Papyriflavonol A from *Broussonetia papyrifera* Inhibits the Passive Cutaneous Anaphylaxis Reaction and Has a Secretory Phospholipase A₂-Inhibitory Activity

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Papyriflavonol A, a new prenylated flavon isolated from *Broussonetia papyrifera*, selectively inhibits recombinant human secretory phospholipase A₂ (sPLA₂)s. Papyriflavonol A was found to inhibit human group IIA and V sPLA₂s potently and irreversibly in a dose-dependent manner, with respective IC₅₀ values of 3.9 and 4.5 μM. The inhibitory effects of papyriflavonol A against bovine group IB (IC₅₀ of 76.9 μM) and the human group X (IC₅₀ of 225 μM) sPLA₂s were weaker than those against human group IIA and V sPLA₂s, and human group IIF sPLA₂ was not inhibited. In addition, papyriflavonol A potently inhibited the stimulus-induced production of leukotriene C₄ with an IC₅₀ value of approximately 0.64 μM in mouse bone marrow-derived mast cells. In addition, papyriflavonol A significantly reduced IgE-dependent passive cutaneous anaphylaxis in rats. These results indicate that papyriflavonol A provides a basis for novel types of antiinflammatory drugs.

Key words: papyriflavonol A; phospholipase A₂; leukotriene C₄; passive cutaneous anaphylaxis

Phospholipase A₂ (PLA₂) is a growing family of distinct enzymes that exhibits different substrate specificities, cofactor requirements, subcellular localization, and cellular functions. ¹ Secretory PLA₂s (sPLA₂)s have low molecular weights (14–18 kDa) with a rigid tertiary structure configured by 6–8 disulfide bridges. They require millimolar concentrations of Ca²⁺ to exert their enzymatic action and have little fatty acid selectivity when assayed *in vitro*. ² Thus far, 10 genes coding for structurally related and enzymatically active sPLA₂s have been identified in mammals (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII). ³–⁷

Group IB sPLA₂ (sPLA₂-IB), known as pancreatic PLA₂, is abundant in the pancreatic juice, where it catalyzes the breakdown of dietary phospholipids. Group IIA sPLA₂ (sPLA₂-IIA), known as an inflammatory PLA₂, is expressed in a variety of tissues and is markedly induced following challenge with proinflammatory stimuli ⁸–¹⁰ sPLA₂-IIA is thought to play a role in inflammation, ¹¹,¹² exocytosis, ¹³,¹⁴ blood coagulation, ¹⁵ and atherosclerosis. ¹⁶,¹⁷ sPLA₂-IIA involvement in the inflammatory process has also been investigated by the administration of particular inhibitors or antibodies that are specifically to sPLA₂-IIA into inflamed sites. For example, injecting a monoclonal antibody that neutralizes sPLA₂-IIA activity into rats with carrageenan-induced pleurisy reduced the pleural exudate volume and the intrapleural leukocyte number significantly. ¹⁸ Group V sPLA₂ (sPLA₂-V) is expressed mainly in rats and the human heart ¹⁹ and various mouse tissues. ²⁰ This enzyme may compensate for sPLA₂-IIA under certain conditions since it is induced in many tissues by proinflammatory agents ²⁰,²¹ in a similar manner as sPLA₂-IIA ⁸–¹⁰ sPLA₂-IIA and -V, the genes for which are clustered on the same chromosome locus, share several enzymatic and functional features. ²²,²³ These enzymes are therefore referred to as the group II sub-family of sPLA₂, into which several novel sPLA₂ enzymes, including group IIC, IID, IIE and IIF, are also classified. ³–⁶ Group X sPLA₂ (sPLA₂-X) is constitutively expressed in the immune and digestive organs. ²⁴ Current cellular biological evidence suggests that sPLA₂-IIA, -IID, -V, and -X are capable of promoting cellular arachidonate release. ⁹,¹⁰,¹²–¹⁵,²⁵,²⁶ This fact, together with the observation that sPLA₂s belongs to the group II subfamily (IIA, IID, IIE, and V) are stimulus inducible, ³,⁶,⁸–¹⁰,²¹ implies the possible involvement of these enzymes in the inflammatory process. If this is the case, one might anticipate that the inhibition of the inducible group II subfamily of sPLA₂s can attenuate the severity of inflammation.

Prenylated flavonoids have a limited distribution in the plant kingdom, i.e., the Moraceae. Most of these plants have various types of prenylated flavonoids as major constituents and have been used as antiinflammatory agents from antiquity. ²⁷ These molecules have the chemical constituents with a limited distribution in the plant kingdom, i.e., the Moraceae. Most of these plants have various types of prenylated flavonoids as major constituents and have been used as antiinflammatory agents from antiquity. ²⁷ These molecules have the chemical constituents with an isoprenyl (3,3-dimethylallyl), geranyl (E-3,7-dimethyl-2,6-octadienyl), 1,1-dimethylallyl, and/or lavandulyl (5-methyl-2-isoprophenyl-hex-4-enyl) moiety added to their flavonoid backbone structure. ²⁸ During investigations of the effects of various flavonoid derivatives on sPLA₂s, papyriflavonol A isolated from *Broussonetia papyrifera* was found to inhibit sPLA₂-IIA and -V more potently than other sPLA₂ enzymes. The inactivation patterns of various mammalian sPLA₂s *in vitro* by this new prenylated flavonoid and its antiinflammatory activity *in vivo* were also examined.

MATERIALS AND METHODS

**Enzyme Sources** The cDNAs for human sPLA₂-IIA, -V, and -X were cloned into an expression vector and transfected...
into human embryonic kidney 293 cells (HEK293 cells) using LipofecAMINE PLUS (Gibco BRL, Gaithersburg, MD, U.S.A.) as described previously.22,23) Human sPLA2-IIIF was expressed by the baculovirus/insect cell expression system (Murakami et al., unpublished results). The culture supernatants were used as enzyme sources. Bovine porcine pancreatic sPLA2-IIB was purchased from Boehringer (Mannheim, Germany).

Assay of Phospholipase A₂ Inhibitory Activity by Papyriflavonol A  The standard reaction mixture (200 μl) contained 100 mM Tris–HCl (pH 9.0), 6 mM CaCl₂, 1% bovine serum albumin, 2.5 μM of radiolabeled 1-acyl-2-[1-14C] arachidonyl-sn-glycerol phosphoethanolamine (48 mCi/m mole, NEN, Boston, MA, U.S.A.), and papyriflavonol A. The reaction was started by the addition of an aliquot of the culture medium as an enzyme source and carried out at 37 °C for 20 min, and 14C arachidonic acid released was extracted from the medium as an enzyme source and carried out at 37 °C for 20 min, and [14C] arachidonic acid released was extracted by the method described previously.11) Under these conditions, the reaction mixture without papyriflavonol A released 10% free fatty acid. Inhibition was expressed as a percentage; with enzyme control as 100% reaction, i.e., 0% inhibition. Papyriflavonol A was dissolved in dimethylsulfoxide (DMSO) and added to the enzyme assay tubes at 2% of the final volume. Control experiments showed that DMSO at concentrations up to 2% had no effect on enzymatic activity. All data are expressed as the mean of duplicate determinations.

Preparation of Mouse Bone Marrow-Derived Mast Cells Bone marrow cells from male BALB/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 100 units/ml penicillin, 100 μg/ml of streptomycin, 10 mg/ml of gentamycin, 2 μM of L-glutamine, 0.1 mM of nonessential amino acids, and 10% fetal calf serum) and a 50% WEHI-3 cell-conditioned medium as a source of interleukin-3. After 3 weeks, more than 98% of the cells in the culture were bone marrow-derived mast cells (BMMCs)29–31.

Determination of Leukotriene C₄  Leukotriene C₄ production was determined using an LTC₄ enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer’s instructions. BMMC suspended in enriched medium at cell density of 1×10⁶ cells/ml were pre-treated with papyriflavonol A for 30 min at 37 °C and stimulated with stem cell factor (SCF; 100 ng/ml), which was re-combinantly expressed by the baculovirus/insect cell expression system.29–31) After 20-min stimulation, the supernatants were isolated for further analysis by enzyme immunoassay. Papyriflavonol A was solved in DMSO and added to the cells at 0.05% of the final volume.

Passive Cutaneous Anaphylaxis  Male Sprague–Dawley rats weighing 180–200 g (Hyochang Science, Daegu, Korea) were used in the passive cutaneous anaphylaxis test. The animals were housed in cages at least 1 week before the experiments. In brief, 100 μl/site of monoclonal anti-DNP mouse IgE (1:1000 dilution; Seikagaku-Kougyo, Japan) was intra-dermally injected in the shaved back of the rat. After 48 h, PCA was evoked by intravenous injection of the antigen (Ag; 1 mg of DNP-BSA; Sigma Chemical, St. Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) containing 1 ml of 1% Evans blue (1:4). Papyriflavonol A (12.5 to 50 mg/kg body weight, i.p.) was administered 10 h before Ag challenge. After 30 min, the skin was removed and the dye leaking into the skin was extracted and quantified, as described previously.32)

RESULTS

Papyriflavonol A Inhibits Basic Group II Subfamily sPLA₂s in Vitro  A comparative test was performed to determine how papyriflavonol A (Fig. 1) affects various mammalian sPLA₂s. Figure 2 shows that papyriflavonol A inhibits recombinant human sPLA₂-IIA and -V (IC₅₀ of ca. 3.9 and ca. 4.5 μM respectively) more potently than bovine pancreatic sPLA₂-IIB (IC₅₀ of ca. 76.9 μM). Inhibition of sPLA₂-X was not inhibited by papyriflavonol A even at 1 mM. Thus the inhibitory effect of papyriflavonol A was in the order of II A>V>I B>X>II F. This suggests that papyriflavonol A inhibits basic sPLA₂s in preference to neutral and acidic sPLA₂s.

Papyriflavonol A Inhibits LTC₄ Production by Mast Cells  BMMCs stimulated for 15 min with SCF produced ca. 500 pg/ml of LTC₄, and preincubation of cells with papyriflavonol A resulted in the dose-dependent suppression of LTC₄ biosynthesis with an IC₅₀ value of 0.65 μM and with almost complete inhibition at 7.2 μM (Fig. 3). This particularly low IC₅₀ value may reflect the inhibition of two regulatory steps for LTC₄ biosynthesis, namely arachidonate release by sPLA₂-V and its conversion to LTs by 5-lipoxygenase (5-LO), by papyriflavonol A.

Papyriflavonol A Inhibits the PCA Reaction in Vivo  To explore the antiinflammatory activity of papyriflavonol A in vivo, the effect of this compound on IgE-induced PCA was examined in a rat model (Fig. 4). Papyriflavonol A showed...
potent antiinflammatory activity.

DISCUSSION

Extracellular sPLA2-IIA activity has been detected in the inflamed sites of several experimental animal models, and in human diseases such as pancreatitis and rheumatoid arthritis. The correlation between sPLA2-IIA levels and disease status has led to the development of pharmacological agents that are capable of inhibiting sPLA2-IIA and possibly the activities of other sPLA2, which would be expected to be beneficial for the mitigation of disease. As a result of our ongoing efforts to develop new sPLA2 inhibitors from natural products using an activity-guided isolation procedure, we isolated a new human sPLA2-IIA and -V inhibitor, papyriflavonol A, from B. papyrifera. Although the primary structures of the sPLA2 isozymes show some homology (30—50%), they contain two well-conserved regions comprising the “active site” and “the Ca$^{2+}$-binding site.” It is noteworthy that the IC$_{50}$ values of papyriflavonol A and of sPLA2-IIA and sPLA2-V are almost the same sPLA2-IIA and sPLA2-V exhibit redundant functions under certain conditions and their genes are clustered in the same chromosome locus, whereas sPLA2-IB and sPLA2-X are more distant. 2) sPLA2-IIF is unique (a region where basic amino acids are clustered; see below) among the group II subfamily of sPLA2s in that it is an acidic protein and has an unusually long C-terminal extension. 5) Papyriflavonol A may recognize the subtle structural differences between these enzymes and bind preferentially to a unique region present only in sPLA2-IIA and sPLA2-V, because it exhibits stronger inhibitory activity against sPLA2-IIA and -V than against sPLA2-IB, -X, and -IIF.

It is worth noting that papyriflavonol A can carry negative charges in neutral or alkaline solution because of the presence of multiple phenolic hydroxyl groups (Fig. 1). Both sPLA2-IIA and -V are highly cationic proteins, whereas sPLA2-IB is neutral and both sPLA2-X and -IIF are acidic (the pI values for sPLA2-IIA, V, IB, X, and IIIF were found to be 9.4, 8.7, 6.9, 5.1, and 4.7, respectively). 3) Several basic amino acids in human sPLA2-IIA (and probably in -V) are essential for its interfacial recognition, and therefore the attendant hydrolysis, of negatively charged substrates. 3) It is therefore conceivable that papyriflavonol A binds preferentially to basic sPLA2s via a charge-based interaction, whereas it binds poorly to the charge-neutral sPLA2-IB and acidic sPLA2-X and -IIF.

Papyriflavonol A was recently reported to exert an inhibitory effect on 5-LO, but not the cyclooxygenases (COX), with an IC$_{50}$ of 7 µM (when homogenates of rat polymorphonuclear mononuclear cells were used as the enzyme source). However, whether papyriflavonol A has an inhibitory effect on LT production at the cellular level, or whether it has antiinflammatory activity in vivo has not been reported. Therefore the effect of papyriflavonol A on LTC$_4$ generation in BMMCs was examined. This cell model appears to be suitable for assessing the effect of papyriflavonol A, since immediate LTC$_4$ generation elicited by IgE-dependent or cytokine-initiated stimulus occurs in BMMCs through the functional coupling between sPLA2-V in cooperation with cPLA2 and 5-LO.

As shown in Fig. 4, the suppression of the IgE/anti-IgE-induced PCA reaction by papyriflavonol A is dose dependent, and an injection of 12.5 mg/kg of papyriflavonol A 10 h before Ag challenge significantly reduced this reaction. However, its in vivo dynamics and metabolism await future study. Our results suggest that sPLA2s may be involved in the PCA reaction by augmenting LTC$_4$ production by tissue mast cells (or other inflammatory cells), and that papyriflavonol A blocks that process. However, considering the general concept that the participation of COX or 5-LO products in the PCA reaction in the rat is relatively weak, we cannot rule out the possibility that papyriflavonol A can affect other sPLA2-regulatory processes, such as histamine release. We have previously shown that sPLA2s (sPLA2-V in particular) have the ability to augment both histamine release and LTC$_4$ production by mast cells. Although our preliminary data demonstrated that degranulation of BMMCs was suppressed only minimally by papyriflavonol A in vitro (data not shown), it is possible that degranulation of rat skin mast cells (connective tissue-type mast cells) is susceptible to sPLA2s (and is therefore suppressed by papyriflavonol A), since histamine release from connective tissue-type mast cells, but not from BMMCs, is markedly enhanced by lyosphosphatidylserine, an sPLA2 reaction product. 4) The more profound inhibitory effect of papyriflavonol A on LTC$_4$ production by mast cells than on in vitro sPLA2 activity suggests that the inhibition of 5-LO activity by papyriflavonol A also contributes to reduced LTC$_4$ production. Nonetheless, our present results provide a new insight into the role of sPLA2 in acute allergic re-
action and demonstrate the potential of papyriflavonol A to serve as a leading developmental compound for novel antiinflammatory and antiallergic drugs.

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