INHIBITION OF 15-LIPOXYGENASE BY OROBOL

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There is growing evidence that oxidative modification of low density lipoprotein (LDL), the major cholesterol-carrying lipoprotein in human, plays an important role in the pathogenesis of atherosclerosis[1]. Monocyte-derived macrophages avidly ingest oxidized LDL, but not native LDL, and accumulate large amount of cholesteryl esters, thereby being converted into foam cells and contributing to the atherogenesis. 15-Lipoxygenase is implicated in LDL oxidation under physiological conditions, since LDL can be oxidatively modified by 15-lipoxygenase[2] and the enzyme is abundant in atherosclerotic lesions[3].

In the course of the search for microbial metabolites that inhibit 15-lipoxygenase, we found that a fungal strain, *Tritirachium* sp. F3707, produced an active metabolite. This compound was identified to be orobol [5,7-dihydroxy-3-(3,4-hydroxyphenyl)-4*H*-1-benzopyran-4-one], an isoflavonoid originally isolated as a glucoside from a plant *Orobus tuberosus*[4]. Later, it has been characterized as an inhibitor of dihydroxyphenylalanine decarboxylase by UMEZAWA *et al.*[5], while its effects on 15-lipoxygenase have not been studied. We report here the orobol inhibition of 15-lipoxygenase and formation of 15-hydroxyeicosatetraenoic acid (15-HETE) from arachidonic acid in mouse peritoneal macrophages.

Orobol was isolated from cultures of *Tritirachium* sp. F3707, which was isolated from a soil sample collected in Tokushima, Japan. Quercetin and 15-lipoxygenase (soybean lipoxygenase, type V) were purchased from Sigma. Linoleic acid was supplied from Wako Pure Chemical, Japan. [1-^{14}C]Arachidonic acid was obtained from American Radiolabeled Chemicals. Enzyme activity was determined photometrically at 25°C by monitoring the absorbance of conjugated diene at 234 nm ($\varepsilon_{max}=25{,}000$). A mixture (475 μl) containing 0.1 M potassium phosphate, pH 7.5, 0.01% (wt/vol) Triton X-100, 50 μM linoleic acid and 5 μl of either dimethylsulfoxide or a test sample dissolved in dimethylsulfoxide was preincubated at 25°C for 5 minutes. Subsequently, enzyme reaction was initiated by introducing 25 μl of lipoxygenase (2.7 μg/ml) dissolved in 0.1 M potassium phosphate, pH 7.5 and 0.01% Triton X-100 into the mixture. 15-Lipoxygenase activity in mouse peritoneal macrophages was determined as described by SPARROW and OLSZEWSKI[6] using [^{14}C]arachidonic acid as a substrate.

Orobol, at concentrations ranging from 15 to 100 μM, inhibited soybean lipoxygenase activity in a concentration-dependent manner; the inhibition was 50% at 26 μM. This inhibition appeared to be reversible as judged from an experiment in which enzyme activity was determined before and after ultrafiltration of preincubated reaction mixture. Thus, as shown in Table 1, lipoxygenase which was preincubated with 1.4 mM orobol showed 15% of control activity, while enzyme activity recovered to 87% of control activity after ultrafiltration of the preincubated mixture. From Lineweaver-Burk plots for the inhibition of soybean lipoxygenase by orobol (Fig. 1), it was suggested that the inhibition was noncompetitive with respect to linoleic acid. Apparent K_I value was calculated to be 44 μM. These results suggest that orobol reversibly binds to a site on the enzyme other than the active site. Some

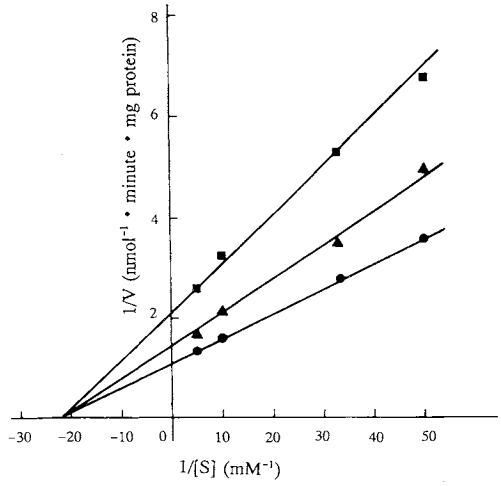
Table 1. Reversible inhibition of soybean lipoxygenase by orobol.

Orobol at preincubation (μM)	Lipoxygenase activity (nmol·minute^{-1}·mg protein^{-1})	
	Before ultrafiltration	After ultrafiltration
0	218 (100%)[a]	144 (100%)
70	32 (15%)	125 (87%)

[a] Percentage of control values are shown in parentheses.

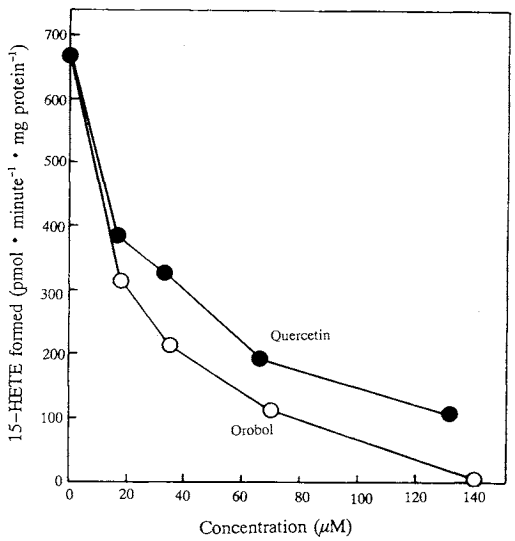
Soybean lipoxygenase (2.7 μg/ml) was preincubated at 25°C for 5 minutes in a mixture (2 ml) containing 0.1 M potassium phosphate, pH 7.5, 0.01% Triton X-100, 5% (vol/vol) ethanol in the absence or presence of 1.4 mM orobol. Aliquot (1 ml) of the mixture was either stored at 4°C or subjected to ultrafiltration using Ultracent-30 (TOSOH, Japan). Unfiltered materials (approximately $M_r > 30{,}000$) were washed twice by ultrafiltration with 0.5 ml of buffer containing 0.1 M potassium phosphate, pH 7.5 and 0.01% Triton X-100, followed by suspending with 1 ml of the same buffer. Enzyme activity was determined using 25 μl of the preincubated mixture before and after the ultrafiltration. Each value represents the average of duplicate determinations.

Fig. 1. Lineweaver-Burk plots for the inhibition of soybean lipoxygenase by orobol.



Soybean lipoxygenase activity was determined with varying concentrations of linoleic acid in the presence of orobol at concentrations of 0 (●), 5 (▲) and 20 μM (■). Each value represents the average of duplicate determinations.

Fig. 2. Inhibition of 15-HETE formation from [^{14}C]-arachidonic acid in mouse peritoneal macrophages by orobol and quercetin.



Mouse peritoneal macrophages were incubated in 0.2 ml of Ham's F-10 medium containing 10 μM of [^{14}C]arachidonic acid (12,100 dpm/nmol) at 37°C for 5 minutes in the absence or presence of the indicated concentrations of either orobol (○) or quercetin (●). Subsequently, [^{14}C]15-HETE released into the medium was determined. Each value represents the average of duplicate determinations.

O-methylated lipophilic flavonoids[7] as well as quercetin[8] have been reported to inhibit soybean lipoxygenase. The mechanism for the inhibition of 15-lipoxygenase by orobol seems to be distinct from that for these flavonoids, since it has been reported that these compounds are uncompetitive inhibitors of the enzyme[7,8], and that quercetin acts as a reductant of fatty acid radicals[8], thereby inhibiting fatty acid oxygenation.

Effect of orobol on 15-lipoxygenase activity was further characterized using mouse peritoneal macrophages, in which 15-lipoxygenase activity predominates over other arachidonate-metabolizing activities, and which are able to mediate oxidative modification of LDL *in vitro*[9]. Under the conditions employed in our experiment, the major metabolite of [^{14}C]arachidonic acid found in medium was 15-HETE. Orobol reduced the formation of 15-HETE by 50% at a concentration of 17 μM (Fig. 2). However, quercetin, which was as active as orobol in soybean lipoxygenase inhibition (50% inhibition at a concentration of 26 μM), showed 1/2 the activity of orobol, giving 50% inhibition at 33 μM. Under these conditions, both agent caused neither cytolysis nor cell detachment.

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