Inhibitory Effect of Glycyrrhetinic Acid Derivatives on Capsaicin-Induced Ear Edema in Mice

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ABSTRACT—We examined the effect of glycyrrhetinic acid (Ia) and its derivatives on ear edema induced by topical application of capsaicin in mice. Three dihemiphthalate compounds: di-sodium salt of 18β-olean-12-ene-3β,30-diol (deoxoglycyrrhetol, IIa) di-O-hemiphthalate (IIb); 18β-olean-9(11),12-diene-3β,30-diol di-O-hemiphthalate (IIla); and olean-11,13(18)-diene-3β,30-diol di-O-hemiphthalate (IVa) inhibited capsaicin-induced edema with ED50 values of 52.6, 41.0 and 51.8 mg/kg (p.o.), respectively. However, glycyrrhetinic acid and deoxoglycyrrhetol at a dose of 200 mg/kg (p.o.) had no effect. Compound IIIa (100 mg/kg, p.o.) also inhibited the edema response to capsaicin in mast cell-deficient mice. Furthermore, compounds IIb, IIIa and IVa (25-100 mg/kg, p.o.) prevented ear edema in response to intradermal injection of substance P (SP) and compound 48/80. In addition, these compounds at a high dose of 100 mg/kg (p.o.) produced a significant inhibition of the plasma extravasation in ear skin induced by i.v. administration of SP. The above results suggest that the effect of these compounds on capsaicin-induced ear edema is due at least in part to an inhibition of the increase of vascular permeability induced by vasoactive agents released from mast cells. Moreover, it seems likely that these compounds at a high dose can suppress vasodilatation and plasma extravasation induced by SP involved in capsaicin-induced edema.

Keywords: Capsaicin, Ear edema, Glycyrrhetinic acid derivative, Substance P

Glycyrrhetinic acid (Ia) (Fig. 1), the aglycone of glycyrrhizin isolated from licorice root (Glycyrrhiza), has been established to have various pharmacological activities such as anti-inflammatory (1, 2), anti-tumorigenic (3) and anti-hepatotoxic (4) as well as inhibitory activities against hepatic and renal 11β-hydroxysteroid dehydrogenase (5) and growth of mouse melanoma (6). Glycyrrhetinic acid derivatives have been prepared to enhance the therapeutic effects and suppress adverse actions (pseudo-aldosteronism) of the parent compound (7). Clinically, glycyrrhetinic acid 3β-O-hemiphthalate sodium (Ib) has been found to reduce pain with a transdermal 10% lidocaine base gel (8). We have previously reported that deoxoglycyrrhetol dihemiphthalate (IIb) and the related compounds (IIla and IVa) inhibit leukotrienes and prostaglandin E2 synthesis in vitro (9) and in vivo (10, 11), and edema formation induced by inflammatory agents such as arachidonic acid (10), 12-O-tetradecanoylphorbol-13-acetate (TPA) (12) and carrageenan (13). Furthermore, these compounds have been found to prevent experimental gastric ulcer by strengthening gastric mucosal defense mechanisms, which were independent of the protective effect of prostaglandins (14). Recently, some other workers have reported that the di-sodium salts of the dihemiphthalates of urs-12-ene-3β,28-diol and ursa-9(11),12-diene-3β,28-diol derived from uvaol are effective anti-ulcer agents (15).

Neurogenic inflammatory responses such as axon reflex vasodilatation, plasma extravasation and mast cell activation are induced by stimulation of capsaicin-sensitive afferent neurons that contain neuropeptides including substance P (SP) as neurotransmitters (16, 17). Topical application of capsaicin, the primary pungent ingredient of red peppers, to the ear of mouse produces neurogenic skin inflammation (18–20). Previous studies have demonstrated that capsaicin-induced mouse ear edema is inhibited by tachykinin NK1-receptor antagonists, histamine and/or serotonin antagonists, but not by inhibitors of arachidonate metabolites such as indomethacin, nortic, hydroxyparic acid and AA 861 (20–22). In this study, we examined the effect of glycyrrhetinic acid and its derivatives on capsaicin-induced mouse ear edema.
**MATERIALS AND METHODS**

**Animals**

Six-week-old male ddY mice weighing 30–35 g (Japan SLC, Hamamatsu) or nine-week-old male WBB6F1-W/- mice weighing 25–31 g (Japan SLC) were used for the experiments. The animals were kept in an environmentally controlled room (24±1°C, 55±10% humidity) and allowed free access to food and water.

**Capsaicin-induced mouse ear edema**

Induction of mouse ear edema was performed as reported previously (20). Animals were conscious when tested. Capsaicin was dissolved in acetone at a concentration of 12.5 mg/ml, and 20 μl (250 μg/ear) was then applied topically to both surfaces of an ear of each mouse. The magnitude of edema was assessed by measuring the thickness at the edge of ear before and 30 min after capsaicin treatment in units of 0.001 mm with dial calipers.

Fig. 1. The structures of glycyrrhetinic acid derivatives.
Ear edema was expressed as the increase in ear thickness.

**Histological examination**

Thirty minutes after capsaicin treatment, ears were removed by cutting horizontally across the indentation at the base of the ear and fixed in 10% neutral-buffered formaldehyde. Sections of the tissue were stained with hematoxylin/eosin for light microscopy.

**Mouse ear edema induced by intradermal injection of SP and compound 48/80**

Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Abbott Laboratories, North Chicago, IL, USA). Intradermal injections were made into the central site of the ear with a hypodermic needle (0.28 x 18.00 mm) and a repeating dispenser (Hamilton Co., Reno, NV, USA). A blister was formed in the outer surface of the ear by injection of 5 μl of saline containing SP (100 pmol/site) and compound 48/80 (5 μg/site). The ear thickness was measured at the edge of the ear with dial calipers before and 30 min after injections.

**Plasma extravasation in mouse ear induced by SP**

Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Plasma extravasation was induced by i.v. injection of SP at a dose of 5 μg/kg, together with Evans blue (60 mg/kg; Wako Pure Chemical Industries, Osaka) used as a marker. Control mice for SP received saline and Evans blue. Thirty minutes later, the animals were killed by cervical dislocation, and an ear was removed by cutting across the indentation at the base of the ear. The ear was weighed and dissolved with 0.7 ml of 1 N potassium hydroxide at 37°C for 18 hr, and then it was added with 4.3 ml of a mixture of 0.6 N phosphoric acid and acetone (5:13). After vigorous shaking, the mixture was centrifuged at 3,500 x g for 20 min. The dye in the supernatant was measured by spectrophotometry at 620 nm. Plasma extravasation was expressed as μg of Evans blue per 100 mg of wet tissue.

**Drug administration**

Glycyrrhetinic acid and its derivatives were orally administered 30 min before treatment of capsaicin, SP and compound 48/80. RP 67580 and chlorpheniramine were intravenously given to a tail vein 15 min before irritant agents. Control mice received the vehicle by the corresponding route.

**Drugs**

We used the following compounds: glycyrrhetinic acid and its derivatives (Minophagen Research Laboratory, Kanagawa); capsaicin, chlorpheniramine maleate salt.
substance P and compound 48/80 (Sigma Chemical Co., St. Louis, MO, USA). RP 67580 ((3$\alpha$R,7$\alpha$R)-7,7-diphenyl-2-(1-imino-2-(2-methoxyphenyl) ethyl) perhydroisindol-4-one) was the generous gift of Rhône-Poulenc Rorer, Vitry sur Seine, France. Glycyrrhetinic acid and its derivatives were dissolved or suspended with 1% polyoxyethylene sorbitan mono-oleate (Tween 80; Tokyo Kasei Chemical Industry, Tokyo). RP 67580 was initially dissolved in 0.1 N methanesulfonic acid and diluted in saline as required.

Fig. 3. Representative light microphotography of capsaicin-induced ear edema in mice treated with compound IIIa. Compound IIIa (50 mg/kg, p.o.) was administered 30 min before capsaicin application (250 $\mu$g/ear). A thin section was obtained from mouse ear tissue 30 min after capsaicin treatment (hematoxylin-eosin staining, ×100). a: control, b: compound IIIa-treated.

Data analyses
Results are expressed as the mean±S.E.M. The ED$_{50}$ values of test compounds were calculated by linear regression analysis. Statistical significance of differences between control and test groups was determined by Student's $t$-test or the Cochran-Cox test after analysis of variance.
Table 2. Effects of Compound IIIa and RP 67580 on capsaicin-induced ear edema in mast cell-deficient mice

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg/kg)</th>
<th>Increase in ear thickness (mm)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.142±0.009</td>
<td></td>
</tr>
<tr>
<td>Compound IIIa</td>
<td>100</td>
<td>0.069±0.009***</td>
<td>51</td>
</tr>
<tr>
<td>RP 67580</td>
<td>0.5</td>
<td>0.044±0.008***</td>
<td>69</td>
</tr>
</tbody>
</table>

Compound IIIa was orally administered 30 min, and RP 67580 was intravenously given 15 min before capsaicin treatment. Ear edema was examined 30 min after the treatment. Values represent the means±S.E.M. of 5 animals. ***P<0.001, statistically significant compared with the control group.

RESULTS

Effects of glycyrrhetic acid derivatives on capsaicin-induced mouse ear edema

Deoxoglycyrrhetol dihemiphthalate (IIb) and the related compounds (IIIa and IVa) at a p.o. dose of 25 or 50 mg/kg significantly inhibited ear swelling induced by topical application of capsaicin (250 μg/ear) (Table 1). The ED₅₀ values were 52.6 (39.5-70.1) mg/kg for IIb, 41.0 (33.0-51.1) mg/kg for IIIa and 51.8 (40.3-66.7) mg/kg for IVa (Fig. 2), respectively. Glycyrrhetic acid (Ia), glycyrrhetic acid 3β-O-hemiphthalate sodium (Ib) and deoxoglycyrrhetol (IIa) at a dose of 200 mg/kg (p.o.) had little effect on the edema formation. Chlorpheniramine (4 mg/kg, i.v.), a histamine H₁ blocker, exhibited a slight, but significant inhibition of capsaicin-induced edema. Furthermore, RP 67580 (0.1 and 0.5 mg/kg, .
i.v.), a tachykinin NK₁-receptor antagonist, was very effective in suppressing the edema response to capsaicin. The ED₅₀ value of RP 67580 is 0.19 mg/kg (21). Compounds IIb, IIIa and IVa had no effect on ear thickness in normal mice.

Examination of the hematoxylin- and eosin-stained sections clearly revealed the edema response in the dermis following topical application of capsaicin (Fig. 3a). Compound IIIa at a dose of 50 mg/kg (p.o.) markedly diminished the increase in the thickness of the epidermis (Fig. 3b). Application of capsaicin to the ear causes degranulation of mast cells in skin connective tissue, although capsaicin itself does not induce mast cell degranulation directly (22). However, toluidine blue sections showed that compound IIIa did not prevent degranulation of mast cells in the tissue of capsaicin-treated ears (H. Inoue, unpublished data).

Effects of compound IIIa on ear edema induced by capsaicin in mast cell-deficient mice

The edema response to capsaicin in mast cell-deficient mice (WBB6F₁⁻⁻) was decreased up to 36%, compared with that in the control mice (WBB6F₁⁺⁺) (H. Inoue, unpublished data). Compound IIIa at a dose of 100 mg/kg (p.o.) diminished the response to capsaicin in mast cell-deficient mice (Table 2). Furthermore, RP 67580 (0.5 mg/kg, i.v.) suppressed capsaicin-induced ear edema by 70%.

Effects of deoxoglycyrrhetol dihemipthalate (IIb) and the related compounds (IIIa and IVa) on edema and plasma extravasation induced by SP in mouse ear

Test compounds IIb, IIIa and IVa (25–100 mg/kg, p.o.) dose-dependently inhibited ear edema induced by intradermal injection of SP (100 pmol/site) with ED₅₀ values of 69.5 (57.8–83.6) mg/kg for IIb, 67.2 (58.6–77.0) mg/kg for IIIa and 61.6 (55.3–68.6) mg/kg for IVa (Fig. 4). Moreover, these compounds at a dose of
100 mg/kg prevented plasma extravasation induced by i.v. administration of SP (5 μg/kg) (Fig. 5). However, glycyrrhetinic acid at 200 mg/kg (p.o.) (Ia) was ineffective in suppressing both responses to SP. The test compounds themselves did not modify the base level of plasma extravasation (H. Inoue, unpublished data). Chlorpheniramine (4 mg/kg, i.v.) significantly inhibited SP-induced ear edema but had little effect on plasma extravasation. Both responses to SP were markedly suppressed by pretreatment with RP 67580 (0.5 mg/kg, i.v.).

**Effects of deoxoglycyrrhetol dihemiphthalate (IIb) and the related compounds (IIa and IVa) on compound 48/80-induced mouse ear edema**

Pretreatment with compound IIb, IIa or IVa at doses of 50 and 100 mg/kg (p.o.) produced a significant inhibition of compound 48/80 (5 μg/ear)-induced ear edema (Fig. 6). Chlorpheniramine (4 mg/kg, i.v.) significantly inhibited the edema response to compound 48/80. In contrast, glycyrrhetinic acid (200 mg/kg, p.o.) did not suppress the edema.

**DISCUSSION**

Deoxoglycyrrhetol dihemiphthalate (IIa) and the related compounds (IIa and IVa), the glycyrrhetic acid derivatives with hemiphthalate groups at the 3- and 30-positions of rings A and E in the oleanane skeleton, inhibited mouse ear edema induced by capsaicin, SP and compound 48/80, whereas glycyrrhetic acid (Ia) (and deoxoglycyrrhetol (IIa)), the parent compounds of the dihemiphthalate derivatives, had no effect on these models. Thus, it appears that the anti-inflammatory profile of the three compounds (IIb, IIa and IVa) is quite
different from that of glycyrrhetinic acid. Compound (Ib) with a hemiphthalate group at the 3-position of ring A was weakly effective in inhibiting capsaicin-induced ear edema. These results suggest that the dihemiphthalate substitution on the oleanane skeleton of these compounds is essential for the potent inhibition of the edema response to capsaicin. This agrees with the results previously obtained from biochemical and pharmacological studies on the inhibitory effect of these compounds on lipoxygenase and cyclooxygenase activities (9) and on the inhibition of mouse ear edema induced by arachidonic acid (10) and TPA (12). In addition, the effect of these three compounds has been suggested to be a direct action and does not involve the anti-inflammatory action of steroids mediated by the formation of a reactive protein (10).

It has been suggested that SP released from sensory neurons plays an important role as a chemical mediator in capsaicin-induced skin inflammation (20, 21, 23). In contrast, arachidonate metabolites are minor factors in the edema response to capsaicin (20). The response to SP and compound 48/80 is also unaffected by pretreatment with indomethacin (20, 24). Hence it appears that the inhibitory effect of three compounds on capsaicin-induced edema is not due to an inhibition of the arachidonate pathway at the inflammatory site.

SP is well-known to cause an inflammatory response by mediation of NK1-receptors (23, 25, 26) and by releasing vasoactive amines from mast cells (27, 28). Indeed, peripheral nerve endings and mast cells are in close spatial and functional association (29–31). Deoxoglycyrrhetol dihemiphthalate and the related compounds, at the same doses which prevented capsaicin-induced edema, exhibited a significant inhibition of ear edema induced by intradermal injection of SP. However, capsaicin-evoked mast cell degranulation in skin connective tissue was unaffected when compound IIIa was administered before capsaicin application (H. Inoue, unpublished data). These observations provide evidence that the three compounds act at the postsynaptic level, but not at the presynaptic site, to inhibit the edema response to capsaicin. A previous study has shown that these compounds can prevent mouse paw edema and the contraction of isolated guinea pig ileum induced by histamine (13). This suggests that the inhibitory effect of the compounds on edema formation evoked by capsaicin and intradermal SP is due at least in part to a prevention of the increase of vascular permeability induced by histamine released from mast cells. This is supported by the finding that the three compounds inhibited ear edema induced by compound 48/80, a histamine liberator.

We have shown that compound IIIa prevented capsaicin-induced ear edema in mast cell-deficient mice. This indicates that the mode of action of the three compounds is not merely due to the suppression of the inflammatory response to mediators released from mast cells. Furthermore, we found that deoxoglycyrrhetol dihemiphthalate and related compounds at a high dose are able to inhibit the i.v. SP-induced plasma extravasation in ear skin. Chlorpheniramine exhibited a partial inhibition of the response induced by capsaicin and intradermal injection, but not by i.v. administration, of SP. It is, therefore, conceivable that histamine cannot primarily mediate the response to i.v. SP. On the other hand, in addition to the inhibition of the edema response to topical capsaicin and SP, RP 67580 (32), a non-peptide NK1-receptor antagonist, blocked the ability of i.v. SP to augment vascular permeability in ear skin. Thus it seems that the response to i.v. SP is dependent on the mediation of NK1-receptors on postcapillary venules and arterioles (33). These findings suggest that three compounds can modulate vaso dilatation and plasma extravasation induced by SP involved in the capsaicin-induced ear edema, and this effect of the derivatives may play a role in the suppression of the response to capsaicin. However, in the present study, it is unclear whether the compounds affect the NK1-receptor in mouse skin.

On the basis of the present study, we conclude that deoxoglycyrrhetol dihemiphthalate and the related compounds, the glycyrrhetinic acid derivatives, may be useful for the treatment of skin diseases including the neurogenic inflammatory response.

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