Inhibitory Effect of Stevioside on Tumor Promotion by 12-*O*-Tetradecanoylphorbol-13-acetate in Two-Stage Carcinogenesis in Mouse Skin

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Four steviol (*ent*-kaurene-type diterpenoid) glycosides, stevioside, rebaudiosides A and C, and dulcoside A, have been isolated from *Stevia rebaudiana* BERTONI. These compounds showed strong inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. The 50% inhibitory dose of these compounds for TPA-induced inflammation was 54.1—291.6 μg/ear. Furthermore, at 1.0 and 0.1 mg/mouse of stevioside mixture, the mixture of these compounds markedly inhibited the promoting effect of TPA (1 μg/mouse) on skin tumor formation initiated with 7,12-dimethylbenz[*a*]anthracene (50 μg/mouse).

Key words stevioside; antitumor promoter; stevia; two-stage carcinogenesis; 12-*O*-tetradecanoylphorbol-13-acetate; *ent*-kaurene-type diterpene glycosides

The leaves of *Stevia rebaudiana* BERTONI (Compositae), known colloquially as Caa-ehe, have been used for centuries by the Paraguayan Indians as a sweetening agent with maté tea.[1] The steviol (*ent*-kaurene type diterpene) glycosides, stevioside, rebaudiosides A, C, and F, and dulcoside A have been isolated from *S. rebaudiana* and are widely used as sweeteners.[2,3] In a previous paper we reported the inhibitory effect of the methanol extract of *S. rebaudiana* and its active component, lupeol palmitate, on inflammatory ear edema caused by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin.[4] Steviol inhibited Epstein-Barr virus early antigen (EBV-EA) induced by TPA in Raji cells, although stevioside did not inhibit the induction of EBV-EA by TPA.[5] In this paper we examine the antiinflammatory effect of steviol glycosides, stevioside, rebaudiosides A and C and dulcoside A against TPA-induced inflammation and found these compounds to possess a marked inhibitory effect. The 50% inhibitory dose (ID_{50}) of these compounds for TPA-induced inflammation was 54.1—291.6 μg/ear. Furthermore, the stevioside mixture (the steviol glycosides from *S. rebaudiana*) was found to suppress markedly the tumor-promoting effect of TPA on skin tumor formation initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) in mouse skin.

MATERIALS AND METHODS

Chemicals Stevioside, rabaudiosides A and C, dulcoside A, and stevioside mixture was obtained from Tokiwa Phytochemical (Chiba, Japan). These compounds were identified by chromatographic and spectroscopic comparison with authentic samples.[6] The stevioside mixture contained stevioside (48.9%), rebaudioside A (24.4%), rebaudioside C (9.8%), dulcoside A (5.6%), and unidentified components (11.3%). DMBA, indomethacin, hydrocortisone, quercetin, and dimethylsulfoxide were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). TPA was obtained from Chemicals for Cancer Research (Minneapolis, MN, U.S.A.).

Animals Female ICR mice (7 weeks old) were obtained from the Japan SLC (Shizuoka, Japan). The animals were housed in an air-conditioned specific pathogen-free room (22—23 °C), lit from 08:00 to 20:00. Food and water were available *ad libitum*.

Assay of TPA-Induced Inflammation TPA (1 μg/ear) dissolved in acetone (20 μl) was applied to the right ear only of ICR mice using a micropipette. A volume of 10 μl was delivered to both the inner and outer surfaces of the ear. The sample, or its vehicle (20 μl), chloroform–methanol–water (1 : 2 : 1) or methanol–water (1 : 1), as a control, was applied topically about 30 min before each TPA treatment. For thickness determinations, a pocket thickness gauge (Mitsutoyo, Tokyo, Japan) with a range of 0—9 mm, graduated at 0.01-mm intervals and modified so that the contact surface area was increased to reduce the tension, was applied to the tip of the ear.

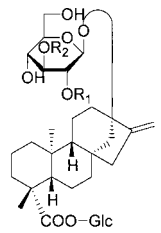
The ear thickness was measured before treatment (*a*). Edema was measured 6 h after TPA treatment (*b*: TPA alone; *b′*: TPA plus sample). The following values were then calculated:

Edema A: edema induced by TPA alone ($b-a$)

Edema B: edema induced by TPA plus sample ($b'-a$)

$$\text{inhibitory ratio (\%)} = \frac{\text{Edema A} - \text{Edema B}}{\text{Edema A}} \times 100$$

Each value represents the mean of individual determinations from 5 mice. The ID_{50} values were determined by pro-



	R_1	R_2
Stevioside	Glc	H
Rebaudioside A	Glc	Glc
Dulcoside A	Rha	H
Rebaudioside C	Rha	Glc

Chart 1. Chemical Structures of Stevioside and Related Compounds

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bit-graphic interpolation for four dose levels.

Two-Stage Carcinogenesis Experiment The backs of mice (7 weeks old) were shaved with electric clippers. Initiation was accomplished by a single topical application of DMBA 50 μg. Promotion with TPA 1 μg, applied twice weekly, was begun 1 week after the initiation. Stevioside mixture (0.1, 1.0 mg), or its vehicle, acetone–dimethylsulfoxide (9 : 1; 100 μl), were applied topically 30 min before each TPA treatment. DMBA and TPA were dissolved in acetone and applied to the shaved area in a volume of 100 μl using a micropipette. The back of each animal was shaved once a week to remove hair. The number and diameter of skin tumors were measured every other week, and the experiment continued for 20 weeks. Experimental and appropriate control groups each consisted of 15 mice.

Statitstical Analysis Statistical analysis was performed using Student's *t*-test.

RESULTS

Effect of *ent*-Kaurene-Type Diterpenoid Glycosides on TPA-Induced Inflammation As can be seen in Table 1, TPA-induced inflammation, for which the inhibitory ratio was calculated at the time of the 6-h maximum edema, was inhibited by *ent*-kaurene-type diterpenoid glycosides, stevioside, rebaudiosides A and C, and dulcoside A. Application of a sample (1.0, 0.2, 0.04, or 0.008 mg/ear) inhibited the TPA-induced inflammation in a dose-dependent manner. All assayed steviol glycosides inhibited the inflammatory activity induced by TPA. The inhibitory effects were compared with the literature values for the antitumor-promoting agent quercetin and the two commercially available antiinflammatory drugs indomethacin and hydrocortisone. All compounds examined markedly inhibited the TPA-induced inflammation, with 0.06—0.36 μM being the ID_{50}. In comparison with antiinflammatory drugs, rebaudiosides A and C and dulcoside A were similar in activity to hydrocortisone, and stevioside was more effective than indomethacin. Rebaudioside C showed 50% inhibition of the swelling at doses 10 and 100 times smaller than those of indomethacin and quercetin, an antitumor-promoting agent, respectively.

Table 1. Inhibitory Effect of Steviol Glycosides on TPA-Induced Inflammatory Ear Edema in Mice

Compound	ID_{50}		I.R.[a] (%)
	μg/ear	μM/ear	
Stevioside mixture	239.9	—	40*
Stevioside	291.6	0.36	35*
Rebaudioside A	92.2	0.10	57*
Rebaudioside C	54.1	0.06	67*
Dulcoside A	92.5	0.12	64*
Quercetin	1600	6.6	12
Indomethacin	300.1	0.84	36*
Hydrocortisone	30.0	0.08	70*

a) The inhibitory ratio was at 200 μg/ear, $p<0.01$ by Student's *t*-test as compared with the control group.

Effect of Stevioside Mixture on TPA-Induced Skin Tumor Formation Figure 1A shows the time course of skin tumor formation in the group treated with DMBA plus TPA, with or without stevioside mixture. The first tumor appeared at week 6 in the group treated with DMBA plus TPA. In the group treated with DMBA plus TPA and stevioside mixture 0.1 mg and 1.0 mg, the first tumor appeared at weeks 9 and 16, respectively. The percentage of tumor-bearing mice treated with DMBA plus TPA was 80% at week 20, whereas the percentage in the groups treated with DMBA plus TPA and stevioside mixture 0.1 mg and 1.0 mg was 20% and 13%, respectively. Figure 1B shows the average number of tumors/mouse. The group treated with DMBA plus TPA produced 8.1 tumors/mouse at week 20, whereas the groups treated with DMBA plus TPA and stevioside mixture 0.1 mg and 1.0 mg had 2.2 and 0.3 tumors/mouse, respectively. The treatment with stevioside mixture 0.1 mg and 1.0 mg caused 73% and 96% reductions, respectively, in the average number of tumors/mouse at week 20.

DISCUSSION

A series of naturally occurring components of Compositae plants has been found to possess antitumor-promoting activities.[7] In our previous studies, a sesquiterpenoid from Atractylodis Rhizoma,[8] sterols from Carthami Flos,[9] triterpenoids from the flowers of edible Chrysanthemum,[10,11] and *syn*-alkane-6,8-diols from Carthami Flos[12] inhibited TPA-induced tumor promotion in two-stage carcinogenesis in mouse skin. In this study, *ent*-kaurene-type diterpenoid glycosides from *S. rebaudiana* inhibited the inflammatory activity induced by TPA in mouse skin.

On the other hand, in our previous studies, the inhibitory activities of TPA-induced inflammation were shown roughly to parallel their inhibitory activities against TPA-induced

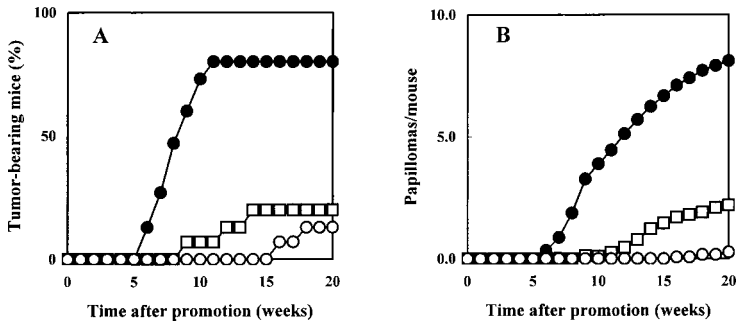

Fig. 1. Inhibitory Effect of Stevioside Mixture on the Promotion of Skin Papillomas by TPA in DMBA-Initiated Mice

From 1 week after initiation with a single topical application of DMBA 50 μg, TPA 1 μg was applied twice weekly. Topical application of stevioside mixture 0.1 mg and 1.0 mg and vehicle was performed 30 min before each TPA treatment. Data are expressed as percentage of mice bearing papillomas (A), and as average number of papillomas/mouse (B). ●, +TPA with vehicle alone; ○, +TPA with 1.0 mg/mouse stevioside mixture; □, +TPA with 0.1 mg/mouse stevioside mixture.

tumor promotion.[13,14] Stevioside and related compounds inhibited inflammatory ear edema and tumor-promoting activities induced by TPA in mouse skin. The *in vitro* assay for EBV-EA by TPA in Raji cells is usually used as a primary screening assay for antitumor-promoting agents. Many compounds that are active in the EBV-EA assay have been confirmed to be inhibitors of tumor promotion in two-stage carcinogenesis tests *in vivo*.[15] Stevioside did not inhibit the induction of EBV-EA by TPA,[5] although stevioside inhibited TPA-induced inflammatory ear edema in mice. These results suggested that the assay of the inhibitory effect against TPA-induced inflammation in mouse skin was better than the assay of EBV-EA induction by TPA.

The inhibitory activities of these compounds were more effective than that of indomethacin in TPA-induced inflammatory ear edema. Rebaudiosides A and C and dulcoside A were similar in activity to hydrocortisone in inflammatory ear edema induced by TPA. In our previous studies, some triterpenoids, heliantriol C,[11] pachymic acid, 3-*O*-acetyl-16α-hydroxytrametenolic acid, and poricoic acid B[16] were most effective in the naturally occurring components from plants and fungi on tumor promotion by TPA in mouse skin. Rebaudiosides A and C and dulcoside A were similar in activity to these triterpenoids in TPA-induced inflammation in mouse skin. Stevioside mixture was similar in activity to these triterpenoids against the tumor-promoting effect by TPA following initiation with DMBA. In addition, these compounds are widely used as sweeteners, although these triterpenoids are contained in the crude drugs. This suggests that stevioside was better than these triterpenoids for the chemoprevention of cancer.

Orally administered stevioside (2.5, 5%) was not carcinogenic in F344 rats of both sexes.[17] In addition, stevioside did not produce acute toxicity in mice, rats, and hamsters of both sexes.[18] The reported pharmacological activities of stevioside and related compounds can be summarized as follows: 1) sweetener in diet for diabetes patients; 2) no abnormalities at 2.5 g/kg in hamsters[19]; 3) has an antiinflammatory effect; and 4) has an antitumor-promoting effect. However, the mechanism by which these compounds exert these effects remains to be elucidated.

Stevioside and related compounds are widely used as sweeteners. It is of interest that food factors contained in many foods inhibited the tumor-promoting activity of TPA in two-stage carcinogenesis in mouse skin. This is of great importance from the viewpoint of the chemoprevention of cancer.

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