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Biochemistry

Characterization of a platelet-activating factor receptor antagonist isolated from haifenteng (*Piper futokadsura*): Specific inhibition of *in vitro* and *in vivo* platelet-activating factor-induced effects

(platelet aggregation/neutrophil activation/lysosomal enzyme release/vascular permeability/Chinese herb)

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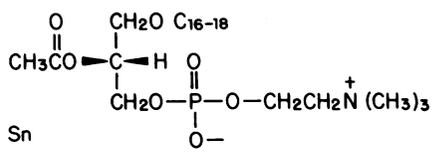
ABSTRACT **Platelet-activating factor (PAF) is a potent lipid mediator of inflammation and asthma. Using a receptor preparation of rabbit platelet membranes, we identified a novel antagonist of PAF in the methylene chloride extract of a Chinese herbal plant, haifenteng (*Piper futokadsura*). The active antagonist, kadsurenone, was isolated and characterized in several *in vitro* and *in vivo* assays. It is a specific and competitive inhibitor of PAF binding to its receptor with a K_i of 5.8 $\times 10^{-8}$ M vs. a K_i of 6.3 $\times 10^{-9}$ M for PAF itself. It inhibits PAF-induced aggregation of rabbit platelets and human neutrophils at 2.4–24 μM, without showing any PAF agonistic activity. It potently inhibits PAF-induced degranulation of human neutrophils at 2.5–50 μM, also without any agonist activity. Kadsurenone is active orally at 25–50 mg/kg of body weight in blocking PAF-induced cutaneous permeability in the guinea pig. It also inhibits PAF-induced increases of hematocrit and circulating *N*-acetylglucosaminidase in the rat at >10 mg/kg i.p. in a dose-dependent manner. Kadsurenone does not interfere with the function of several pharmacological mediators and receptors tested. Its structural specificity is evidenced by the poor PAF-antagonistic activities of three related structures isolated from the same haifenteng extract.**

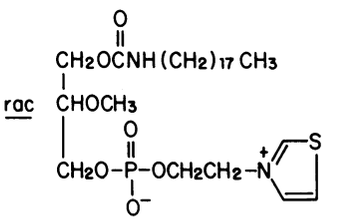
Platelet-activating factor (PAF, PAF-acether, or AGEPC) is a potent lipid mediator produced by stimulated basophils, neutrophils, platelets, macrophages, endothelial cells, and isolated tissue preparations (1). It is also released from IgE-sensitized bone marrow mast cells (2). Chemically, PAF has been identified as an unusual phospholipid derivative with the structure of 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine (structure I) (3–5). PAF was initially recognized by



Structure I

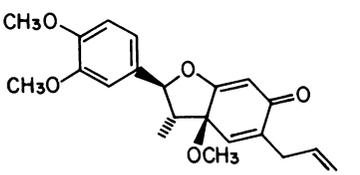
its great potency in causing the aggregation and degranulation of platelets at very low concentrations (1 pM to 10 nM) (1). Recent studies have shown that PAF exerts a myriad of biological actions *in vitro* and *in vivo* (6). *In vitro*, PAF induces smooth-muscle contraction, chemotaxis, aggregation, and degranulation of neutrophils and heightened metabolic activity of macrophages. It reduces coronary blood flow and contractile force of isolated guinea pig heart (7), leading to cardiac anaphylaxis (8). In various animal models, PAF induces thrombocytopenia, neutropenia, hypotension, bronchoconstriction, hyperalgesia, increased cutaneous vascular permeability, increased hematocrit (9, 10), and lysosomal enzyme secretion (10). In man, intradermal injection of PAF at 100 ng per site elicits a biphasic inflammatory response, which is potentiated by prostaglandin E_2, and further demonstrates the mediatory role of PAF in acute and persisting inflammation (11, 12).

The structural requirement for such potent biological actions is highly specific. Alterations of the chemical groups—e.g., the alkyl ether group at C_1, acetyl group at C_2, or phosphorylcholine at C_3—or the stereochemical configuration of the molecule invariably leads to significant reductions of potency (6). A phospholipid analog, CV-3988 (structure II),



Structure II

was recently reported to be a PAF antagonist (12). A cell-surface receptor that specifically binds PAF is generally believed to mediate various cellular responses to PAF. In our laboratory a rabbit platelet membrane preparation containing specific binding sites for PAF has been characterized (13). Such a preparation allows a search for PAF-receptor antagonists, which may inhibit the biological actions of PAF in various experimental systems. This paper describes the isolation and characterization of an orally active, specific PAF-receptor antagonist, kadsurenone (structure III), from



Structure III

a Chinese herbal plant, haifenteng. Kadsurenone at 0.1–1 μM potently and specifically inhibits PAF-induced platelet and neutrophil aggregation and degranulation. At an oral

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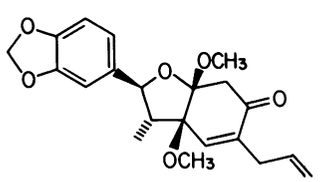
Abbreviations: PAF, platelet-activating factor; pA_2, negative logarithm of the molar concentration of an antagonist that reduces the effect of a double dose of PAF to that of a single dose.
‡Deceased Feb. 10, 1984.

dose of 25 mg/kg of body weight, this compound inhibits *in vivo* PAF-induced cutaneous vascular permeability. Intraperitoneal injection of kadsurenone (8–42 mg/kg) significantly inhibits the vascular permeability and lysosomal enzyme secretion elicited by PAF infused intravenously.

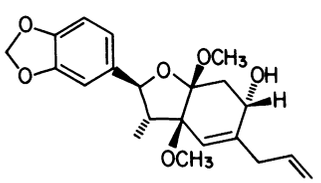
MATERIALS AND METHODS

Materials. The synthetic ^{3}H-labeled PAF, 1-*O*-[*alkyl*-1',2'-^{3}H]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine was purchased from New England Nuclear with a specific activity of 45 Ci/mmol (1 Ci = 37 GBq) and was stored at −80°C in ethanol/toluene, 50:50 (vol/vol). Unlabeled PAF was obtained from Calbiochem or Bachem Fine Chemicals (Torrance, CA).

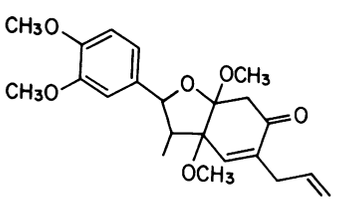
Haifenteng is an herbal preparation of the stem of *Piper futokadsura* Sieb and Zucc (Piperaceae), a medicinal plant grown in the southeast of China (Fu-Chien province) and Taiwan. The dried slices of haifenteng were ground into small pieces and extracted with methylene chloride. Evaporation of the solution yielded a brown gum that accounts for 2.3% of dry weight of haifenteng. The crude extract was fractionated by silica gel (kiesel gel 60, Merck) column chromatography with a hexane system containing increasing amounts of ethyl acetate. Fractions that showed inhibitory activity in the PAF-binding assay (see below) were further purified on a Waters Associates 500 high-performance liquid chromatograph using silica columns and were eluted with hexane/ethyl acetate, 3:1 (vol/vol), to yield kadsurenone (structure **III**) as a colorless syrup, two minor components, kadsurin A (structure **IV**) and kadsurin B (structure **V**), and a known compound, piperenone (structure **VI**). Details of the



Structure **IV**



Structure **V**



Structure **VI**

isolation procedure and physical and chemical characterizations of these compounds will be published elsewhere.

Methods. *Inhibition of PAF–receptor binding.* The inhibition of [^{3}H]PAF binding to the PAF receptor on isolated platelet plasma membranes was carried out by a filtration technique to separate the free and bound ligand. The difference between the total amounts of [^{3}H]PAF bound in the absence and in the presence of excess unlabeled PAF (×1000 excess) was defined as specific binding of [^{3}H]PAF. The percent inhibition of PAF–receptor binding in the presence of a known amount of compound was expressed as:

$$\% \text{ inhibition} = \frac{\text{total binding} - \text{total binding with compound}}{\text{specific binding}} \times 100\%.$$

The detailed procedure for purification of platelet plasma membranes, the characterization of the PAF-specific receptor, and the *in vitro* assay of the PAF–receptor binding have been published (13, 14).

Inhibition of rabbit platelet aggregation. Nine volumes of blood were drawn from the central ear artery directly into 1 volume of 3.8% sodium citrate solution. The blood was centrifuged at 270 × *g* for 10 min, and the top platelet-rich plasma was carefully removed. The erythrocyte pellet was further centrifuged at 1100 × *g* for 10 min. The supernatant (platelet-poor plasma) was used as the reference for platelet aggregation. Aggregation of platelets in plasma was monitored by measuring the changes in turbidity with a Chronolog Lumi-Aggregometer, model 400 (Havertown, PA) at 37°C. The percentage of platelet aggregation was calculated from the maximum transmittance change by assigning the transmittance of unstimulated platelet-rich plasma to be 0% and that of platelet-poor plasma to be 100%. The percent platelet aggregation was then plotted against the PAF concentration with or without the PAF antagonist. pA_2 was defined here as the negative logarithm of the molar concentration of an antagonist that reduces the effect of a double dose of PAF to that of a single dose.

Inhibition of PAF-induced aggregation and degranulation of isolated human neutrophils. Human neutrophils were isolated from freshly drawn blood by using the previously published procedure of dextran sedimentation, centrifugation through lymphocyte separation medium, and erythrocyte lysis (15). Neutrophil aggregation, similar to platelet aggregation (see the last section), was monitored with a Chronolog Lumi-Aggregometer at 37°C. Percent aggregation was calculated as above except that Hanks' bovine serum albumin solution was used as the reference. For neutrophil degranulation, the isolated neutrophils (3.1×10^6 cells per ml per tube) in Hanks' balanced salt solution (0.5% albumin/5 μg of cytochalasin B per ml/0.6 mM $CaCl_2$/1.0 mM $MgCl_2$) were preincubated at 37°C for 10 min, during which time the test compound in dimethyl sulfoxide was added. Final dimethyl sulfoxide concentration was 0.5% and was not harmful to the cells. PAF was then added for a further 10-min incubation. The neutrophils were then pelleted, and the supernatants were retained for subsequent enzyme assay. Lactate dehydrogenase (16) was determined as a measure of cytotoxicity, and myeloperoxidase (17) and β-glucuronidase (18) were measured as indicators of degranulation.

In vivo *inhibition of PAF-induced cutaneous vascular permeability in guinea pigs.* PAF-induced cutaneous vascular permeability was measured by the skin-blueing method as described by Humphrey *et al.* (19), except that the exuded Evans blue at the injection site was extracted with a mixture of 14 ml of acetone and 6 ml of 0.5% aqueous solution of sodium sulfate (20). Thus, the skin edema, or the amount of Evans blue in each injection site, can be expressed quantitatively as the volume of the exuded plasma by comparing the dye content in the plasma. Detailed procedures will be published elsewhere. The test compound was suspended in 3 ml of CM 438 (0.9% NaCl/0.5% carboxymethylcellulose/0.4% Tween 80/0.9% benzyl alcohol) and was given orally 1 hr before PAF challenge. An equal volume of CM 438 was given alone to the control animals.

The percent inhibition of PAF-induced cutaneous vascular permeability was calculated from the paper weight under the

skin edema dose–response curve of the control animal (A_C) and the paper weight under the plasma exudation curve of the experimental animal (A_D) according to the following equation:

$$\% \text{ inhibition} = \frac{A_C - A_D}{A_C} \times 100\%.$$

Inhibition of PAF-induced vascular permeability and enzyme release in rats. Female Wistar rats (Charles River Breeding Laboratories; 190–210 g) were given an i.p. injection of 0.2 ml of kadsurenone dissolved in polyethylene glycol 400 or 0.2 ml of polyethylene glycol alone for controls. Fifteen minutes later, the rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). Thirty minutes after kadsurenone or control injection, a basal blood sample was taken from a 2.5-cm 23-gauge needle inserted into the tail vein, and 10 nmol of PAF/kg in 0.5 ml were then infused into the tail vein with the same needle. A second blood sample was taken by cardiac puncture 11 min after PAF infusion, the time of maximum increase in hematocrit and enzyme secretion. For both basal and 11-min blood samples, the hematocrit, as a measure of vascular permeability, and the plasma *N*-acetylglucosaminidase (18), as a measure of lysosomal enzyme secretion, were determined.

RESULTS

Inhibition of PAF-Receptor Binding. Fig. 1 shows the percent inhibition of [^{3}H]PAF binding to the PAF receptor on isolated platelet plasma membranes in the presence of kadsurenone. The IC_{50} of kadsurenone was calculated to be 0.1 μM. The equilibrium dissociation constant (K_d) for [^{3}H]PAF binding to its specific receptor site in the same membrane preparation under the same experimental conditions was $1.36 \pm 0.05 \times 10^{-9}$ M at 0°C (13). By assuming competitive inhibition, the inhibition coefficient (K_i) of kadsurenone is 5.8×10^{-8} M according to the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[^3\text{H}]\text{PAF}}{K_d}},$$

in which [^{3}H]PAF is the concentration (1 nM) used in the receptor binding inhibition. To show the structural specificity of kadsurenone in the inhibition of PAF-receptor binding, several related compounds isolated from the same plant were tested also in the same assay. Piperenone (5), a 7α-methoxy analog of kadsurenone, showed 25% inhibition at 1 μM. Kadsurin A (4) and kadsurin B (5) were also weakly

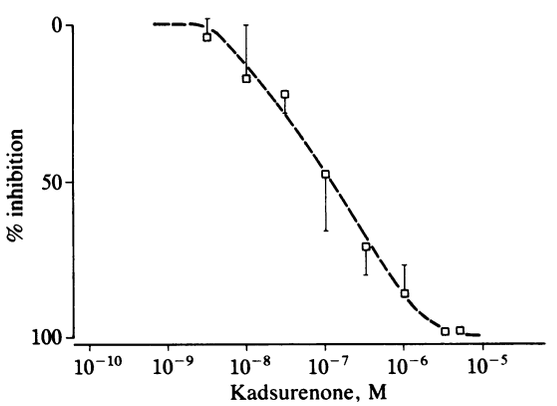


FIG. 1. Inhibition of PAF-specific receptor binding by kadsurenone. Each data point is the average of results from three independent experiments, and each sample was performed in triplicate in each experiment. Error bars show the standard deviation.

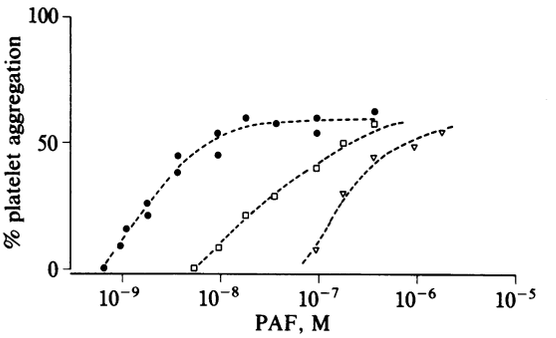


FIG. 2. Inhibition of PAF-induced aggregation of rabbit platelets in plasma. Dose–response curves are shown in the absence of kadsurenone (control) (●) and in the presence of kadsurenone at 2.4 μM (□) and at 24 μM (△).

active, both showing 25% inhibition only at 3 μM, as compared to 90% inhibition by kadsurenone at 1 μM.

Inhibition of Aggregation of Rabbit Platelets in Plasma. Kadsurenone inhibited aggregation of rabbit platelets in plasma effectively (Fig. 2). PAF induced platelet aggregation with a half-maximum response (30%, the maximum response is 60% aggregation in Fig. 2) at 2.2 nM. In the presence of 24 μM kadsurenone, the PAF concentration required to obtain the same degree of aggregation increased almost 100-fold to 200 nM. By reducing the amount of kadsurenone to 2.4 μM, the PAF concentration required to obtain 30% aggregation was shifted to about 27 nM. The pA_2 of kadsurenone to inhibit the aggregation of rabbit platelet in plasma calculated from Fig. 2 is 6.28. In all experiments addition of kadsurenone, prior to the addition of PAF, did not cause any platelet aggregation.

Inhibition of Aggregation of Human Neutrophils. At the high PAF concentration of 1 μM, <10% (about 1/10 of the transmittance change of platelet aggregation) scattering change was observed in the aggregometer (Fig. 3). This is due to the low number of cells per aggregate in the case of neutrophils. In this assay, ≈4 nM PAF was required to produce 50% of the maximum aggregation of isolated human neutrophils. In the presence of 24 μM kadsurenone, the concentration of PAF needed to obtain an equivalent degree of aggregation was increased ≈100-fold to 400 nM. The pA_2 was calculated to be 6.32 for kadsurenone in the inhibition of PAF-induced isolated human neutrophil aggregation. This value is very close to the pA_2 for the inhibition of rabbit platelet aggregation (6.28).

Inhibition of PAF-Induced Degranulation of Isolated Human Neutrophils. Kadsurenone potently inhibited the degranulation of isolated human neutrophils stimulated with different concentrations of PAF (Fig. 4). The IC_{50}s for kad-

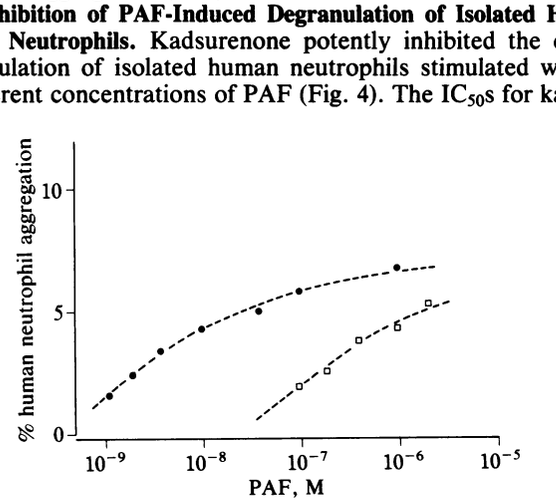


FIG. 3. Kadsurenone inhibition of PAF-induced human neutrophil aggregation. ●, Control; □, in the presence of 24 μM kadsurenone.

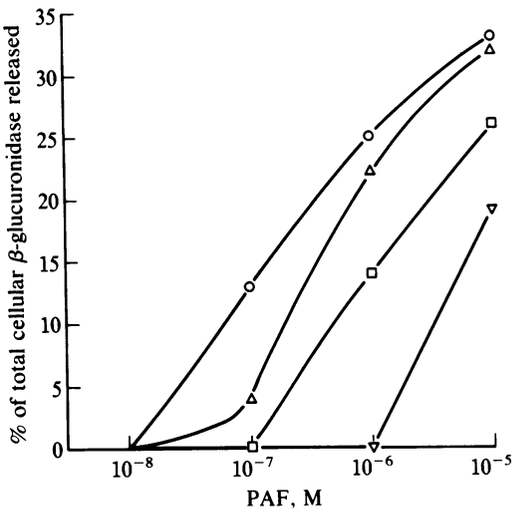


FIG. 4. Inhibition of PAF-induced human neutrophil degranulation by kadsurenone. Neutrophils were isolated and incubated as described. The concentrations of kadsurenone are 0 μM (○), 2.5 μM (△), 12.5 μM (□), and 50 μM (▽).

surenone were 2.3 μM with 0.1 μM PAF and 14.5 μM with 1 μM PAF. Almost identical results were obtained regardless of whether secretion of β-glucuronidase or myeloperoxidase was followed. Kadsurenone did not affect lactate dehydrogenase secretion, which was low (<7% of total) for all samples, indicating that kadsurenone was not cytotoxic in this assay. This PAF-inhibitory effect of kadsurenone on neutrophils appears specific as 10 μM levels of this compound did not exhibit any inhibition of the extensive neutrophil degranulation induced by high concentrations of the known neutrophil-degranulating agents precipitating immune complexes, fMet-Leu-Phe, or the calcium ionophore A23187.

Inhibition of PAF-Induced Cutaneous Vascular Permeability. In the guinea pig, intradermal injection of 1 pmol of PAF in 0.1 ml of solution A (150 mM NaCl/10 mM Tris/0.25% bovine serum albumin, pH 7.5) resulted in the exudation of ≈40 μl of plasma at the injection site, which was significantly larger than that produced by the vehicle alone (≈16 μl, including the background). The amount of plasma exudation

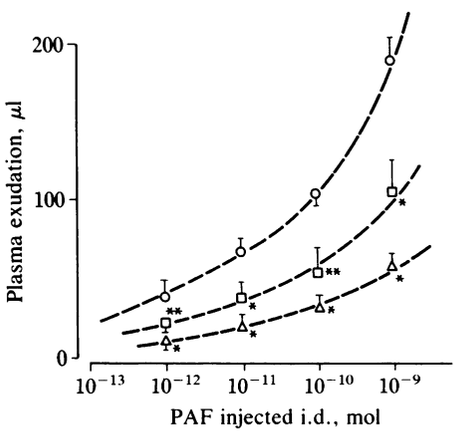


FIG. 5. Inhibition by kadsurenone of PAF-induced guinea pig cutaneous vascular permeability. Guinea pigs were pretreated orally with kadsurenone 1 hr before intradermal injection of PAF. Each animal received duplicate injections of 0.1 ml containing 5×10^{-10} g, 5×10^{-9} g, 5×10^{-8} g, and 5×10^{-7} g of PAF (C_{16}) for both control and treated guinea pigs. Each data point is the average of data from 2–5 guinea pigs, and the error bar is the standard error. Dose–response curves are shown in the absence of kadsurenone (control) (○) and in the presence of kadsurenone at 25 mg/kg (□) and at 50 mg/kg (△). *, $P < 0.005$; **, $P < 0.01$.

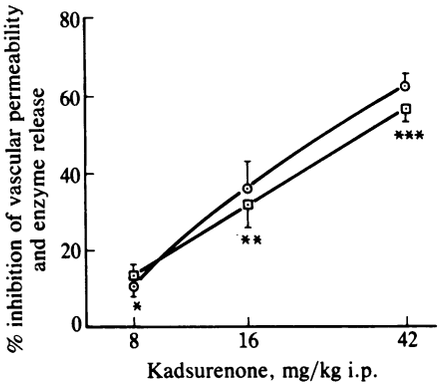


FIG. 6. Kadsurenone inhibition of i.v. PAF-induced vascular permeability (○) and enzyme release (□) *in vivo* in rats. Kadsurenone was completely dissolved in polyethylene glycol 400 and then given as an i.p. injection to rats 30 min before an i.v. PAF infusion (four rats per dose level and controls). The individual points are the mean ± SEM. The results at the different dose levels are significantly different from the control responses according to the following *P* values: $P < 0.0001$ (***), $P < 0.01$ (**), and $P < 0.1$ (*).

increased with increasing intradermal dosage of PAF, up to ≈200 μl of plasma at 1 nmol of PAF per injection site (Fig. 5). Oral administration of kadsurenone at 25 and 50 mg/kg gave 40% and 60% inhibition of PAF-induced cutaneous vascular permeability, respectively.

Inhibition by Kadsurenone of i.v. PAF-Induced Vascular Permeability and Enzyme Release *in Vivo* in Rats. Intravenous infusion of nanomole quantities of PAF into rats induces dramatic increases in vascular permeability throughout the circulation as evidenced by large hematocrit increases (9, 10) and stimulates lysosomal enzyme secretion in the blood as indicated by increased plasma *N*-acetylglucosaminidase activity (10). Intraperitoneal injection of different amounts of kadsurenone (8–42 mg/kg) significantly inhibited the PAF-induced hematocrit increase and *N*-acetylglucosaminidase secretion in a dose-dependent manner (Fig. 6). For the control rats, PAF induced a hematocrit increase from a mean of 45 to 72 and an increase in the plasma activity of *N*-acetylglucosaminidase from a mean of 480 to 806 nmol/hr per ml.

DISCUSSION

PAF is a recently described mediator derived from membrane phospholipids of many cell types under a variety of pathological conditions. Its pronounced biological effects are comparable to those of leukotrienes and prostaglandins produced in the arachidonic acid cascade but often at much lower concentrations. It is also of interest to note that ether phospholipids constitute a significant portion of the total phospholipids in neutrophils (21), lymphocytes (22), macrophages (23, 24), and platelets (25)—cells that play dominant roles in inflammatory and hypersensitivity responses. A major portion of these ether phospholipids carry an arachidonyl group at the C-2 position (26, 27) and, thus, may serve as a common source for the two substrates, arachidonic acid and lyso-PAF, for the arachidonic acid cascade and PAF biosynthesis, respectively. In addition, prostaglandins, leukotrienes, and PAF may potentiate the cellular and vascular effects of each other. PAF also has been shown to activate the 5-lipoxygenase pathway in the guinea pig lung, macrophages, and neutrophils.

To delineate the dynamic roles of PAF and to modulate its actions in various pathological conditions, obviously it would be highly desirable to have a specific and *in vivo* active receptor antagonist. The rabbit platelet membrane re-

ceptor for PAF used in this study has been well characterized (13). The binding of [^{3}H]PAF to this receptor preparation is highly specific, as it is only minimally affected by stereoisomers or close analogs of PAF or by several representatives of pharmacological agents (e.g., cyproheptadine, pyrilamine, cimetidine, and indomethacin). The physiological significance of this receptor preparation was demonstrated by a parallel and semiquantitative correlation of receptor binding and platelet activation for a series of closely related PAF analogs and putative receptor antagonists including kadsurenone (unpublished data). Kadsurenone was shown to have specificity for the PAF receptor as it did not interfere with the ligand-binding or with the function of receptors for histamine (H_1), leukotriene D_4, or benzodiazepine. It is also inactive as an inhibitor in several enzymatic systems (e.g., cyclooxygenase, 5-lipoxygenase, etc.). The structural specificity of kadsurenone was illustrated by the low PAF–receptor blocking activity of three closely related derivatives, piperenone and kadsurin A and B, isolated from the same plant extract. Kadsurenone is a competitive inhibitor of PAF binding to its receptor with a K_i of 5.8×10^{-8} M, $\approx$10 times higher than that for PAF itself ($K_i = 6.3 \times 10^{-9}$ M; unpublished data). Kadsurenone effectively inhibits the aggregation of rabbit platelets induced by PAF. In these experiments, prior to the addition of PAF, the presence of 2.4–24 μM of kadsurenone apparently has no effect on platelets *per se*. This shows that, despite its high affinity for the PAF receptor, kadsurenone does not possess any significant PAF agonistic activity. The *in vivo* activity of kadsurenone after oral or parenteral administration in several animal models at 25–50 mg/kg further substantiated its PAF-antagonistic property. While some of the *in vivo* effects of PAF have been attributed to its activation of the 5-lipoxygenase pathway, which generates leukotrienes as mediators, the *in vivo* effect of kadsurenone apparently does not involve this mechanism. Since the vascular permeability and enzyme release induced in rats is minimally inhibited by inhibitors of cyclooxygenase or lipoxygenase or receptor antagonists of histamine (H_1 and H_2) or serotonin (10), the substantial inhibitory action of kadsurenone in this model constitutes a clear indication of its *in vivo* PAF antagonism.

Haifenteng is a Chinese herbal preparation of *P. futokadsurae* for the general relief of bronchoasthma and the stiffness, inflammation, and pain of rheumatic conditions. It is interesting that an active ingredient of this traditional remedy has now been identified as a novel receptor antagonist for a potent lipid mediator whose structure was elucidated only 5 years ago.

The authors are grateful to Dr. Harry Dougherty for advice on human neutrophil isolation and myeloperoxidase assay.

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