A Useful Method for Differential Evaluation of Anti-Inflammatory Effects Due to Cyclooxygenase and 5-Lipoxygenase Inhibitions in Mice

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Received October 25, 1993 Accepted April 22, 1994

ABSTRACT—This study was performed to establish a useful method for monitoring the effects of inhibitors of 5-lipoxygenase (5-LO) and/or cyclooxygenase (CO) and for differential evaluation of these inhibitors. After oral dosing, CO inhibitors such as indomethacin (20–40 mg/kg) and ketoprofen (40–80 mg/kg), zileuton (5-LO inhibitor, 20–80 mg/kg) and MK886 (5-LO-activating-protein inhibitor, 640 mg/kg) potently suppressed arachidonic acid (AA, 0.25 mg)-induced ear edema in mice. Methysergide (serotonin antagonist, 20 mg/kg) showed a slight anti-edematous effect, while mepyramine (160 mg/kg) and bromelain (320 mg/kg) had no effect. The anti-edematous effects of indomethacin and ketoprofen were reduced by concomitant topical application of prostaglandin E2 (PGE2, 1 µg/ear), but not by concomitant intradermal application of leukotriene C4 (LTC4, 0.1 µg/ear). On the contrary, the anti-edematous effects of zileuton and MK886 were reduced by LTC4, but not by PGE2. Dual (5-LO and CO) inhibitors such as phenidone (80–160 mg/kg) and BW755C (40–80 mg/kg), which inhibited the biosynthesis of LTB4 13–15 times more potently than that of PGE2 in rat peritoneal exudate cells, also showed anti-edematous effects that were reduced by LTC4, but not by PGE2. These results suggest that the AA (0.25 mg)-induced ear edema in mice is mainly mediated by LTs and PGs and is suitable for evaluating inhibitors of 5-LO and/or CO, and that an application of LTC4 or PGE2 with AA is a useful method for differential evaluation of these inhibitors.

Keywords: Arachidonic acid-induced ear edema, Prostaglandin E2, Leukotriene C4, Indomethacin, Zileuton

It is well established that prostaglandins (PGs), cyclooxygenase (CO) products of arachidonic acid (AA), are involved in inflammatory reactions as important inflammatory mediators, and inhibitors of PG biosynthesis have been developed as non-steroidal anti-inflammatory drugs. Recently, it has been shown that leukotrienes (LTs), 5-lipoxygenase (5-LO) products of AA, are also involved in inflammatory reactions as proinflammatory mediators. LTC4 and LTD4 cause edema together with increased microvascular permeability (1, 2), and LTB4 causes leukocyte chemotaxis (3, 4). These findings have prompted interest in developing dual inhibitors of 5-LO and CO as anti-inflammatory drugs.

Several experimental models of inflammation such as carrageenan-induced paw edema and AA-induced ear edema have been widely used for the discovery and evaluation of anti-inflammatory drugs. The carrageenan-induced paw edema in rats is known to be sensitive to CO inhibitors, but not to 5-LO inhibitors (5, 6). On the other hand, the AA-induced ear edema in mice is known to be suitable for evaluating 5-LO inhibitors (7–10), but the effects of CO inhibitors on ear edema are not consistent. It has been demonstrated that indomethacin, piroxicam and naproxen suppressed AA-induced ear edema (7, 9, 10), while aspirin, ibuprofen and naproxen failed to suppress it (8, 10). Griswold et al. (10) showed that the suppressive effect of indomethacin on ear edema may be mediated through a mechanism other than CO inhibition. Thus, the involvement of PGs in the AA-induced ear edema and the mechanism of the anti-inflammatory effect of CO inhibitors in ear edema are still unclear, and there is no simple animal model of inflammation for evaluating dual inhibitors of 5-LO and CO.

The aim of this study is to establish a useful method for evaluating inhibitors of 5-LO and/or CO, and for differentially evaluating these inhibitors. We examined
the effects of CO and/or 5-LO inhibitors on the ear edema induced by low and high doses of AA and the effect of a local application of PGE₂ or LTC₄ on their anti-inflammatory effects to clarify the involvement of PGs and LTs in the ear edema and to differentially evaluate these inhibitors.

MATERIALS AND METHODS

Animals

Male ICR mice and Wistar rats were obtained from Nihon Clea, Inc. (Tokyo) and Nihon SLC, Inc. (Hamamatsu), respectively. They were housed in temperature-controlled rooms (23 ± 2°C) with a 12-hr light-dark cycle, and they were allowed free access to food and water. The experiments were carried out at a room temperature of 23 ± 2°C. The animals were randomly assigned to the treatment groups.

Drugs

The following drugs were used: zileuton, MK886 and BW755C were synthesized at Dainippon Pharmaceutical Co., Ltd. The other drugs were obtained from the following commercial sources: indomethacin, ketoprofen, phenidone, mepyramine maleate, dexamethasone and AA (Sigma, St. Louis, MO, USA); bromelain (Nacalai Tesque, Kyoto); PGE₂ and LTC₄ (Funakoshi, Tokyo); PGE₂ RIA kit (DuPont/NEN, Wilmington, DE, USA); and LTB₄ RIA kit (Amersham, Tokyo). Drugs were suspended in a 0.5% tragacanth solution for oral administration. In an in vitro test, drugs were dissolved in ethanol and diluted with the medium used. The doses of drugs described in this paper refer to the forms presented above.

Biosynthesis of PGE₂ and LTB₄ in isolated rat peritoneal cells

Rat peritoneal cells were obtained from the peritoneal cavity of male Wistar rats (300–350 g) that had been injected intraperitoneally with 50 ml/kg of 5% sterilized soluble starch solution containing 5% bactopeptone 4 days before (11). The peritoneal exudate was centrifuged at 1,000 x g for 5 min. Contaminating erythrocytes were lysed by exposing the cell pellet to 0.2% NaCl solution for 30 sec. The cells were washed with saline solution 3 times and then resuspended in modified Eagle’s medium containing 10% calf serum to be a density of 2 x 10⁶ cells/ml. The cell suspension (0.9 ml) was incubated at 37°C for 1 hr after addition of a drug (0.1 ml) that was dissolved in 3% ethanol. After cooling the tubes, 0.5 N HCl (1 ml) and ethyl acetate (1 ml) were added to the cell suspension (1 ml). The cell suspension was shaken for 10 min and centrifuged at 1,700 x g for 10 min. A 30-μl aliquot of the ethyl acetate layer was removed and evaporated under reduced pressure. The residue was stocked at −80°C for subsequent analysis of PGE₂ and LTB₄ levels by radioimmunoassay.

AA-induced ear edema

The method described by Young et al. (7) was used with minor modifications. Briefly, 20 μl of the acetone solution of AA was applied to both the inner and outer surfaces of the right ears of mice (20–23 g). One hour later, the mice were killed by inhalation of CO₂ gas. A circular tissue sample (5.5 mm in diameter) from each ear was removed with a metallic punch and weighed. In some experiments, mice were killed 0.5, 1, 1.5, 2, 3, 4, 5 or 6 hr after AA application. In some experiments to elucidate the involvement of PGs or LTs in the anti-inflammatory effects of test drugs, PGE₂ (1 μg/20 μl/ear) was topically applied to the right ears together with AA solution or LTC₄ saline solution (0.1 μg/10 μl/ear) was intradermally administered to the right ears immediately after AA application. The left ears of mice served as the control. Drugs were orally administered to mice 1 hr before AA application. The ear swelling rate was calculated according to the following equation. The anti-edematous effect of drugs was expressed as percent inhibition of the ear swelling compared with the vehicle control.

\[ \text{Ear swelling (}% = \frac{(a - b)}{b} \times 100 \]

a: weight of a right ear treated with AA

b: weight of a left ear untreated

Statistical analyses

Results are presented as means ± S.E.M. and IC₅₀ values. Statistical significance between groups was analyzed by Student’s t-test and Duncan’s multiple-range test. The IC₅₀-value, the concentration that is necessary to obtain 50% inhibition of the responses, was determined from the best fit regression line of a dose-response curve.

RESULTS

Effect of drugs on LTB₄ and PGE₂ biosynthesis in rat peritoneal cells

Zileuton and MK886 potently reduced the LTB₄ level (mean LTB₄ level ± S.E.M. in the matched control groups: 936 ± 20 pg/ml and 1513 ± 163 pg/ml, respectively) in rat peritoneal cells with IC₅₀-values of 0.20 μM and 10.8 μM, respectively, but did not affect the PGE₂ level even at a dose of 50 μM (Table 1). On the other hand, indomethacin and ketoprofen reduced the PGE₂ level (mean PGE₂ level ± S.E.M. in the matched control groups: 3248 ± 170 pg/ml and 2597 ± 114 pg/ml, respectively) in the peritoneal cells with IC₅₀-values of 0.51 μM and 0.50 μM, respectively, but did not reduce the LTB₄
level up to 50 μM. BW755C and phenidone reduced both the levels of LTB₄ and PGE₂, and their effects on LTB₄ level were 13–15 times more potent than those on PGE₂ level (Table 1).

### Table 1. Effects of inhibitors of 5-LO and/or CO on biosynthesis of LTB₄ and PGE₂ in isolated rat peritoneal cells

| Drugs                | LTB₄ biosynthesis IC₅₀ (μM) | PGE₂ biosynthesis IC₅₀ (μM) |
|----------------------|-----------------------------|-----------------------------|
| 5-LO inhibitor       |                             |                             |
| Zileuton             | 0.20                        | >50                         |
| MK-886               | 10.8                        | >50                         |
| CO inhibitor         |                             |                             |
| Indomethacin         | >50                         | 0.51                        |
| Ketoprofen           | >50                         | 0.50                        |
| Dual (5-LO and CO) inhibitor | 2.83                        | 36.4                        |
| BW755C               | 2.36                        | 37.2                        |
| Phenidone            |                             |                             |

**Dose-response and time-course of AA-induced ear edema**

A topical application of AA (0.025–2.0 mg/ear) to mouse right ear caused edema dose-dependently; ear edema was observed at doses over 0.05 mg/ear, and it was maximal at 1 mg/ear (Fig. 1). The values of the mean weight and standard error of mouse AA-treated right and untreated left ears were 8.7±0.59 mg and 6.5±0.13 mg, respectively, for 0.05 mg/ear of AA; and they were 16.2±0.28 mg and 6.3±0.14 mg, respectively, for 1 mg/ear of AA.

Fig. 1. Dose-response relationships for AA-induced ear edema in mice. Various doses (0.025–2.0 mg/ear) of AA dissolved in acetone were topically applied to the right ears of mice. One hour later, a circular portion of the ear was punched out and weighed. Each value represents the mean±S.E.M. of 8 mice. Calculation of ear edema (%): see Methods.

AA (0.25 mg/ear)-induced ear edema almost reached the maximum at 1 hr after AA application (Fig. 2), and the effect lasted for 2 hr. LTC₄ (0.1 μg), when intradermally administered immediately after AA (0.25 mg) application, did not enhance the ear edema (ear swelling: 143±5.0% for AA alone and 148±7.0% for AA plus LTC₄) and neither did PGE₂ (1 μg) concomitantly applied with AA (ear swelling: 128±5.0% for AA alone and 129±7.0% for AA plus PGE₂). An intradermal administration of LTC₄ (0.1 μg) caused ear edema (ear swelling: 66%) more potently than that of saline (ear swelling: 23%), while a topical application of PGE₂ (1 μg) did not cause it (ear swelling: 5%).

### Effects of zileuton and indomethacin on AA (0.25 and 2 mg)-induced ear edema

The effects of zileuton and indomethacin on ear edema induced by 0.25 mg and 2 mg of AA were compared. As shown in Fig. 3, zileuton and indomethacin at 20 mg/kg, p.o. suppressed ear edema induced by 0.25 mg of AA, but not by 2 mg of AA. Zileuton and indomethacin at 40 mg/kg, p.o. suppressed AA (0.25 mg)-induced ear edema more potently than AA (2 mg)-induced ear edema. The 0.25 mg/ear dose of AA was chosen for the following experiments.

**Effect of various drugs on AA (0.25 mg)-induced ear edema**

As shown in Fig. 4, zileuton (40 and 80 mg/kg, p.o.), a 5-LO inhibitor, and MK886 (640 mg/kg, p.o.), an inhibitor of 5-lipoxygenase activating protein (FLAP), dose-
Fig. 3. Effect of zileuton (A) and indomethacin (B) on mouse ear edema induced by 0.25 mg and 2 mg of AA. Drugs were orally administered to mice at 1 hr before AA application. The effect of drugs was expressed as percent inhibition of the ear swelling compared with the vehicle control. Each value represents the mean ± S.E.M. of 7 mice. A) Open and closed columns: 20 and 40 mg/kg of zileuton, respectively. B) Open and closed columns: 20 and 40 mg/kg of indomethacin, respectively. *P<0.05 and **P<0.01, significantly different from the vehicle control.

Fig. 4. Effect of drugs on AA-induced ear edema in mice. Drugs were orally administered to mice 1 hr before AA (0.25 mg) application. The effect of drugs was expressed as percent inhibition of the ear swelling compared with the vehicle control. Each value represents the mean ± S.E.M. of 7–8 mice. *P<0.05 and **P<0.01, significantly different from the vehicle control.
dependently suppressed the ear edema. MK886 was less potent than zileuton in suppressing the ear edema. Indomethacin (20 and 40 mg/kg, p.o.) and ketoprofen (40 and 80 mg/kg, p.o.), CO inhibitors, BW755C (40 and 80 mg/kg, p.o.) and phenidone (80 and 160 mg/kg, p.o.), 5-LO and CO dual inhibitors, also suppressed the ear edema. Methysergide (20 mg/kg, p.o.), a serotonin antagonist, suppressed the ear edema by 35%, while mepyramine (160 mg/kg, p.o.), a histamine antagonist, and bromelain (320 mg/kg, p.o.), a bradykinin depletor, did not.

Fig. 5. Influence of PGE2 on anti-edematous effects of 5-LO and/or CO inhibitors in AA-induced ear edema in mice. PGE2 dissolved in acetone (1 µg/20 µl/ear) was topically applied together with AA to the right ears of mice. Drugs were orally administered to rats 1 hr before AA application. Each value represents the mean±S.E.M. of 8 mice. **P<0.01. Open column: AA-induced edema, Closed column: AA plus PGE2-induced edema.

Influence of PGE2 and LTC4 on the anti-edematous effects of 5-LO and/or CO inhibitors in AA-induced ear edema

As shown in Fig. 5, a topical application of PGE2 (1 µg/ear) to mouse ear markedly reduced the suppressive effect of indomethacin (40 mg/kg, p.o.) and ketoprofen (80 mg/kg, p.o.) on AA-induced ear edema, but did not reduce those of zileuton (80 mg/kg, p.o.), MK886 (640 mg/kg, p.o.), BW755C (80 mg/kg, p.o.) and phenidone (160 mg/kg, p.o.). On the other hand, an intradermal application of LTC4 (0.1 µg/ear) reduced the anti-edema-
tous effect of zileuton, phenidone and BW755C in the model, and it completely abolished that of MK886. However, an intradermal application of LTC4 did not reduce those of indomethacin and ketoprofen (Fig. 6).

DISCUSSION

The present study showed that a topical application of AA to mouse ear caused ear edema dose-dependently, and that the 0.25 mg dose of AA exerted the maximal edema of the ear. At this dose of AA, both the PG and LT mechanisms must be fully operating in inducing edema, since PGE2 (1 µg/ear) and LTC4 (0.1 µg/ear), which enhance mutually the AA-induced ear edema in mice (8), did not enhance the ear edema. The AA (0.25 mg) dose used for evaluating the 5-LO and/or CO inhibitors in the present study was 8 times less than the dose (2 mg/ear) used in the previous reports (8–10). In the present study, zileuton and indomethacin at 20 mg/kg suppressed ear edema induced by 0.25 mg of AA, but not that by 2 mg of AA. Accordingly, the anti-edematous effects of inhibitors of 5-LO and CO can be detected more sensitively under our conditions.

Before the effects of CO and/or 5-LO inhibitors on the AA-induced ear edema in mice were examined, we confirmed the effects of these agents, especially dual inhibitors of CO and 5-LO, on CO and 5-LO activities of rat peritoneal exudate cells, as it has been demonstrated that there was little difference in the inhibition of leukotriene production by drugs such as MK886, zileuton and BW755C between mouse and rat peritoneal leukocytes (12). Zileuton (13), a 5-LO inhibitor, and MK886 (14, 15), a 5-LO activating protein inhibitor, markedly inhibited LTB4 biosynthesis, but did not inhibit PGE2 biosynthesis in rat peritoneal cells up to 50 µM. As well known, indomethacin and ketoprofen, CO inhibitors, markedly inhibited PGE2 biosynthesis, but did not inhibit LTB4 biosynthesis up to 50 µM. Phenidone (16) and BW755C (17), dual inhibitors of 5-LO and CO, were found to inhibit LTB4 biosynthesis 13–15 times more potently than PGE2 biosynthesis.

Orally administered zileuton, MK886, phenidone and BW755C potently suppressed the AA-induced ear edema in mice. These results, being consistent with those of locally applied 5-LO inhibitors in AA-induced ear edema in mice (7, 9), support the findings that LTs are involved in AA-induced ear edema in mice. In addition, indomethacin and ketoprofen were found to markedly suppress the ear edema, although the higher doses of these drugs were necessary for suppressing the ear edema as compared with the doses at which these drugs exerted anti-inflammatory effects in other models (18). On the other hand, it has been demonstrated that CO inhibitors such as ibuprofen, aspirin and naproxen did not suppress the AA-induced ear edema in mice (8, 10). In the present study, indomethacin at 40 mg/kg suppressed ear edema induced by 0.25 mg of AA more potently than that induced by 2 mg of AA. Young et al. (7) showed that naproxen suppressed ear edema induced by 0.5 mg of AA, but failed to suppress ear edema induced by 2 mg of AA. Therefore, the inconsistent effects of the CO inhibitors between their experiments and ours may be explained by the difference of AA dose used as described above. The high doses of mepyramine (160 mg/kg), a histamine antagonist, and bromelain (320 mg/kg), a bradykinin depletor, did not suppress the ear edema, while methysergide (a serotonin antagonist) at 20 mg/kg (19), suppressed it slightly. These results suggest that PGs as well as LTs are mainly involved in the AA-induced ear edema in mice, although serotonin may also partly contribute to it.

Griswold et al. (10) reported that indomethacin suppressed the AA-induced ear edema in mice and that its effect might be mediated through a mechanism other than CO inhibition. Thus, we examined the mechanism of the anti-inflammatory effect of indomethacin and zileuton in the AA-induced ear edema in mice. If indomethacin and zileuton suppress the ear edema through reducing the levels of endogenous PGs and LTs, respectively, exogenous supplementary PGs and LTs could reduce the anti-edematous effect of these drugs. In the present experiments, the suppressive effects of indomethacin and ketoprofen on the AA-induced ear edema were found to be reduced by a topical application of PGE2 (1 µg/ear), which did not cause ear edema by itself, but not to be reduced by an intradermal administration of LTC4 (0.1 µg/ear), which caused ear edema by itself. A topical application of LTC4 (1 µg/ear) did not cause ear edema and did not reduce the suppressive effects of zileuton on the AA-induced ear edema, suggesting that LTC4 enough to cause ear edema was not absorbed from the skin. The results are in line with the findings that the suppressive effect of topically applied indomethacin on AA-induced ear edema in mice is abolished by concomitant application of PGE2 (9), and they suggest that their suppressive effects are mediated through the well-known CO inhibition. As it has been suggested that PGE2 causes vasodilatation, which is responsible for the potentiation of plasma exudation into tissues produced by other inflammatory mediators (20), the reduction of the anti-edematous effects of indomethacin and ketoprofen by PGE2, which did not cause ear edema by itself, may probably be due to the synergistic effect of PGE2 with LTs and serotonin. On the other hand, the suppressive effects of zileuton and MK886 on the AA-induced ear edema were reduced by LTC4, but not by PGE2. These results suggest that the suppressive effects of these drugs on the ear edema are mediated through the inhibi-
tion of 5-LO. Reduction of the anti-edematous effects of zileuton and MK886 by LTC₄ may be probably due to the increased vascular permeability caused by LTC₄.

We demonstrated that inhibitors of 5-LO and/or CO are differentially evaluated by a local application of LTC₄ or PGE₂ with AA. Using this method, we examined the anti-inflammatory effects of dual inhibitors and their mechanism. Phenidone and BW755C suppressed the AA induced ear edema in mice, and their suppressive effects were reduced by LTC₄, but not by PGE₂. These results suggest that the anti-edematous effects of phenidone and BW755C are mediated through the inhibition of LT biosynthesis but not through the inhibition of PG biosynthesis.

In conclusion, our results suggest that AA (0.25 mg)-induced ear edema in mice is mediated mainly by LTs and PGs, and this is a suitable model for evaluating inhibitors of 5-LO and/or CO, and that a local application of LTC₄ or PGE₂ with AA makes the differential evaluation of inhibitors of 5-LO and/or CO possible.

Aknowledgments
We wish to thank Dr. J. Matsumoto and Dr. T. Karasawa for their helpful advice, and Dr. Y. Nishikawa for drug synthesis.

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