Effects of Flavonoids and Related Compounds from Mulberry Tree on Arachidonate Metabolism in Rat Platelet Homogenates

YOSHIYUKI KIMURA,*, a HIROMICHI OKUDA, a TARO NOMURA, b TOSHIO FUKAI b and SHIGERU ARICHI c

2nd Department of Medical Biochemistry, School of Medicine, Ehime University, a Shigenobu-cho, Onsen-gun, Ehime 791-02, Japan, Faculty of Pharmaceutical Sciences, Toho University, b Funabashi, Chiba 274, Japan and The Research Institute of Oriental Medicine, Kinki University, c Sayama-cho, Minamikawachi-gun, Osaka 589, Japan

(Received August 23, 1985)

The effects of various flavonoids and related compounds isolated from the root bark of mulberry tree on rat platelet lipoxygenase and cyclooxygenase products formed from [1-14C] arachidonic acid were studied. Morusin was found to inhibit the formations of 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B2 (cyclooxygenase products) more strongly than the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (12-lipoxygenase product). Oxydihydromorusin and kuwanon C were also found to inhibit the formation of thromboxane B2 more strongly than the formations of HHT and 12-HETE. Mulberrofuran A inhibited the formations of HHT and thromboxane B2, but it increased the formation of 12-HETE. Albanol B and mulberrofuran F did not affect arachidonate metabolism in rat platelet homogenates.

Keywords — flavonoid; mulberry tree; thromboxane B2; rat platelet homogenate; arachidonate metabolism

Mulberry trees have been widely cultivated in China and Japan, and the leaves are used to feed silkworms. On the other hand, the root bark of the mulberry tree (Morus alba L. and other plants of the genus Morus) has been used as an anti-phlogistic, diuretic, expectorant, and laxative in Chinese traditional medicine. In a pharmacological study, the extract of the root bark was reported to show a marked hypotensive effect.1 Nomura et al.2 have isolated many phenolic constituents from this plant.

Platelet cyclooxygenase is known to catalyze the initial reaction that leads to the formation of prostaglandin H2 (PGH2), which is converted to thromboxane A2 (TXA2) by thromboxane synthetase and to other eicosanoids such as PGD2 and PGE2.3 TXA2 is readily transformed to TXB2, which is a stable form. TXA2 is known to be a potent leukocyte chemotactic substance4 and a potent platelet aggregator.5 A number of non-steroidal anti-inflammatory drugs (e.g., aspirin and indomethacin) have been found to inhibit the formation of cyclooxygenase products such as 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), TXB2 and prostaglandins, but do not inhibit the lipoxygenase enzyme.6,7

In the present work, we examined the effects of various flavonoids and related compounds isolated from the root bark of mulberry tree on arachidonate metabolism in rat platelet homogenates.

Materials and Methods

Materials — Rat blood was obtained from normally fed Wistar–King strain rats (300–400 g). Washed platelets were prepared by differential centrifugation. [1-14C] Arachidonic acid was purchased from Amersham Co. Morusin, oxydihydromorusin, kuwanon C, mulberrofuran A, mulberrofuran F and albanol B were isolated from the root bark of mulberry tree as described by Nomura et al.2 The chemical structures of these compounds are shown in Fig. 1.
Precoated Silica gel 60 TLC plastic sheets were obtained from Merck Co. Other chemicals were of reagent grade.

**Measurements of the [1-14C] Arachidonic Acid Cascade in Homogenates of Rat Platelets**—Sonication of rat platelets was performed using a Sonifier Cell Disruptor (Branson Sonic Power, Co.). An ethanol solution of [1-14C] arachidonic acid (10 μCi/ml) was preserved at −40 °C, and then 0.1 ml of the solution was diluted by the addition of 0.9 ml of Heps/saline buffer (pH 7.4) and used for this study (1 μCi/ml). Test compounds were suspended in Heps/saline buffer (pH 7.4) by using the sonicator. Sonicated platelets (5 mg protein/ml) (130 μl) were preincubated with test compounds (20 μl) for 5 min at 37°C. Then, [1-14C] arachidonic acid (50 μl, 0.05 μCi/tube) was added to give a final concentration of 0.84 nmoi/0.2 ml tube and the mixture was incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 N formic acid (200 μl) and the products were extracted with 8 volumes of EtoAc. The EtoAc phase was evaporated under N2 gas. The residue was dissolved in a small amount of EtoAc (40 μl), applied to precoated Silica gel 60 TLC plastic sheets, and developed with EtoAc–2,2,4-trimethylpentane–acetic acid–water (100:50:20:100, v/v, upper phase) or CHCl3–MeOH–acetic acid–water (135:12:1.5:1.2, v/v). There was no effect of the organic solvent at the concentrations used on the response of arachidonate metabolism in rat platelet homogenates. These metabolites were identified by comparison with authentic compounds and by gas chromatography–mass spectrometry as described previously.8) Radioactive spots were detected by autoradiography, cut out with scissors and counted in a liquid scintillation counter. Protein was determined by the method of Lowry et al.9) with bovine serum albumin as a standard.

**Results**

When arachidonic acid was incubated with sonicated rat platelet homogenates, it was converted to three major compounds, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), HHT and thromboxane B2. The radioactivities of 12-HETE (12-lipoxygenase product), HHT and thromboxane B2 (cyclooxygenase products) formed in the control were 34.5 ± 1.43, 15.8 ± 1.04 and 13.1 ± 1.01 (× 104 cpm) (means ± standard errors for 18 experiments), respectively. The amounts of 12-HETE, HHT and thromboxane B2 formed after a 5 min incubation were found to be proportional to the amount of homogenate present (data not shown).

Figures 2(a)—(e) show the effects of morusin, oxydihyromorusin, kuwanon C, mulberrofuran A and indomethacin on the formations of 12-HETE, HHT and thromboxane B2. As shown in Fig. 2(a), the formations of HHT and thromboxane B2 were inhibited by morusin dose-dependently, while the formation of 12-HETE was slightly stimulated at low concentrations (10−6—10−5 M) but was inhibited at high concentrations (10−4—10−3 M).
Platelets (5 mg protein/ml) (130 µl) sonicated in 25 mM HEPES/125 mM NaCl buffer (pH 7.4) containing 2 mM EDTA were preincubated with various flavonoids and related compounds (20 µl) for 5 min at 37°C. After addition of [1-14C] arachidonic acid (50 µl, 0.05 µCi/tube), the mixture was incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 N formic acid (200 µl). The products were extracted with ethyl acetate, and chromatographed on a silica gel TLC plastic sheet in ethyl acetate–2,2,4-trimethylpentane–acetic acid–water (100:50:20:100, v/v, upper phase). Radioactivities in the arachidonic acid, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B2 fractions counted by liquid scintillation spectrometry.

Values are the means ± standard errors for 3 experiments. ●, 12-HETE; ●, HHT; ○, thromboxane B2.

Oxydihydromorusin also inhibited the formations of HHT and thromboxane B2 dose-dependently, while it showed no effect on the formation of 12-HETE. (Fig. 2(b)). Furthermore, oxydihydromorusin inhibited the formation of thromboxane B2 more strongly than the
formation of HHT. As shown in Fig. 2(c), the formations of 12-HETE, HHT and thromboxane B₂ were inhibited by kuwanon C in a dose-dependent manner. On the other hand, mulberrofuran A also inhibited the formations of HHT and thromboxane B₂, though less strongly, while it stimulated the formation of 12-HETE from arachidonic acid in platelet homogenates at concentrations of $10^{-6} - 10^{-3}\text{M}$. Mulberrofuran F and albanol B did not affect arachidonate metabolism in rat platelet homogenates (data not shown). An anti-inflammatory drug, indomethacin, also inhibited the formation of HHT and thromboxane B₂ dose-dependently, while it stimulated the formation of 12-HETE (Fig. 2(e)).

**Discussion**

The present investigation has demonstrated that various phenolic compounds isolated from the root bark of the cultivated mulberry tree significantly affect arachidonate metabolism in rat platelet homogenates. A number of non-steroidal anti-inflammatory drugs such as aspirin and indomethacin have been shown to inhibit the formation of cyclooxygenase products, but lipooxygenase enzymes are not inhibited.⁶,⁷ Among the compounds used in this study, morusin and kuwanon C inhibited both cyclooxygenase and 12-lipoxygenase at high concentrations ($10^{-4} - 10^{-3}\text{M}$), but oxydihydromorusin selectively inhibited the formation of the cyclooxygenase product, thromboxane B₂ without affecting the formation of 12-HETE (12-lipoxygenase product). These findings suggest that a 2,2-dimethylchromene ring of angular type in the A-ring of the flavone and a free phenolic hydroxyl group at the C-5 position in the flavone skeleton may be essential for selective inhibition of the formation of thromboxane B₂. Generally, the inhibitory effects of prenylflavones such as morusin, oxydihydromorusin and kuwanon C on the formations of HHT and thromboxane B₂ were stronger than those of 2-arylbenzoferan derivatives such as mulberrofuran A, mulberrofuran F and albanol B.

In terms of structure–activity relationship, two elements appear to be important. Both the 2,2-dimethylchromene ring of angular type in the A-ring and the γ,γ-dimethylallyl group at the C-3 position in the γ-pyrones ring of the flavone skeleton might be essential for the inhibition of the formation of cyclooxygenase products, HHT and thromboxane B₂. In the previous paper,¹⁰ we reported that a number of flavonoids, (2S),2',5,6',7-tetrahydroxyflavanone, (2R,3R),2',3,5,6',7-pentahydroxyflavanone, 2',5,5',7-tetrahydroxy-6',8-dimethoxyflavone, wogonin (5,7-dihydroxy-8-methoxyflavone) and skullcapflavone II (2',5,5'-dihydroxy-6',6',7,8-tetramethoxyflavone) isolated from Scutellariae Radix inhibited the formation of the cyclooxygenase product, HHT, in leukocyte homogenate. Therefore, a free phenolic hydroxyl group at C-5 in the A-ring and a free phenolic hydroxyl group at the C-2' position in the B-ring of the flavone skeleton might be required for the inhibition of the formation of the cyclooxygenase products, HHT and thromboxane B₂. Among 2-arylbenzoferan derivatives, mulberrofuran A only inhibited the formations of HHT and thromboxane B₂, while the other 2-arylbenzoferan derivatives, mulberrofuran F and albanol B had no effect. These results suggest that the geranyl group having a two double bond system at the C-2' position in the benzene ring may be essential for the inhibition of the formations of thromboxane B₂ and HHT.

In this study, it was found that the inhibitory effects of indomethacin on the formations of HHT and thromboxane B₂ were stronger than those of various flavonoids and related compounds isolated from the root bark of mulberry tree.

Thromboxane A₂ is known to be involved in various inflammatory processes, such as formation of leukocyte chemotactic substance⁴ and platelet aggregation.⁵ The anti-inflammatory action of the root bark of the cultivated mulberry tree may be due to the inhibition of formation of the cyclooxygenase product, thromboxane B₂, by various phenolic
compounds.

References