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Inhibitory Effect of Glycyrrhetinic Acid Derivatives on Arachidonic Acid-induced Mouse Ear Oedema

HIDEO INOUE, TAKEO MORI, SHOJI SHIBATA* AND YASUKO KOSHIHARA†

*Research Laboratory, Minophagen Pharmaceutical Co., Komatsubara, Zama-shi, Kanagawa 228, *Laboratory of Natural Medicinal Materials, Minophagen Pharmaceutical Co., Yotsuya, Shinjuku-ku, Tokyo 160 and † Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo 173, Japan*

Abstract—The inhibitory effects of glycyrrhetinic acid and its derivatives were examined on arachidonic acid (AA)-induced ear oedema in mice. Of the compounds, dihemiphthalate derivatives of 18β-olean-12-ene-3β, 30-diol (IId, IId′), 18β-olean-9(11)12-diene-3β, 30-diol (IIIa, IIIa′) and olean-11,13(18)-diene-3β, 30-diol (IVa, IVa′) showed a strong inhibition of ear oedema on both topical (ID50, 1·9, 2·8 and 1·7 mg/ear, respectively) and oral (ID50, 90, 130 and 88 mg kg^{-1}, respectively) administration. Topical ID50 values were approximately the same potency as nordihydroguaiaretic acid (ID50, 2·1 mg/ear). Given topically these compounds were also capable of inhibiting PGE_2 and LTC_4 formation at an early stage of AA-induced ear oedema. However, glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa), the fundamental skeletons of the derivatives, showed no detectable inhibition of oedema at a dose of 1 mg/ear (topical) or 200 mg kg^{-1} (oral). The most effective time for the topical administration of the compound IId against ear oedema was 0–30 min before AA application; this is different from dexamethasone which requires a time lag for reaction. The results suggest that the inhibitory effect of the hemiphthalate compounds (IId, IId′, IIIa, IIIa′, IVa and IVa′) is a direct action, and does not involve the anti-inflammatory action of steroids mediated by the secondary formation of a reactive protein.

Glycyrrhetinic acid (Ia) is the aglycone of glycyrrhizin which was isolated from the root of liquorice (*Glycyrrhiza* spp.) and confirmed to have anti-inflammatory (Sotomatsu et al 1959), anti-allergic (Kuroyanagi & Sato 1966) and interferon-inducing (Shinada et al 1986) activities as well as anti-viral effects (Pompei et al 1979) including those against AIDS virus (Ito et al 1987). Pharmacological and biological activities of glycyrrhetinic acid such as anti-inflammation (Finney & Tarnoky 1960; Capasso et al 1982), anti-tumour promotion (Nishino et al 1986) and inhibition of intercellular junctional communication (Davidson et al 1986), have been reported. Clinically, carbenoxolone sodium (glycyrrhetinic acid 3-0-hemisuccinate sodium salt) has been used to treat gastric ulcer (Doll et al 1962). Derivatives of glycyrrhetinic acid have been made to enhance the therapeutic activity of the parent compound with the object of suppressing the unfavourable side effect of pseudo-aldosteronism (Takahashi et al 1980; Shibata et al 1987). A derivative of glycyrrhetinic acid, deoxoglycyrrhetol (IIa), has been shown to prevent gastric ulcer and allergic action (Takahashi et al 1980). Furthermore, three kinds of hemiphthalate compounds (disodium salt of 18β-olean-12-ene-3β, 30-diol di-*o*-hemiphthalate (IId), 18β-olean-9(11), 12-diene-3β, 30-diol di-*o*-hemiphthalate (IIIa), olean-11, 13(18)-diene-3β, 30-diol di-*o*-hemiphthalate (IVa)) derived from glycyrrhetinic acid (Ia) have been found to inhibit gastric ulcer more strongly (Yano et al unpublished). Previously, we reported that these compounds inhibited lipoxygenase and cyclooxygenase activities in a cell-free system using cloned mastocytoma cells (Inoue et al 1986), and showed anti-nociceptive and anti-type IV allergic effects (Inoue et al 1987).

The arachidonic acid (AA)-induced mouse ear oedema has been applied for screening of lipoxygenase inhibitors in the arachidonate pathway (Young et al 1983, 1984; Carlson et al 1985). Recently, Inoue et al (unpublished data) and others (Opas et al 1985; Chang et al 1986) found that leukotriene (LT)C_4 and prostaglandin (PG)E_2 are important mediators for ear oedema.

We now describe three kinds of dihemiphthalate compounds derived from glycyrrhetinic acid that inhibited AA-induced ear oedema on both topical and oral application.

Correspondence to: Hideo Inoue, Research Laboratory, Minophagen Pharmaceutical Co. 2-5233, Komatsubara, Zama-shi, Kanagawa 228, Japan.

Materials and Methods

Test compounds

Glycyrrhetinic acid and its derivatives were synthesized or prepared according to Shibata et al (1987). Compounds used as reference were purchased as follows: aspirin (Nakarai Chemical Co., Japan), nordihydroguaiaretic acid (NDGA) and dexamethasone (Sigma Chemical Co., USA). AA 861 (2,3,5-timethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) was a generous gift from Takeda Chemical Industry Co., Japan.

Assay for AA-induced mouse ear oedema

Male ddY mice, 6 weeks old (Shizuoka Laboratory Animal Center, Japan), were assigned to eight treatments, and housed together. Animals were acclimatized under standard conditions for one week before use, with free access to food and water. Induction of ear oedema was based on the method of Carlson et al (1985). AA (Sigma Chemical Co., USA) was dissolved in acetone at a concentration of 100 mg mL^{-1}. Each mouse was treated with 20 μL (2 mg/ear) of AA on both surfaces of an ear. Test compounds were dissolved in acetone or ethanol for topical application, and were suspended with 1% polyoxyethylene sorbitan mono-oleate (Tween 80, Tokyo Kasei Chemical Industry, Japan) in saline (0·9%

NaCl) for oral administration. Both treatments were performed 30 min before AA application. Control mice received the vehicle only. Ear oedema was measured 1 h after AA application by a dial thickness gauge (Ozaki Factory, Japan) with the unit of 0·01 mm, and Student's *t*-test was used to determine the statistical significance.

Determination of LTC_4 and PGE_2 in ear oedema

Animals tested were killed 5 min for PGE_2 and 10 min for LTC_4 after AA application, and each ear was removed immediately and stored on dry-ice. It was homogenized with ice-cold 80% ethanol for LTC_4 and 95% ethanol for PGE_2 by a motor driven homogenizer (5000 rev min^{-1}, 30 s). The homogenate was centrifuged at 3000 *g* for 15 min at 4°C. LTC_4 in 80% ethanol extract or a further purified fraction by a Sep-Pak C_{18} cartridge was measured by radioimmunoassay using an LTC_4-radioimmunoassay kit purchased from New England Nuclear, Boston, MS, USA. PGE_2 in 95% ethanol extraction was absorbed by ODS resin and eluted with ethyl acetate. The elution was purified and evaporated, and PGE_2 was measured by a radioimmunoassay kit (New England Nuclear, USA).

Results

Effect of glycyrrhetinic acid derivatives on AA-induced mouse ear oedema

The structures of glycyrrhetinic acid derivatives used are shown in Fig. 1. A single application of AA (2 mg/ear) resulted in a maximal peak of swelling around 1 h after treatment. The effects of test compounds applied topically on AA-induced ear oedema are summarized in Table 1. These compounds were assayed at a dose of 1 mg/ear. Of glycyrrhetinic acid derivatives, three kinds of hemiphthalate compounds having hemiphthalate groups at the 3- and 30-positions of ring A and E in oleanane skeleton, 18β-olean-12-ene-3β, 30-diol di-*o*-hemiphthalate (IId), 18β-olean-9(11)12-diene-3β, 30-diol di-*o*-hemiphthalate (IIIa) and olean-11, 13(18)-diene-3β, 30-diol di-*o*-hemiphthalate (IVa) significantly inhibited ear oedema about 30% ($P < 0{\cdot}01$). However glycyrrhetinic acid (Ia), the parent compound showed no remarkable inhibition in this system. In addition, 3β-*o*-mono-succinate compounds, Ib and IIb, also had little effect. Aspirin gave no detectable suppression of oedema, even at a dose of 3 mg/ear, whereas NDGA inhibited ear oedema

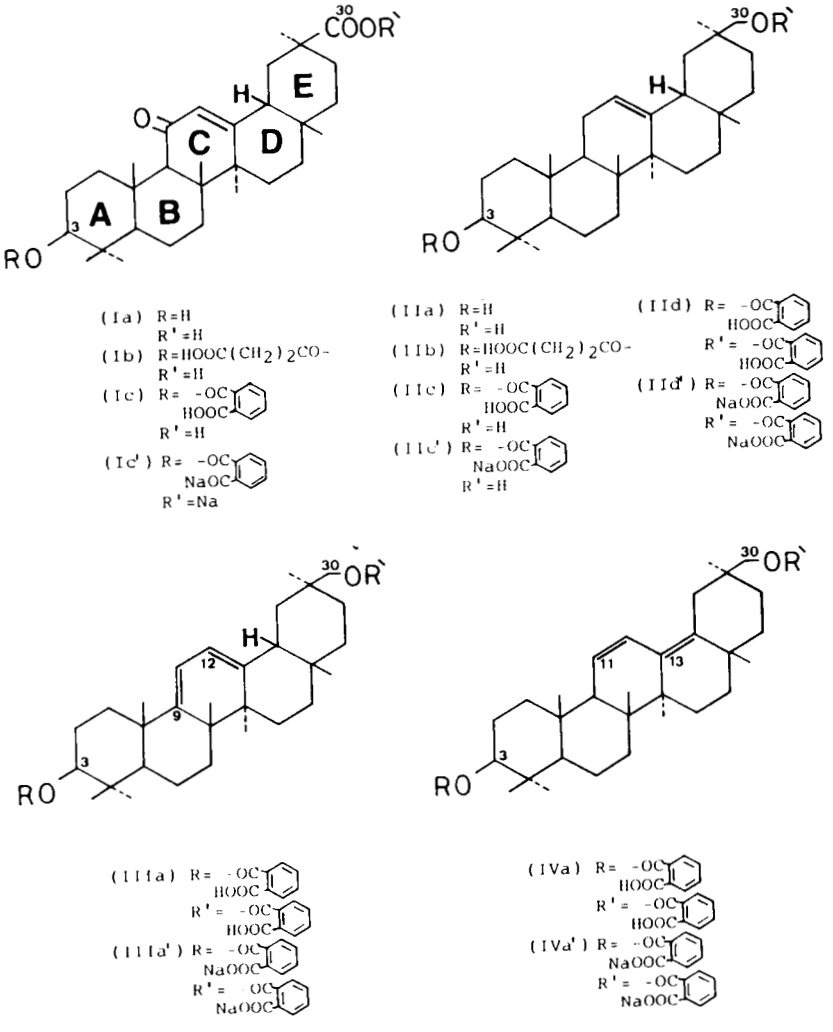

FIG. 1. Chemical structures of glycyrrhetinic acid derivatives.

Table 1. Inhibition of glycyrrhetinic acid derivatives applied topically on arachidonic acid-induced mouse ear oedema.

Compound	Dose (mg/ear)	Increase of ear thickness ($\times 10^{-2}$ mm) Control	Treated	Inhibition (%)
Ia	1	35·7±1·2	31·8±1·6	11
Ib	1	28·8±1·1	31·1±0·9	0
Ic	1	24·4±3·0	24·7±2·4	0
IIb	1	32·6±0·7	31·5±0·8	3
IIc	1	28·8±1·1	28·8±1·1	0
IId	1	28·9±2·0	20·2±1·2**	30
IIIa	1	33·0±2·2	24·9±1·4**	25
IVa	1	27·6±2·5	17·6±2·2**	36
Aspirin	1	28·2±1·0	29·5±0·4	0
NDGA	1	30·3±0·6	17·4±0·6***	43
AA 861	1	29·5±1·6	17·2±2·4***	42

Values are expressed as mean±s.e. of 8 animals.
Significant difference from the control at $^{**}P<0.01$ and $^{***}P<0.001$.

about 40% ($P<0.01$). A selective lipoxygenase inhibitor, AA 861, showed the same inhibitory potency as NDGA. The inhibitory effects of three kinds of dihemiphthalate compounds (IId, IIIa, IVa) and NDGA on the oedema formation were further investigated. AA-induced ear oedema was inhibited dose-dependently, as shown in Fig. 2. The ID50 values were 1·9±0·2 mg/ear for the compound IId, 2·8±0·3 mg/ear for IIIa and 1·7±0·2 mg/ear for IVa, while the ID50 value of NDGA was 2·1±0·5 mg/ear.

The effect of some glycyrrhetinic acid derivatives on AA-induced ear oedema by oral administration was examined (Table 2). In this case, the disodium salts of the dihemiphthalate compounds (IIc', IId', IIIa', IVa') were used because of their ready solubility in water. The compounds IId', IIIa' and IVa' inhibited ear oedema dose-dependently at doses of 25–100 mg kg^{-1} (p.o.). The ID50 values were 90 mg kg^{-1} for IId', 130 mg kg^{-1} for IIIa' and 88 mg kg^{-1} for IVa'. However, glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa), which are the parent compounds of IId', showed no detectable inhibition of oedema at 200 mg kg^{-1} (p.o.). Aspirin also did not show any significant inhibition in this system.

Optimum time for topical application of the compound IId to AA-induced ear oedema

To find the optimum time for topical application of the dihemiphthalate compounds to AA-induced oedema, IId was administered to the ear at an appropriate time before or after AA application (Fig. 3). The compound IId inhibited ear oedema most strongly when administered during the 30 min before AA application, while IId was also effective when applied 15 min after the AA treatment, though its potency was decreased (18% inhibition, $P<0.05$).

The comparative studies on inhibition mechanism of compound IId and dexamethasone

Inhibitory effects of compound IId and dexamethasone administered topically on AA-induced ear oedema are summarized in Table 3. Both compounds were applied 0·5 or

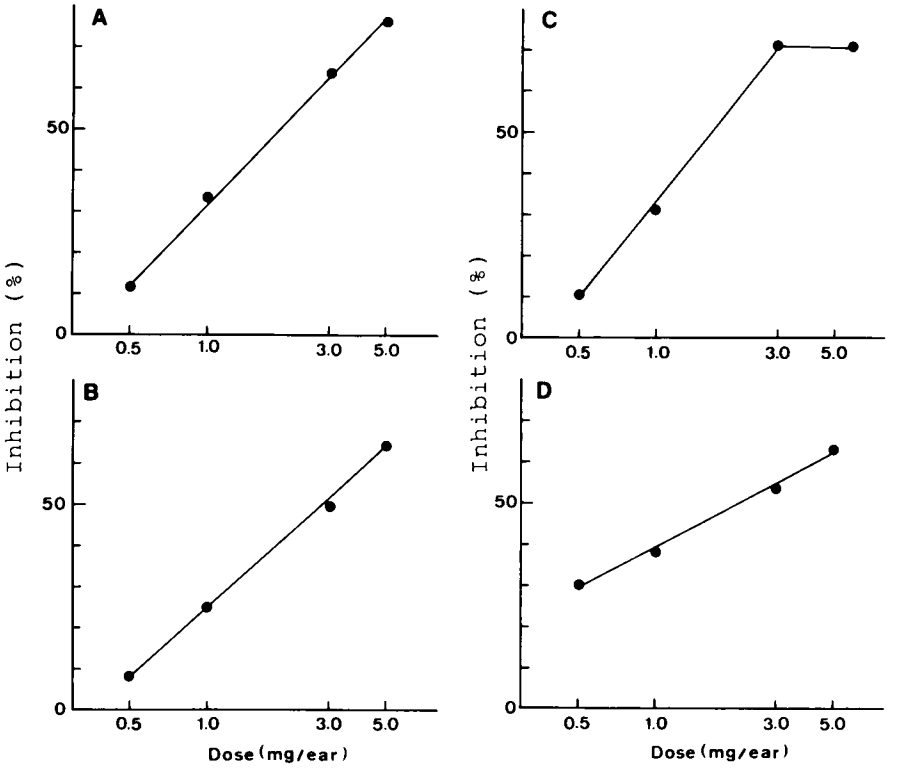

FIG. 2. Dose-dependent inhibition of dihemiphthalate compounds and NDGA on AA-induced ear oedema. Various doses of test compounds were applied topically to the ear 30 min before AA (2 mg/ear) treatment as described in Materials and Methods. Each point represents as percent of control and the mean of three separate experiments (n = 8 of each point). Key: A, compound IId; B, compound IIIa; C, compound IVa; D, NDGA.

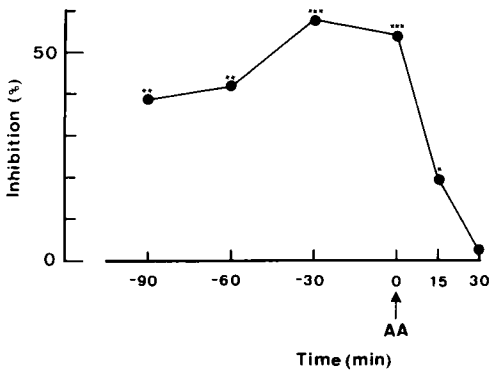

FIG. 3. Time effect of topical application of compound IId (18β-olean-12-ene-3β,30-diol di-*o*-hemiphthalate) on AA-induced ear oedema. The compound was administered various times before and after AA application at 3 mg/ear. Each point represents as percent of control and the mean of 8 animals.

3·0 h before AA application. The inhibitory effect of dexamethasone was weak (6% inhibition) when administered 30 min before AA application, while it showed 50% inhibition ($P<0.001$) against oedema when applied 3 h before AA treatment. It is noticeable that oedema-inhibition by dexamethasone was suppressed when cycloheximide was applied at a dose of 0·1 mg/ear 30 min after treatment with the steroid. The inhibitory potency of compound IId on ear oedema was decreased by 13% when it was applied 3 h before AA treatment.

Inhibition of the dihemiphthalate derivatives on PGE_2 and LTC_4 production in AA-induced ear oedema

The effects of the dihemiphthalate derivatives, IId, IIIa and IVa, when administered topically were examined on PGE_2 and LTC_4 production in AA-induced ear oedema. As shown

Table 2. Inhibition of glycyrrhetinic acid derivatives administered orally on arachidonic acid-induced mouse ear oedema.

Compound	Dose (mg kg^{-1})	Increase of ear thickness (x 10^{-2} mm) Control	Treated	Inhibition (%)
Ia	200	29·5±1·6	29·1±1·9	2
IIa	200	29·5±1·6	30·7±1·3	0
IIc′	200	29·5±1·6	29·2±0·7	1
IId′	12·5	28·3±1·3	31·3±0·8	0
	25	30·1±1·9	24·0±1·6*	20
	50	28·3±1·8	16·6±1·4***	41
	100	29·0±2·0	13·8±2·1***	52
IIIa′	12·5	28·3±1·3	26·0±1·8	6
	25	30·1±1·9	24·2±1·9*	20
	50	28·3±1·8	20·9±2·6*	26
	100	28·4±1·7	15·7±1·3***	45
IVa′	12·5	28·3±1·3	31·3±0·3	0
	25	30·1±0·9	24·1±2·0*	20
	50	28·3±1·8	20·1±2·6*	29
	100	29·0±2·0	13·4±1·8***	54
Aspirin	200	28·3±1·8	23·1±2·0	18

Values are expressed as mean±s.e. of 8 animals.
Significant difference from the control at $*P<0.05$ and $***P<0.001$.

in Fig. 4, compounds IId, IIIa and IVa significantly reduced PGE_2 and LTC_4 production at a dose of 3 mg/ear. These compounds inhibited LTC_4 more effectively than they did PGE_2. The inhibition of LTC_4 production was over 70% ($P<0.001$). However, the sodium salt of dihemiphthalate compounds, IIIa′ and IVa′, on oral administration of 100 mg kg^{-1}, showed no significant inhibition of PGE_2 or LTC_4, while compound IId′ inhibited PGE_2 production (data not shown). On the topical application of aspirin at 3 mg/ear, PGE_2 production was completely inhibited whereas LTC_4 production was promoted. NDGA at 1 mg/ear suppressed

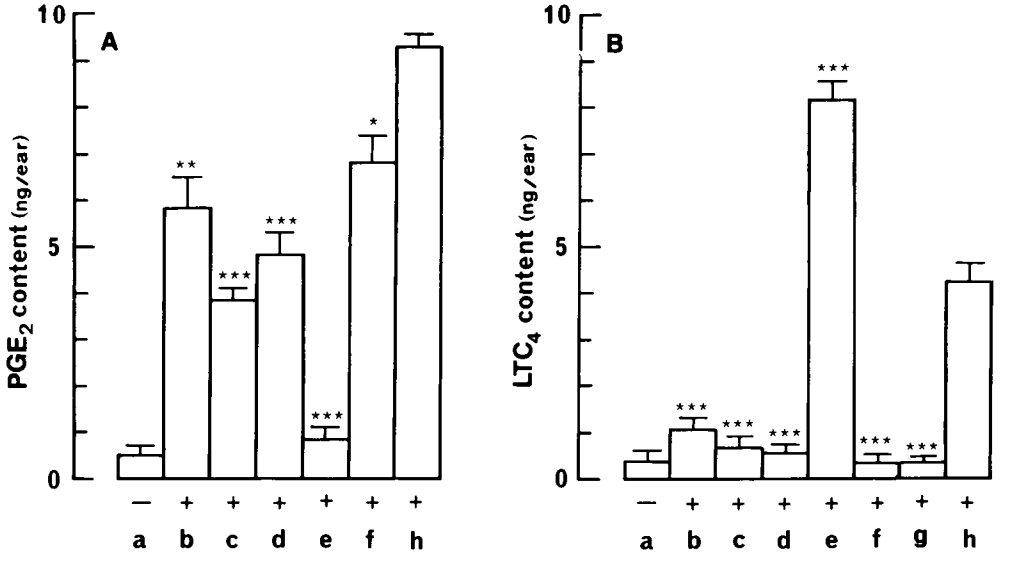

FIG. 4. Effects of diphthalate compounds on PGE_2 and LTC_4 production in AA-induced ear oedema. Test compounds were topically applied 30 min before AA application. PGE_2 (A) and LTC_4 (B) were extracted from the ear 5 and 10 min after AA application, respectively. Each value represents the mean±s.e. of 6 animals. a; untreated group, b; compound IId (3 mg/ear), c; compound IIIc (3 mg/ear), d; compound IVa (3 mg/ear), e; aspirin (3 mg/ear), f; NDGA (1 mg/ear), g; AA 861 (1 mg/ear), h; control group (AA, 2 mg/ear), +; AA-treated, −; AA-untreated. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with the control group by the paired Student's *t*-test.

PGE_2 by 26% and LTC_4 by 94%, while AA 861 also showed a strong inhibition of LTC_4 production ($P < 0.001$).

Discussion

The inhibitory effects of glycyrrhetinic acid derivatives have been examined on AA-induced mouse ear oedema. Among them, the dihemiphthalate derivatives (IId, IId′, IIIa, IIIa′, IVa and IVa′) having hemiphthalate groups at the 3- and 30-positions of ring A and E in the oleanane skeleton were found to inhibit ear oedema on both topical and oral administration (Table 1, 2). Topical inhibition (ID50) of these compounds was at approximately the same potency as NDGA (Fig. 2). In contrast, glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa), the parent compounds of the derivatives, showed no detectable inhibition. Glycyrrhetinic acid hemiphthalate (Ic or Ic′) and deoxoglycyrrhetol 3-*o*-monohemiphthalate (IIc or IIc′) were not potent. Glycyrrhizin also had no effect against AA-induced ear oedema (data not shown). These results suggest that dihemiphthalate substitution on the oleanane skeleton is required for potent inhibitory effect on ear oedema.

The products of the lipoxygenase pathway are known as being primarily related to AA-induced ear oedema (Young et al 1984; Chang et al 1986). It was reported that representative non-steroidal anti-inflammatory drugs, such as aspirin, ibuprofen and naproxen, are ineffective, whereas NDGA, AA 861 and EN 105, known inhibitors of lipoxygenase or of both lipoxygenase and cyclooxygenase, strongly inhibit ear oedema (Young et al 1983; Carlson et al 1985; Inoue et al unpublished data). The sodium salt of dihemiphthalate compounds (IId′, IIIa′, IVa′) of glycyrrhetinic acid derivatives have been found to inhibit 5- and 12-lipoxygenase and cyclooxygenase activities in a cell-free system of mastocytoma cells (Inoue et al 1986). In addition, these compounds were more effective in inhibiting lipoxygenase than cyclooxygenase. The ID50 of compound IId′ for 5-lipoxygenase was 5.8×10^{-6} M which is approximately the same potency as the ID50 values of 4.0×10^{-6} M for esculetin (Neichi et al 1983) and of 3.7×10^{-6} M for caffeic acid (Koshihara et al 1984). However, esculetin and caffeic acid had little effect on AA-induced ear oedema. Production of LTC_4 and PGE_2 in ear oedema was reported to reach a maximum up to 15 min after AA application (Opas et al 1985; Chang et al 1986), and we have confirmed that AA-induced ear oedema would be primarily mediated by LTC_4 and promoted by PGE_2 (Inoue et al unpublished data). Actually, a selective inhibitor of 5-lipoxygenase, AA 861 (Ashida et al 1983), showed a strong inhibition of ear oedema and LTC_4 production. NDGA, known as dual inhibitor of lipoxygenase and cyclooxygenase (Morris et al 1980), remarkably suppressed the oedema, while its inhibitory effect was the same as AA 861. Aspirin, a cyclooxygenase inhibitor (Vane 1971), was not effective against ear oedema, though PGE_2 production was completely inhibited. The dihemiphthalate derivatives on topical application effected not only the inhibition of ear oedema but also the reduction of PGE_2 and LTC_4 production at the site. However, we have found no sufficient reason why the sodium salts of dihemiphthalate compounds (IId′, IIIa′ and IVa′), which inhibited ear oedema significantly by oral administration, were weak suppressors of PGE_2 and LTC_4 production at the site. Their inhibitory mechanism may be different from that of topical application.

Table 3. Inhibitory effects of deoxoglycyrrhetol diphthalate (IId) and dexamethasone applied topically on arachidonic acid-induced mouse ear oedema.

Compound	Dose (mg/ear)	Time of treatment (h)	Ear thickness after AA application ($\times 10^{-2}$ mm) 0	1 (h)	Inhibition (%)
None			24·9 ± 0·2	57·6 ± 1·1 (8)	
IId	1	−0·5	24·9 ± 0·2	49·8 ± 0·7 (8)**	24
	1	−3·0	25·0 ± 0·2	53·4 ± 3·3 (8)	13
Dexamethasone	0·1	−0·5	25·0 ± 0·2	55·9 ± 0·9 (9)	6
	0·1	−3·0	25·0 ± 0·2	41·3 ± 1·9 (9)***	50
Dexamethasone + Cycloheximide	0·1	−3·0			
	0·1	−2·5	24·9 ± 0·3	57·1 ± 0·8 (9)	1

Values are expressed as the mean ± s.e. with the number of animals in parentheses. **$P < 0.01$, ***$P < 0.001$ between none and tested.

The optimum time of treatment with compound IId for topical application was observed to be different from that of dexamethasone. Dexamethasone significantly inhibited ear oedema when administered 3 h before AA treatment, but had little effect 30 min before that, whereas IId was effective (Table 3). This is consistent with the result of Carlson et al (1985) that the inhibitory activity of the steroidal drugs against AA-induced oedema has been demonstrated when they were administered 2·5–3·0 h before AA treatment. Tsurufuji et al (1979) found that a time lag was required to obtain anti-inflammatory effect with the steroid in 5-hydroxytryptamine-induced paw oedema to mouse. It is clear that the time lag is needed for the induction of anti-inflammatory proteins, since the inhibition of dexamethasone was prevented when cyclohexamide was applied 30 min after the steroid treatment. On the other hand, compound IId showed no remarkable inhibition of oedema 3 h-pretreatment. Therefore, the hemiphthalate compounds of glycyrrhetinic acid derivatives, IId, IIIa and IVa, have a non-steroidal type anti-inflammatory action which could directly inhibit ear oedema induced by LTC_4 and PGE_2.

On the other hand, the dihemiphthalate compounds, IId′, IIIa′ and IVa′, inhibited both lipoxygenase and cyclooxygenase in a cell-free system induced from cloned mastocytoma cells (Inoue et al 1986), and also inhibited PGE_2 production in macrophage (personal communication from Dr K. Ohuchi). It has been found also that these compounds suppressed vascular permeability to result in anti-inflammatory effect in mice (Inoue et al 1987). Therefore, these compounds could be developed as the drugs having anti-inflammatory activity.

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