A Hydroxyl Group of Flavonoids Affects Oral Anti-inflammatory Activity and Inhibition of Systemic Tumor Necrosis Factor-α Production

Hiroshi UEDA,† Chikako YAMAZAKI, and Masatoshi YAMAZAKI

Department of Medical Life Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Kanagawa 199-0195, Japan

Received July 14, 2003; Accepted September 25, 2003

We previously reported that oral administration of luteolin can inhibit serum tumor necrosis factor (TNF-α) production and several inflammatory and allergic models. We investigated here the effect of various flavonoids which resemble luteolin in structure. Lipo-polysaccharide (LPS)-induced TNF-α production from macrophages was inhibited by treatment with flavone (luteolin, apigenin, and chrys), flavonol (quercetin and myricetin), flavanonol (taxifolin), and anthocyanidin (cyanidin chloride) in vitro. Most of these, however, did not affect mice when administered orally. Serum TNF-α production was inhibited only by luteolin or apigenin, and only luteolin or quercetin inhibited 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema. These results suggest that the structure of luteolin: 3',4',5,7-tetrahydroxyflavone, is most suitable for the oral anti-inflammatory activity and that existence or disappearance of a hydroxy group may cause a loss of efficiency.

Key words: flavonoid; luteolin; tumor necrosis factor-α; anti-inflammation; oral administration

Materials and Methods

Mice. Male ICR mice (4 or 6 weeks old) were purchased from Shizuoka Laboratory Animal Center.
(Shizuoka, Japan). The animals were given a standard laboratory diet and water *ad libitum*. The experiments were done under the control of the guidelines for Animal Experiment in the Law (No. 105) and Notification (No. 6) of the government.

**Chemical reagents.** Luteolin, apigenin, chrysin, kaempferol, and cyanidin chloride were purchased from Funakoshi Co. (Tokyo, Japan). Baicalein, quercetin, and taxifolin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Galangin and myricetin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Their chemical structures are shown in Fig. 1.

Romurtide was generously provided by Daiichi Pharmaceutical (Tokyo) and was dissolved in water just before use. OK-432 (picibanil) was supplied by Chugai Pharmaceutical (Tokyo) and was dissolved in saline just before use. The unit 1 KE, which was used to define the dose of OK-432, means that the reagent contained 0.1 mg of killed *Streptococcus pyogenes* Su.

TPA (12-O-tetradecanoylphorbol-13-acetate) was purchased from Sigma Chemical Co., and acetone and ethanol from Wako Pure Chemical Industries.

**Cells and media.** L929, a transformed murine fibroblast cell line, was grown in Eagle’s minimum essential medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% calf serum (Hyclone Laboratories, Logan, Utah, U.S.A.) and passaged twice a week. RPMI1640 was also purchased from Nissui Seiyaku Co. and used for incubation of peritoneal cells.

**Measurement of TNF-α inhibitory activity in vitro.** Male ICR mice were intraperitoneally injected with 400 μl of 1% glycogen (Wako Pure Chemicals Industries) solution and peritoneal exudate cells were obtained after 18 hours. The obtained peritoneal cells were cultured in 96-well plates at 2.0 × 10⁶ cells in 200 μl of 1%FCS-RPMI/wells at 37°C in 5% CO₂ for 4 hours. The wells were washed three times with phosphate buffered saline (PBS) (−) and 200 μl of 5% FCS-RPMI containing test samples was added. One hour later, the wells were washed again with PBS(−) and were stimulated with 1 μg/ml of *Escherichia coli* O127:B8 lipopolysaccharide (LPS) (Difco, Michigan, U.S.A.) dissolved in 200 μl of 5% FCS-RPMI. Two hours later, TNF-α activity in the culture supernatants was evaluated by the *in vitro* L929 cell cytotoxicity assay(5) using recombinant human TNF-α (National Biological Stand-
ards Board, Hertfordshire, U.K.) as an international standard.

**Measurement of TNF-α inhibitory activity in vivo.** The oral effect of flavonoids on systemic TNF-α production was evaluated by the method previously reported.  
Briefly, mice were orally administered romurtide (500 μg/200 μl/mouse) as a priming agent, and 3 h later were intravenously injected with OK-432 (3 KE/200 μl/mouse) as a triggering agent. A sample was orally administered just before the romurtide administration, and the effect on TNF-α production was observed. Two hours after triggering, the animals were bled to obtain serum, and the serum was stored at −80°C. The TNF-α activity of the serum was evaluated by in vitro L929 cell cytotoxicity using the method of Ruff and Gifford with a slight modification, using international standard recombinant human TNF-α (National Biological Standards Board).

**Anti-inflammatory test for TPA-induced ear edema.** TPA-induced ear swelling in mice was also done by the method of Young et al., with modification. TPA was dissolved in cold acetone at a concentration of 400 μg/ml and stored at −20°C. TPA stock solution was diluted with additional cold acetone at 10 μg/ml just before use.

Male ICR mice were orally administered a test sample and 18 h later were orally administered another one. Three hours after the second administration, 10 μl of TPA solution (10 μg/ml) in acetone was spread on both sides of one ear of each mouse. Four hours later, ear swelling was measured by weighing the pieces of the ears obtained with a 5-mm diameter punch. The inhibition ratio was calculated by the following formula:

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\frac{(S - N)(W - N)}{100}
\]

(S: Sample group weight, W: Water group weight, N: Normal group weight)

**Statistics.** The statistical difference from the control value was analyzed by Student’s t-test or the Dunnett test.

**Results**

**Effect of flavonoids on LPS-induced TNF-α production from macrophage**

In our previous study we found that luteolin inhibits TNF-α production from LPS-stimulated peritoneal macrophages in vitro. Luteolin also inhibits systemic TNF-α production and inflammatory and allergic ear edemas by oral administration to mouse. We therefore investigated here the activities of flavonoids, which resemble luteolin in structure.

To know the influence of the difference of the basal skeleton of flavonoids, we first investigated the in vitro TNF-α inhibitory activities of luteolin, quercetin, taxifolin and cyanidin (chloride), all of which bind hydroxyl group at positions A-5, A-7, B-3′, and B-4′. When macrophage culture was pretreated with luteolin or quercetin (20–500 μg/ml), TNF-α production induced by LPS treatment (1 μg/ml) was reduced in a dose dependent manner (Fig. 2A); taxifolin did not inhibit TNF-α production and cyanidin did augment it. We therefore assumed that the basal structure of flavone or flavonol is required for the TNF-α inhibitory activity, and looked next at the activities of various types of flavone and flavonol to prove this hypothesis. As shown in Fig. 2B, all flavones investigated here inhibited TNF-α production in vitro. The production was completely inhibited by baicalein treatment, but the viability of the macrophages was also decreased to 7% with trypan blue staining. The activity of apigenin was similar to that of luteolin and that of chrysin was weak. In flavonols, myricetin also inhibited TNF-α production to a similar extent as quercetin, however, kaempferol had no activity and galangin oppositely augmented its production. (Fig. 2C).

**Effects of oral administration of flavonoids on serum TNF-α production**

To learn whether the in vitro observation applied to in vivo oral administration, mice were primed with an oral administration of romurtide (500 μg/mouse) and then triggered with an intravenous injection of OK-432 (3 KE/mouse). Excessive TNF-α production was observed in serum, generally amounting to approximately 1000 U/ml. Figure 3 shows the relative value of serum TNF-α activity when various flavonoids were orally administered to mice with romurtide at a concentration of 1 mg/mouse. Flavonoid dose and timing of administration was based on the optimal data of luteolin.

The oral effects of some flavonoids that are different in basal skeleton but bind a common hydroxyl group were also evaluated. Oral administration of luteolin at 1 mg/mouse significantly inhibited the TNF-α production, but quercetin, taxifolin and cyanidin had no effect (Fig. 3A). With regard to the oral effects of certain flavones and flavonols, as shown in Fig. 3B, apigenin had a tendency to inhibit serum TNF-α production, but other flavones did not affect this activity. On the contrary, baicalein augmented serum TNF-α production though it reduced the production in vitro. None of the flavonols inhibited serum TNF-α activity (Fig. 3C), though quercetin and myricetin inhibited the production in vitro (Fig. 2C).

**Effect of oral administration of flavonoids on inflammatory ear edema**

To determine whether oral TNF-α-inhibitory activities are consistent with oral anti-inflammatory activity, selected TPA-induced ear edema as the experimental inflammatory model, having previously shown that perilla leaf extract and its active molecule, luteolin, can inhibit this model.

Figure 4 shows the relative value of ear edema weight
Fig. 2. Effects of Various Flavonoids on LPS-Induced TNF-α Production from Macrophage. Male ICR mice were intraperitoneally injected with 400 μl of 1% glycogen solution and peritoneal exudate cells were obtained after 18 hours. These cells were cultured in 96-well plates at 2.0 × 10^5 cells/200 μl/well at 37°C in 5% CO₂ for 4 hours. The wells were washed three times with PBS(−) and were added with 200 μl of 5% FCS-RPMI containing various flavonoids (20–500 μg/ml). One hour later, the wells were washed again with PBS(−) and were stimulated with 1 μg/ml of E. coli O127:B8 lipopolysaccharide (LPS) dissolved in 200 μl of 5% FCS-RPMI. Two hours later, TNF-α activity in the culture supernatants was evaluated by an in vitro L929 cell cytotoxicity assay as described in Materials and Methods. Each point indicates the mean ± SD. Significant differences from the control: *P < 0.05, **P < 0.01, ***P < 0.001 by Dunnet test.

Fig. 3. Effect of Oral Administration of Various Flavonoids on Systemic TNF-α Production. Male ICR mice (n = 3) were orally administered romurtide (500 μg/400 μl/mouse) and various flavonoids (1 mg/400 μl/mouse). Three hours later, the mice were injected intravenously with OK-432 (3 KE/200 μl/mouse). Two hours later, the animals were bled and the serum TNF-α activity was measured by an L929 cell cytotoxicity assay. Data are the mean ± SD. Experiments were done three times with comparable results. The statistical difference was analyzed by Student’s t-test. Significant differences from the control: *P < 0.05.
of mice that were orally administered various flavonoids (1 mg/mouse). The dose and timing of administration were based on those of luteolin. Oral administration of luteolin inhibited TPA-induced ear edema in addition to TNF-α-inhibitory activity, but taxifolin and cyanidin did not (Fig. 4A). Quercetin also inhibited TPA-induced ear edema, inconsistent with its effect on serum TNF-α production, while other flavones and flavonols did not inhibit this ear edema (Fig. 4B and 4C).

Discussion

Our investigation focused on the anti-inflammatory actions of various flavonoids which resemble luteolin in structure. In vitro study revealed that some flavonoids inhibit LPS-induced TNF-α production (Fig. 2). The results also brought out that the in vitro activities are easily affected by the position of the hydroxyl group. It is suggested that the chemical component necessary to inhibit TNF-α production in vitro is flavone with hydroxyl group at positions A-5, A-7, and B-4', which is a common structure in apigenin, luteolin, quercetin, and myricetin. The hydroxyl groups at positions B-3', B-5', and C-3 had no effect on the activity. Among the tested flavonoids baicalein was seemingly the most effective, but this was because of its deteriorating viability of macrophages. The inhibitory effect of chrysin was less than apigenin and pretreatment with galangin oppositely augmented LPS-induced TNF-α production, suggesting that deletion of hydroxy groups at position B-4' may not only decrease TNF-α production but reverse the action.

The in vivo oral TNF-α inhibitory activities of flavonoids, however, did not coincide with in vitro activities; only luteolin and apigenin inhibited serum TNF-α production when orally administered to mice (Fig. 3). It is therefore suggested that the oral TNF-α inhibitory activity require the 4',5,7-trihydroxyflavone, which is a structure common to apigenin and luteolin. The inhibitory activity is promoted by the existence of the hydroxy group at position B-3', because the inhibitory activity of luteolin was stronger than that of apigenin (Fig. 3B). Myricetin, quercetin, kaempferol, and chrysin did not inhibit serum TNF-α production in spite of the oral effect of luteolin and apigenin, suggesting that the existence of a hydroxyl group at position C-3 or its deletion at position B-4' may affect on metabolism or absorption of flavonoids and, consequently, cause the in vivo oral TNF-α inhibitory activity to disappear.

Only luteolin and quercetin inhibited TPA-induced ear edema by the oral route (Fig. 4). We can confirm, therefore, that the oral anti-inflammatory effects of flavonoids are also not necessarily consistent with in vitro TNF-α inhibitory activity. These results also suggested that oral anti-inflammatory activity requires a common structure to quercetin and luteolin, which is 3',4',5,7-tetrahydroxyflavone, and the activity is elim-
nated by deletion of the hydroxyl group at position B-3' or the binding of the hydroxy group at position B-5'. The oral administration of baicalein augmented serum TNF-α production and aggravated TPA-induced ear edema. Baicalein has been reported to be the flavonoid which inhibits inflammation but most experiments were parenteral and therefore the actual effect when ingested as a food factor may reduce the inflammation.

Thus, only luteolin inhibited both serum TNF-α production and TPA-induced ear edema. This flavone also inhibits arachidonic acid-induced ear edema and production and TPA-induced ear edema. This flavone

References


