Arch. Pharm. Res. Vol.20, No.6, pp. 533-538, 1997

Inhibition of Arachidonate Release From Rat Peritoneal Macrophage By Biflavonoids

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(Received June 28, 1997)

Biflavonoid is one of unique classes of naturally-occurring bioflavonoid. Previously, certain biflavonoids were found to possess the inhibitory effects on phospholipase A_2 activity and lymphocytes proliferation' suggesting their anti-inflammatory/immunoregulatory potential. In this study, effects of several biflavonoids on arachidonic acid release from rat peritoneal macrophages were investigated, because arachidonic acid released from the activated macrophages is one of the indices of inflammatory conditions. When resident peritoneal macrophages labeled with [^{3}H]arachidonic acid were activated by phorbol 12-myristate 13-acetate (PMA) or calcium ionophore, A23187, radioactivity released in the medium was increased approximately 4.1~7.3 fold after 120 min incubation compared to the spontaneous release in the control incubation. In this condition, biflavonoids (10 uM) such as ochnaflavone, ginkgetin and isoginkgetin, showed inhibition of arachidonate release from macrophages activated by PMA (32.5~40.0% inhibition) or A23187 (21.7~41.7% inhibition). Amentoflavone showed protection only against PMA-induced arachidonate release, while apigenin, a monomer of these biflavonoids, did not show the significant inhibition up to 10 uM. Staurosporin (1 uM), a protein kinase C inhibitor, showed an inhibitory effect only against PMA-induced arachidonate release (96.8% inhibition). Inhibition of arachidonate release from the activated macrophages may contribute to an anti-inflammatory potential of biflavonoids *in vivo*.

Key words : Macrophage, Arachidonic acid, Phospholipase A_2, Flavonoid, Biflavonoid, Ochnaflavone, Amentoflavone, Ginkgetin, Isoginkgetin, Anti-inflammation

INTRODUCTION

Biflavonoid is one of the classes of naturally-occurring bioflavonoid. There are diverse families of biflavonoids including chalcone dimer and flavone dimer, etc. Certain biflavonoids were reported to possess inhibitory effects on phosphodiesterase (Ruckstuhl *et al.*, 1979), lens aldose reductase (Iwu *et al.*, 1990; Felicio *et al.*, 1995), and mast cell histamine release (Amella *et al.*, 1985). Others showed anticancer activity (Lin *et al.*, 1989; Sun *et al.*, 1997). Recently, several biflavonoids such as ochnaflavone, amentoflavone, ginkgetin and morelloflavone were reported to possess an inhibitory activity on group II phospholipase A_2 (PLA_2) from platelets and synovial fluid (Chang *et al.*, 1994; Gil *et al.*, 1997). It was also found that ochnaflavone and several other biflavonoids were irreversible inhibitors of lymphocytes proliferation induced by T- or B-cell mitogen (Lee *et al.*, 1995). Since PLA_2 is believed to be involved in various inflammatory disorders and lymphocyte is one of the important cells responsible for chronic inflammation, these properties strongly suggest anti-inflammatory nature of biflavonoids. Therefore, it is meaningful to evaluate effects of biflavonoids on macrophages, another inflammatory and immune responsive cells in the body, for our continuing research to find new anti-inflammatory agents. When macrophages are activated, various mediators of inflammation such as arachidonic acid (AA) and cytokines are released. AA released is converted to inflammatory eicosanoids, prostaglandins (PG), leukotrienes (LT) and hydroxyeicosatetraenoic acids (HETE) by macrophages and other cells nearby. This study was carried out to investigate the effect of biflavonoids on AA release from rat peritoneal macrophages. We found that several biflavonoids clearly inhibited AA release from macrophages activated by phorbol 12-myristate 13-acetate (PMA) or A-23187 up to the concentrations of 10 uM.

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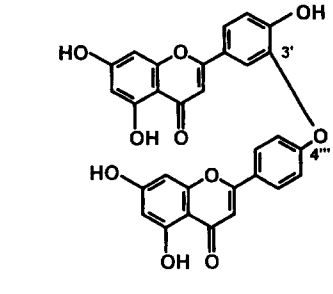
MATERIALS AND METHODS

Materials

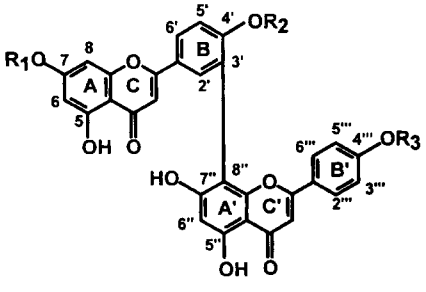
Ochnaflavone was isolated from *Lonicera japonica* (Son *et al.*, 1992). Amentoflavone was purified from *Sellaginella tamariscina* according to the procedure of Shin and Kim (1991). Ginkgetin and isoginkgetin were obtained from *Gingko biloba* leaves (Kang *et al.*, 1990). Purity of all biflavonoids isolated was checked by TLC using at least two different solvent systems and each biflavonoid showed a single spot. Chemical structures of biflavonoids used were shown in Fig. 1. A23187, phorbol 12-myristate 13-acetate (PMA), fatty acid free bovine serum albumin (BSA) and the α-naphthyl acetate kit were provided from Sigma Chem. Co. (St. Loius, MO). Minimum essential medium (MEM), fetal bovine serum (FBS), phosphate buffered saline, glutamine and antibiotic solution were purchased from Gibco Lab. (Grand Island, N.Y.). [^{3}H]Arachidonic acid (100 μCi/mmol) was obtained from NEN (USA). The other reagents used in this study were of highest grade available.

Isolation of rat peritoneal macrophages

Male Splague-Dawley (SD) rats (180~220 g) were euthanized by CO_2. Peritoneal cavity fluid was collected by lavage and centrifuged at 250 g for 10 min. The pellet was washed twice with MEM and finally resuspended to approximately 1×10^6 cells/ml in MEM complete medium containing 2 mM glutamine, 1% antibiotics, 10% FBS and 10 mM Hepes. Macrophages were separated from other cells by incubation at 37°C for 2 hr in 5% CO_2 incubator. The non-adherent cells were removed by repeated washing with MEM. Viability of cells was assessed by the tryphan blue exclusion test. The cell culture showing over 90% viability was only used for further experiments. Macrophages were identified by nonspecific esterase staining according to the manufacture's procedure, which usually gave over 93% positive cells in our preparation.



Ochnaflavone



$R_1 = R_2 = R_3 = H$	Amentoflavone
$R_1 = R_3 = CH_3$, $R_3 = H$	Ginkgetin
$R_1 = H$, $R_2 = R_3 = CH_3$	Isoginkgetin

Fig. 1. Chemical structures of biflavonoids used in this study.

Labeling and release of [^{3}H]arachidonic acid

Adherent macrophages were labeled with [^{3}H]arachidonic acid (0.3 uCi/10^6 cells/well) in 24-well cell culture plates for 24 hr. In this condition, majority of radiolabeled lipids in macrophages was reported to be glycerophospholipid (Triggiani *et al.*, 1994). To study AA release from labeled macrophages, the cells were washed and the medium was replaced by serum-free MEM containing fatty acid free BSA (0.5 mg/ml) to trap the released AA according to the procedure of Balsinde *et al.* (1992). Each biflavonoid was dissolved in DMSO and appropriately diluted to the desired concentration with MEM. The final concentration of DMSO in a well was adjusted to 0.25% (v/v) and the amount of DMSO added did not decrease cell viability. The control wells contained same amount of DMSO without biflavonoid. After 1 hr preincubation with biflavonoid, cells were challenged with PMA (100 ng/ml) or A23187 (5 uM) for the time indicated. Medium was collected and cells were washed twice with serum free MEM containing BSA (1 ml). The medium and washed fluid were collected and radioactivity (released AA and metabolites) was counted using liquid scintillation counter (Pharmacia 1209). Percent inhibition of AA release was calculated as follows:

$$\% \text{ inhibition} = 100\times\left(1-\frac{\text{DPM of tested well}-\text{DPM of control w/o stimulator}}{\text{DPM of control w/ stimulator}-\text{DPM of control w/o stimulator}}\right)$$

The experiments were performed in duplicate and the data were given arithmatic means and standard error (DPM±SE). All study was repeated at least three times and gave similar results. Statistical significance was evaluated using Student's t-test and P values less than 0.05 were considered to be significantly different.

RESULTS AND DISCUSSION

Monocyte/macrophage is one of the important cells participating in inflammatory conditions, especially in chronic diseases. When macrophages are activated,

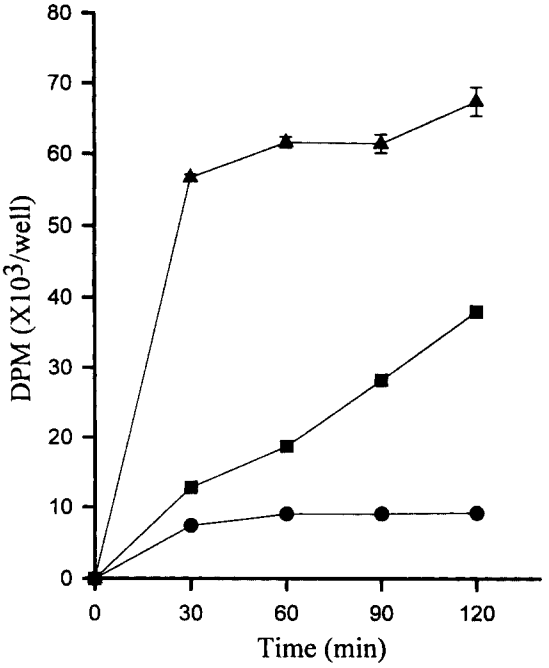


Fig. 2. Arachidonic acid release from macrophages induced by PMA or A23187 Control (●), PMA, 100 ng/ml (■), A 23187, 5 uM (▲).

they secrete various inflammatory mediators in surroundings. Among mediators they secrete, arachidonic acid is a key compound which is converted to proinflammatory eicosanoids such as PGE_2, LTC_4 and 5-HETE depending on locations, species and stimulants (Aderem *et al.*, 1988; M'Rini-Puel *et al.*, 1993; Taylor *et al.*, 1990). Therefore, inhibition of AA release from the activated macrophages is regarded as an index of anti-inflammatory potential. Exogeneous AA was known to be incorporated into membrane lipid, mainly sn-2 position of glycerophospholipid after 24 hr labeling period, although there were some differences of ratios between glycerophopholipid classes in experimental conditions employed (Locati *et al.*, 1994; Triggiani *et al.*, 1994). A wide variety of stimuli such as PMA triggers the release of AA and its metabolites from the activated macrophages. The mechanism of AA release from the activated macrophages has been studied extensively by many investigators (Balsinde *et al.*, 1994; Gewert and Sundler, 1995; Qui and Leslie, 1994).

In order to find an optimum concentration of stimulators, various amount of PMA or A23187 were added and released AA was measured. From this experiment, it was found that PMA (100 ng/ml) or A23187 (5 uM) was optimal for AA release from macrophages (data not shown). When PMA (100 ng/ml) was added to [^{3}H]AA-labeled macrophages, total radioactivity released in the medium was increased approximately 4.1 fold in 120 min incubation, compared to the spontaneous AA release of control (Fig. 2). When A23187 (5 uM) was used as a stimulator, AA was rapidly released in 30 min and maintained 7.3 fold increase up to the incubation time of 120 min. For an inhibition study, 90 min end-point was used. When biflavonoids (1 and 10 uM) were tested, it was revealed that ochnaflavone, amentoflavone, ginkgetin and isoginkgetin showed the similar inhibitory activity in PMA-induced AA release, 21.0~40.0% inhibition at 10 uM (Table I). When macrophages were treated with A23187, ochnaflavone and isoginkgetin exhibited the significant inhibitory activity (41.7~48.4% inhibition at 10 uM), while ginkgetin showed 21.7% inhibition at 10 uM, but not statistically significant. Amentoflavone failed to inhibit AA release significantly in A23187 treatment. In addition, apigenin, a monomer of biflavonoids tested in this study, did not show significant inhibition

Table I. Inhibition of arachidonic acid release from rat peritoneal macrophages by biflavonoids

	Concentration (μM)	PMA		A23187	
		DPM[a] ($\times 10^2$)	% inhibition[b]	DPM[a] ($\times 10^2$)	% inhibition[b]
Control		236.0±25.5		271.7±20.2	
Control/stimulator[c]		575.7±35.8		981.8±90.5	
Apigenin	1	511.3±36.0	18.9	1214.9±220.8	-
	10	541.4±1.7	10.1	1009.3±12.1	-
Ochanaflavone	1	493.1±49.0	24.3	918.6±59.4	8.9
	10	439.8±24.7*	40.0	685.8±40.8*	41.7
Amentoflavone	1	485.3±9.9*	26.6	902.0±87.1	11.2
	10	526.7±23.9	21.0	898.6±23.9	11.7
Ginkgetin	1	507.9±54.7	20.0	1001.5±106.7	-
	10	455.6±2.8*	35.3	827.9±68.9	21.7
Isoginkgetin	1	546.1±9.5	8.7	1026.0±80.5	-
	10	465.1±3.0*	32.5	637.9±22.0*	48.4

[a]Data represented were mean±S.E.
[b]% inhibition calculated as described in experimental section.
[c]PMA (100 ng/ml) or A23187 (5 μM) was used.
*P<0.05, Significantly different from control/stimulator.

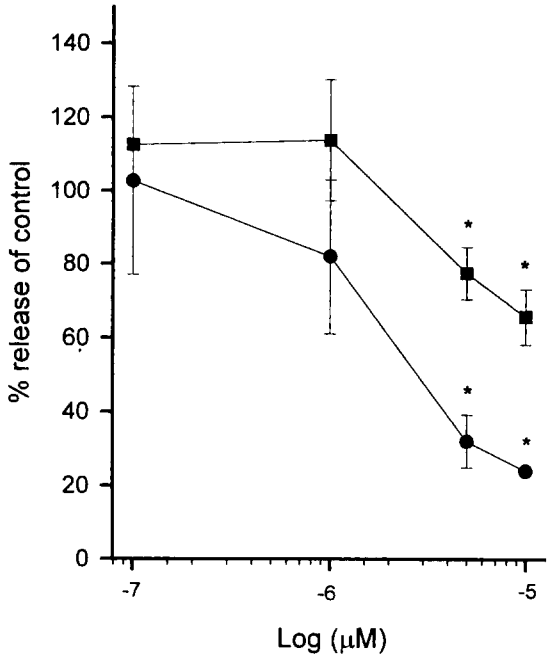


Fig. 3. Inhibition of arachidonic acid release by ochnaflavone PMA-induced release (●), A23187-induced release (■); Media was collected and radioactivity was measured 90 min after stimulatiom., *P<0.05, Significantly different from control/stimulator.

either against PMA or A23187-induced AA release up to the concentration of 10 uM. Ochnaflavone was found to be the most potent one among biflavonoids tested. Ochnaflavone inhibited AA release in a concentration-dependent manner (Fig. 3). IC_{30} values of ochnaflavone were 1.5 uM and 8.3 uM for PMA- and A 23187-induced AA release, respectively. These results clearly indicated that biflavonoids inhibited AA release from the activated macrophages. However, any structural-activity relationship could not be determined because of small numbers of biflavonoids used in this study. When cytotoxicity of these biflavonoids was examined before and after the release experiments, any statistically significant difference in viability of macrophages was not observed between biflavonoid-treated and control cells without biflavonoid (data not shown), which indicated that the inhibition of AA release was not due to the cell cytotoxicity of biflavonoids.

Although it is not fully understood, PMA is known to activate protein kinase C (PKC) and activated PKC initiates sequential activation of several proteins involved in proliferation as well as phospholipases which liberate AA from the membrane glycerophospholipid. A23187 increases the cellular calcium concentration and the increased calcium activates the several calcium dependent enzymes such as PLA_2 (Lloret *et al.*, 1995; Qiu and Leslie, 1994). In order to determine the involvement of PKC in macrophage activation, the inhibitory activity of staurosporin, a well-known PKC inhibitor, was examined in our experiment. Staurosporin potently inhibited PMA-induced AA release at a low concentration (0.1~1 uM) while it did not show significant inhibition in A23187-induced release of AA and its metabolites (Table II). This result conformed the previous finding of Qiu and Leslie (1994) that A23187-induced AA release in peritoneal macrophages did not involve PKC activation. For biflavonoids, our results indicated that they might inhibit common pathway of AA release, which is most likely PLA_2, because ochnaflavone and other biflavonoids such as isoginkgetin inhibited PMA- as well as A 23187-induced AA release. However, it was not possible to find specific inhibitory activity on different isoforms of PLA_2 enzymes in macrophages although $cPLA_2$ was reported to be an enzyme responsible for AA release from macrophages (de Carvalho *et al.*, 1995; Lloret and Moreno, 1994; Vial *et al.*, 1995) and group V $sPLA_2$ was responsible for AA release from macrophage-like cell line P388D1 (Balboa *et al.*, 1996). And we could not exclude other possibilities such as inhibition of phospholipase C or D since these enzymes might be also involved in AA release from macrophages (Chow *et al.*, 1995). Further investigation will unveil precise mechanism of inhibitory activity of biflavonoids on AA release from macrophages.

It was interesting to note that amentoflavone did not exhibit the significant inhibitory activity in A23187-induced AA release. At present, we do not know why amentoflavone was not active in A23187-induced re-

Table II. Inhibition of arachidonic acid release from rat peritoneal macrophages by staurosporin

	Concentration (μM)	PMA		A23187	
		DPM[a] (×10²)	% inhibition[b]	DPM[a] (×10²)	% inhibition[b]
Control		52.3±0.5		85.8± 1.2	
Control/stimulator[c]		137.5±1.4		352.0±28.7	
Staurosporin	0.1	64.3±6.2*	85.9	329.9±11.3	8.3
	1	55.0±4.0*	96.8	335.7±19.0	6.1

[a]Data represented were mean±S.E.
[b]% inhibition calculated as described in experimental section.
[c]PMA (100 ng/ml) or A23187 (5 μM) was used.
*P<0.05, Significantly different from control/stimulator.

lease. It has been suggested that amentoflavone behaves differently from other biflavonoids because amentoflavone possesses different properties in cyclooxygenase inhibition (Kim *et al.*, 1997) and inhibition of lymphocyte proliferation (Lee *et al.*, 1995).

In conclusion, certain biflavonoids inhibited AA release from the activated macrophages induced by PMA or A23187, and their inhibition may contribute to an anti-inflammatory potential of biflavonoids *in vivo*.

ACKNOWLEDGEMENTS

This study was partly supported by the research grant from Ministry of Health and Public Welfare, Korea (1996) and greatly acknowledged.

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