin takes place even in vivo. Blood plasma is known to contain relatively high levels of lysophosphatidylcholine (lysoPC) (e.g. 130 nmol/ml, for rabbit plasma; Mabuchi-Itoh, K., unpublished result) which is assumed to be formed in large part through the action of lecithin:cholesterol acetyltransferase [14] and is usually composed of 1-acyl species [15]. Therefore, the 1-acyl analogue of PAF, which is known to be able to prime human neutrophils [16], may be produced if lysoPC has a chance to contact aspirin. In this case, the influence of serum proteins and serum lipids on the reaction should be carefully taken into consideration, because these other materials may interfere with the reaction to some extent.

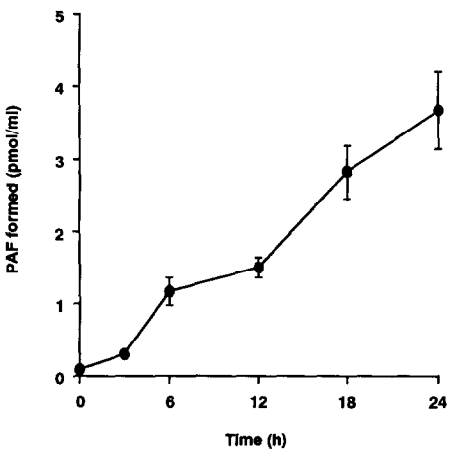

Fig. 4. Formation of PAF upon mixing of lysoPAF with aspirin in HEPES-buffered saline as a function of time. LysoPAF (100 μM) and aspirin (1 mM) were incubated in 0.4 ml of 5 mM HEPES-buffered saline (pH 7.4) at 37°C for the indicated periods. A portion of the mixture was then taken and added to washed rabbit platelets. The amounts of PAF were estimated as described in section 2. Values are the means ± S.D. from three determinations.

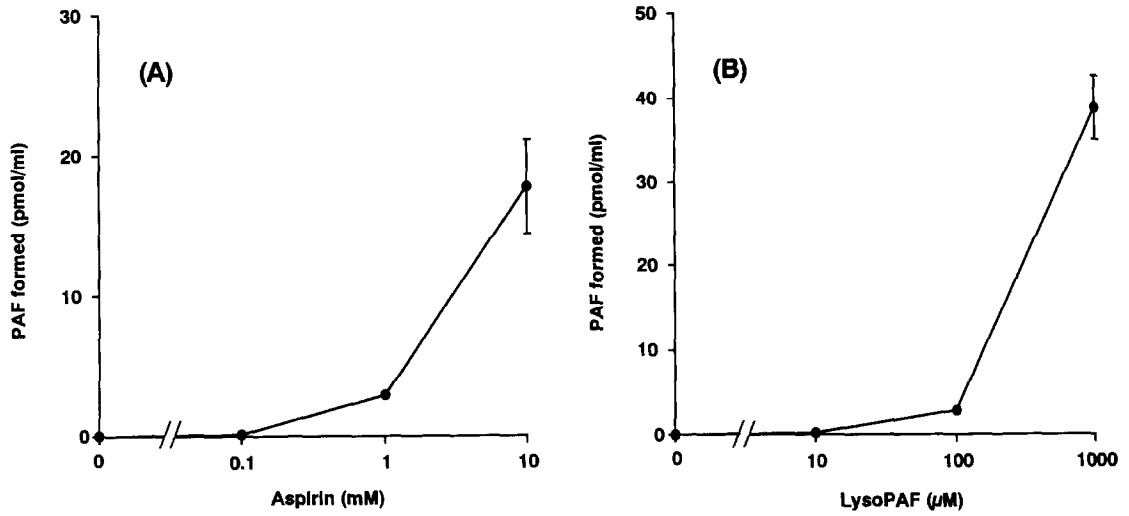

Fig. 5. Effects of different concentrations of aspirin (A) and lysoPAF (B) on the non-enzymatic formation of PAF. (A) Aspirin (0–10 mM) was incubated with lysoPAF (100 μM) in 0.4 ml of 5 mM HEPES-buffered saline (pH 7.4) at 37°C for 24 h. (B) LysoPAF (0–1 mM) was incubated with aspirin (1 mM) in the same buffer for 24 h. The amounts of PAF were estimated as described in section 2. Values are the means ± S.D. from three determinations.

Another possibility may lie in the case of the digestive system. Food-derived phospholipids are subjected to hydrolysis by phospholipases such as pancreatic phospholipase A_2. Such reactions could result in the formation and accumulation of lysophospholipids in the digestive system. If foods contain high levels of alkyl ether-containing PC, such as sea foods, including sea cucumbers, sea urchins and shellfish etc. [6,7], large amounts of lysoPAF may be generated during digestion. Therefore, a simultaneous intake of aspirin and foods enriched in alkyl ether-containing PC may result in the formation of PAF, even if it occurs transiently, in the digestive system. Thus, further detailed studies are indispensable to clarify whether non-enzymatic formation of PAF from lysoPAF and aspirin is actually of practical importance from pharmaceutical and pharmacological viewpoints.

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