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Inactivation of Phospholipase A_2 by Naturally Occurring Biflavonoid, Ochnaflavone

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SUMMARY: Ochnaflavone, a medicinal herb product isolated from *Lonicera japonica*, strongly inhibited rat platelet phospholipase A_2 (IC_{50}, about 3μM). Inactivation was concentration and pH dependent (maximum inactivation occurred between pH 9.0 and 10.0). Ochnaflavone inhibited the enzyme by a noncompetitive manner, with the apparent *Ki* value of 3×10^{-5} M. Reversibility was studied directly by dialysis method; the inhibition was irreversible. In addition, the inhibitory activity of ochnaflavone is rather specific against group II phospholipase A_2 than group I phospholipase A_2(IC_{50}, about 20μM). Addition of excess Ca^{2+} concentration up to 8mM did not antagonize the inhibitory activity of ochnaflavone. These results indicate that the inhibition of phospholipase A_2 by ochnaflvone may result from direct interaction with the enzyme.

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that specifically hydrolyzes the *sn*-2 position of a glycerophospholipid to produce fatty acid , e.g. arachidonic acid(AA), and lysophospholipids. The released AA is converted to prostaglandins, thromboxanes, and leucotrienes (1). PLA_2 exists in both extracellular and intracellular forms (2). The former can be classified into two types, group I and group II, based on their primary structures (3). Group II PLA_2 activities have been detected in inflammatory sites in some experimental animals and also in human diseases, such as glycogen-induced ascitic fluid in rabbits (4), casein-induced peritoneal fluid in rats (5), synovial fluid of patients with rheumatoid arthritis (6), and pleural fluid of patients with tuberculosis(7). In addition, some inflammatory cytokines and lipopolysaccharides dramatically increase group II PLA_2 secretion in several

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tissues of rat through enhancement of gene transcription (8). Recently, Bomalaski et al. (9) reported that the recombinant enzyme of human group II PLA_2 elicits a dramatic inflammatory arthritogenic response when injected into the joint space of healthy rabbits. These findings strongly implicate the importance of group II PLA_2 in the development and possibly in the propagation of inflammatory processes. If such is the case, one might anticipate that inhibition of group II PLA_2 would attenuate the severity of inflammation.

With the use of group II PLA_2 purified from rat platelet, we successfully isolated a new type of PLA_2 inhibitor, ochnaflavone, from the aerial part of *Lonicera japonica*. The present study describes the inactivation mechanism of rat platelet PLA_2 by ochnaflavone.

MATERIALS AND METHODS

Materials:

Ochnaflavone and its methylated derivative were isolated from the aerial part of *Lonicera japonica*. Amentoflavone and its methylated derivatives were isolated the leaves of *Ginko biloba*. Cryptomerin B and isocryptomerin were isolated from the whole plant of *Selaginella tamariscine*. [1-^{14}C]Linoleic acid was purchased from Amersham, U.K. 1-Acyl-2-[1-^{14}C]linoleoyl-*sn*-glycerophosphoethanolamine was prepared by the method described previously (10). Porcine pancreatic phospholipase A_2 was purchased from Boeringer Mannheim Biochemica, Germany. The 14KDa group II phospholipase A_2 were purified from rat platelet (11), and human pleural fluids (12), respectively.

Assay of phospholipase A_2 inhibitory activity by ochnaflavone:

The standard reaction mixture (200 μl), which contained 100mM Tris-HCl(pH9.0), 6mM $CaCl_2$, 20nmole 1-Acyl-2-[1-^{14}C]linoleoyl-*sn*-glycerophosphoethanolamine (1000 cpm/nmole), and ochnaflavone. The reaction was started by addition of 10ng phospholipase A_2. The reaction was carried out at 37°C for 20min and free fatty acid generated was analyzed by the method described previously (13). Under these condition, reaction mixture without the ochnaflavone revealed release of 10% of free fatty acid. Inhibition is expressed as a percent; with enzyme control as 100% reaction, *i.e.*, 0% inhibition. Both ochnaflavone and other biflavonoids were added to assay tube as DMSO solution (2% of the final volume), using a DMSO-enzyme control. Control experiments showed that DMSO at concentration up to 2% has no effect on enzymatic activities. All data are the mean of duplicate determinations.

Dialysis studies:

Rat platelet phospholipase A_2 (150ng) was incubated with 90μM of ochnaflavone as mentioned above for 30min. Equal volume (1ml) were placed in two separated dialysis cellulose tubing with a molecular weight cut off 6000-8000Da. One bag was dialyzed with 1:1000 ratio of enzyme mixture to buffer (20mM Tris-HCl, pH9.0) for 4hr at 4°C with changes of buffer twice, and the other bag was kept at 4°C for 4hr. The hydrolytic activity of the inactivated enzyme was compared between dialyzed and non-dialyzed samples. Control were analyzed in a similar manner.

RESULT AND DISCUSSION

Extracellular group II phospholipase A_2 activity had been detected in inflamed site in some experimental animals and also in human diseases. The regulatory molecule of

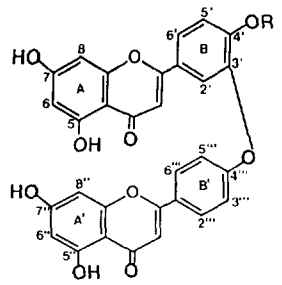

Fig.1. Chemical structures of ochnaflavone and its methylated derivative.

this enzyme activity could be implicated in the control of wide range of physiological states, such as inflammation, pancreatitis and rheumatoid arthritis. Thus the relationship of PLA_2 activation to the inflammatory response has been assumed an increasing role in the development of pharmacological agents which mitigate reversal of diseases.

During screening for PLA_2 inhibitors, biflavonoids, ochnaflavone and its methylated derivative (C-3' of apigenin-O-C-4" of apigenin) from the aerial part of *Lonicera japonica* (Fig.1), exhibit strong inhibitory activity against rat platelet PLA_2 (IC_{50}, about 3μM). Inactivation of PLA_2 by ochnaflavone at pH 9.0 occurred in a concentration-dependent manner (Fig.2A) and the concentration required for virtually complete inhibition

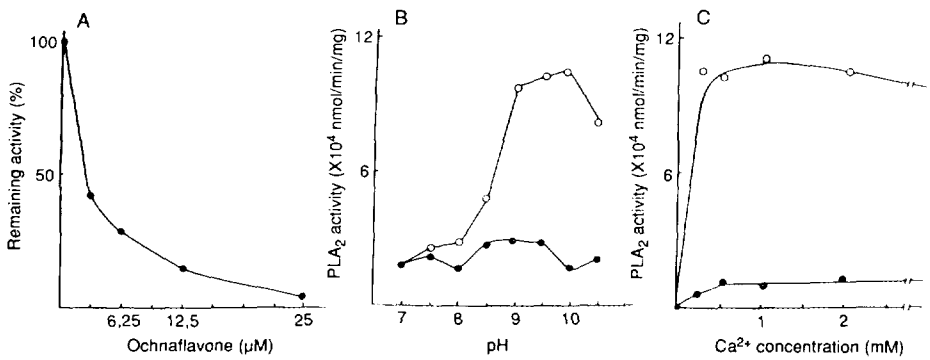

Fig.2A.Concentration-dependent inhibition of rat platelet phospholipase A_2.
Phosphpolipase A_2 was incubated in the presence of indicated concentration of ochnaflavone.
Fig.2B. Effect of pH on the inhibition of phospholipase A_2 by ochnaflavone.
Rat platelet phospholipase A_2 was incubated in the absence (○-○) and in the presence (●-●) of 10μM ochnaflavone. The buffers used were 100mM Tris-HCl (pH 7.0-9.5) and 100mM glycine-NaOH (9.5-10.0).
Fig.2C.Effect of Ca^{2+} on the inhibition of phospholipase A_2 by ochnaflavone.
Rat platelet phospholipase A_2 was incubated in the presence of indicated concentration of Ca^{2+}. Symbols and concentration of ochnaflavone are identical with those in Fig.2B.
The precedure is described in **MATERIALS AND METHODS** in detail. Each value is the mean of duplicate determinations.

of the enzyme activity was 25μM. But its monomer, apigenin, did not inhibit PLA_2 at the concentration up to 100μM (data not shown). In this study, inhibition of rat platelet PLA_2 by ochnaflavone was pH dependent. PLA_2 activity was found to vary with pH as shown in Fig.2B. There was a significant increase in the degree of inactivation according to change in pH from 7.0 to 9.0, indicating that ionization of a functional group(s) in ochnaflavone may be essential for maximum inactivation. Maximal inactivation was achieved between pH 9.0 and 10.0. Because the rat platelet PLA_2 is known to be rich in basic amino acid (14), we propose that ionizable residues in the ochnaflavone may interact with basic amino acid residues in the PLA_2, which contribute to inactive PLA_2 in alkaline pH. However, whether ochnaflavone interacts with basic amino acid residues remains to be clarified. we are now conducting further studies to determine the structure of the complex between rat platelet PLA_2 and ochnaflavone.

Hydrolytic activity of PLA_2 was also dependent on Ca^{2+} which has a specific binding site on the enzyme (15). Some alkaloids and non-steroidal anti-inflammatory agents displace Ca^{2+} and thus the inhibition by some of these agents appear to be dependent on Ca^{2+} concentration (16). However, Ca^{2+} concentrations up to 8mM afford no protection against inactivation of PLA_2 by ochnaflavone (Fig.2C). This result indicates that ochnaflavone do not alter the PLA_2 activity by antagonism of the Ca^{2+} binding site.

Two isoforms of mammalian extracellular PLA_2 are well characterized. Group I PLA_2 is well known to be produced and released by the pancreas and has been supposed to be important for digestion of phospholipids. On the other hand, group II PLA_2 is proposed to have some important roles for inflammtory diseases. A comparative test was done to find out how ochnaflavone affects on various source of PLA_2. Rat platelet PLA_2 and human pleural fluid PLA_2 were used as group II enzyme source, and porcine pancreatic PLA_2 was used as a group I enzyme source. As the result, ochnaflavone showed stronger inhibitory activity against group II PLA_2 than group I PLA_2 (IC_{50}, about 20μM) (Fig.3). Although the primary structure of group II PLA_2 shows only low homology with that of group I PLA_2 (30% and 31% amino acid homology for human and rat, respectively)(17), group II PLA_2 contains some well conserved residues which have been postulated to represent the "active site", "Ca^{2+} binding site", and "surface recognition site" in the group I PLA_2, but it is not clear why ochnaflavone exhibits stronger inhibitory activity against group II PLA_2 than group I PLA_2.

To determine whether the ochnaflavone influence PLA_2 molecules or not, reversibility of the ochnaflavone-PLA_2 complex was investigated directly by dialysis method (Table 1). When ochnaflavone-PLA_2 complex was preincubated at 37°C for 30 min and stored at 4°C for 4 hours, the remaining activity of PLA_2 was not detected. However, when ochnaflavone-PLA_2 complex was preincubated in the same condition and dialyzed, the mixture of ochnaflavone-PLA_2 complex did not reverse the enzymatic activity. This result indicate that the inhibition is apparently irreversible. Furthermore, the double reciprocal plot showed that ochnaflavone behave kinetically as noncompetitive inhibitor for rat platelet PLA_2 with Ki of 3×10^{-5}M (Fig.4). Direct, but non-covalent, interaction of inhibitory agents that

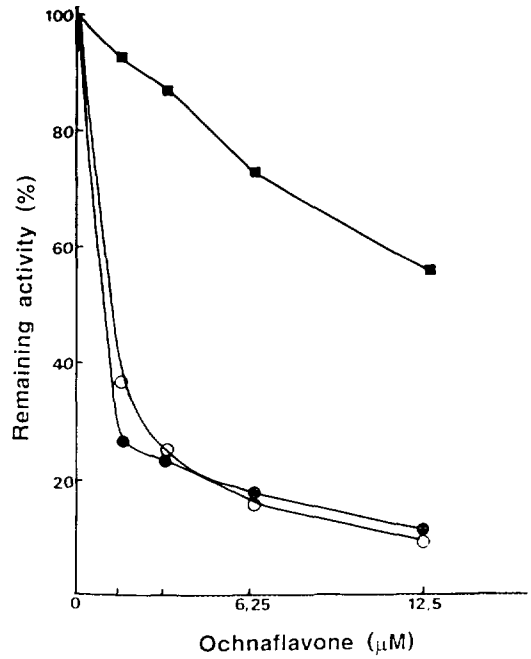

Fig.3. Inhibition of 14KDa group II phospholipase A_2 and 14KDa group I phospholipase A_2 by ochnaflavone.
Standard reaction mixture contained 10ng of each phospholipase A_2 and the indicated concentration of ochnaflavone. Inhibition is expressed as a percent activity remaining of that obtained in control. Rat platelet phospholipase A_2(●), human pleural fluid phospholipase A_2(O) were used as a group II PLA_2 enzyme source, and porcine pancreatic phospholipase A_2(■) was used as a group I enzyme source.

modulate the structure of PLA_2 have been described in only rare instances. In a recent paper, Tanaka *et al.*, (18) reported that specific group II PLA_2 inhibitor, thielocin B3, inhibited group II PLA_2 in a reversible and competitive manner, whereas manolide which was isolated from marine sponge inhibited bee venom PLA_2 directly and covalently (19).

Table.1 Reversibility of the ochnaflavone-rat platelet phospholipase A_2 complex

	Dialysis following 30-min preincubation	
	% Reduction of enzyme activity	
	Before dialysis	After dialysis
Ochnaflavone	85.7	94.7

Ochnaflavone-phospholipase A_2 mixture was preincubated at 37°C for 30min prior to predialysis sampling (Before dialysis) : The remainder of the mixture was dialyzed in cellulose tubing bag. The procedure is described in **MATERIALS AND METHODS** in detail. Enzyme activity is reported as percent reduction in enzyme activity as compared to control (without ochnaflavone) sample which was treated identically to ochnaflavone-phopholipase A_2 mixtures.

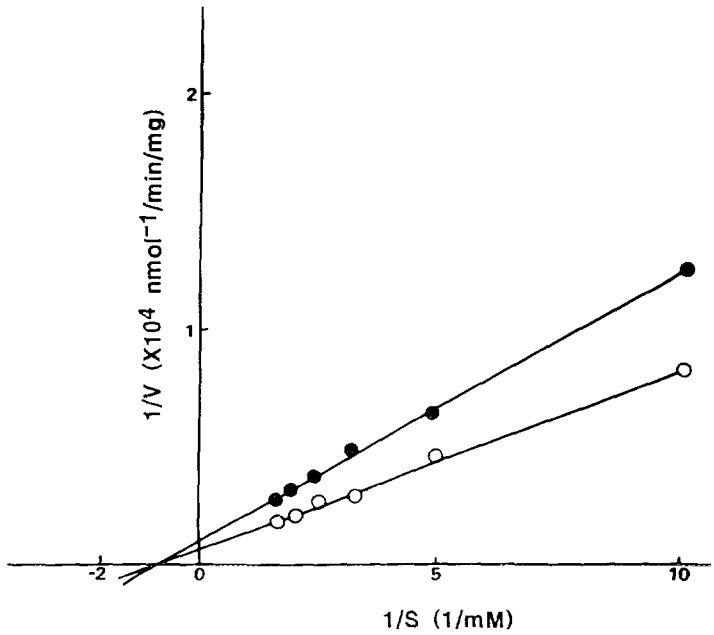

Fig.4. Effect of substrate concentration on the inhibition of rat platelet phospholipase A_2 by ochnaflavone.
Double-reciprocal plot of phospholipase A_2 activity toward 1-Acyl-2-[1-^{14}C]*linoleoyl-sn*-glycerophosphoethanolamine. The data were made according to Linweaver and Burk to obtain *Ki* value. Symbols are identical with those in Fig.2B.

Our results indicate that the inhibition of PLA_2 by ochnaflavone may be the result of a direct binding of the enzyme in a irreversible and noncompetitive manner.

To explore in more detail the inhibitory mechanism of ochnaflavone, we examined the effect of several biflavonoids, such as amentoflavone and 4 of its methylated derivatives, all of which contain apigenin monomer in those molecules. (C-3' of apigenin-C-8" of apigenin). It is noteworthy that other biflavonoids also inhibited group II PLA_2, and the IC_{50} values were nearly the same as that of ochnaflavone (data not shown). Judging from the structure-activity relationship using 9 different biflavonoids, 5 hydroxyl group of A ring, 5"-hydroxyl group of A' ring (all these compounds don't have any substitutions at these positions) and 7"-hydroxyl group of A' ring may play an important role in the PLA_2 inhibition. Among them, only Cryptomerin B (C-4' of apigenin-O-C-6" of apigenin) which has methyl group at C-7" showed about 5 fold decrease in the inhibitory activity compared with the other biflavonoids (data not shown). The experimental findings with biflavonoids as group II PLA_2 inhibitors are somewhat new and thus may be a useful biochemical and pharmacological tool to elucidate the role of group II PLA_2 in certain physiological and pathological events.

Since in several models, low molecular weight extracellular PLA_2 was found to have proinflammatory activity (20,21), the inhibition of this enzyme by ochnaflavone as well as other biflavonoids could serve as an additional rationale to use biflavonoids alone or in

combination with anti-inflammatory agents for treatment of inflammation. The details of *in vivo* results will be reported elsewhere in due course.

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