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# INHIBITION OF PROSTAGLANDIN PRODUCTION IN THE INFLAMMATORY TISSUE BY LOXOPROFEN-Na, AN ANTI-INFLAMMATORY PRODRUG

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**Abstract**—The effect of loxoprofen-Na, a novel non-steroidal anti-inflammatory drug with a prodrug property, on prostaglandin (PG) levels in the inflammatory tissue was investigated with a carrageenin-induced pleurisy model in rats. The intrapleural injection of carrageenin caused a marked increase in the levels of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ in the pleural exudate up to 3 hr after the injection. When [$^{14}$C]$PGE_2$ was injected into the cavity 2 hr after the carrageenin injection, the PG rapidly disappeared from the cavity ($T_{1/2}$ = 5 min). Thus, the PG level determined in the inflammatory exudate represents PG produced in the inflammatory tissue. Loxoprofen-Na, administered orally 2 hr after the carrageenin injection, dose-dependently inhibited the increase in the levels of PGs in the exudate 1 hr after administration ($ID_{50}$ = 0.07 mg/kg for $PGE_2$ and 0.10 mg/kg for 6-keto-$PGF_{1\alpha}$). Indomethacin also inhibited PG production, but was less effective ($ID_{50}$ = 0.24 mg/kg for $PGE_2$ and 0.47 mg/kg for 6-keto-$PGF_{1\alpha}$). Similar results were obtained 3 hr after the administration of these drugs ($ID_{50}$ of $PGE_2$ production = 0.14 mg/kg for loxoprofen-Na and 0.28 mg/kg for indomethacin). The time–course analysis of the effect of loxoprofen-Na showed that this drug had more immediate and stronger inhibitory activity than indomethacin. The relative potencies of suppression of protein leakage and leukocyte infiltration correlated well with the inhibition of PG production, but higher doses were needed for an obvious anti-inflammatory effect. The active metabolite (SRS *trans*-OH) of loxoprofen-Na determined in the inflammatory exudate 1 hr after oral administration of 0.2 and 2 mg/kg of loxoprofen-Na was 0.05 and 0.25 μg/mL, respectively. The concentration was sufficient to suppress PG production in the exudate, because the $IC_{50}$ of the SRS *trans*-OH for PG production *in vitro* with leukocytes was 0.02 μg/mL (0.08 μM). The potency of the SRS *trans*-OH metabolite to inhibit $PGE_2$ production in leukocytes was about 20 times stronger than that of the parent compound and 3 times stronger than that of indomethacin.

Sodium 2-[(4-oxocyclopentylmethyl)phenyl]propionate (loxoprofen-Na) is a novel anti-inflammatory agent, which has marked analgesic and antipyretic activities [1]. These pharmacological effects are observed rapidly after oral administration at relatively low doses.

We previously showed that loxoprofen-Na (CS-600), as well as ten typical non-steroidal anti-inflammatory agents, reduced urinary prostaglandin $E_2$ and $F_{2\alpha}$ ($PGE_2$ and $PGF_{2\alpha}$) in rats after oral administration and that their potencies were in good correlation with their anti-inflammatory activities [2, 3]. Loxoprofen-Na itself has only a weak inhibitory effect on prostaglandin synthetase in bovine seminal vesicle microsomes ($IC_{50}$ = 760 μM), while one of the reduced forms of the cytlopentene moiety of loxoprofen-Na, (2*s*)-2-4-[*trans*-(1*R*,1*S*)-2-hydroxycyclopentylmethyl]phenyl propionic acid (SRS *trans*-OH), potently inhibits this enzyme ($IC_{50}$ = 9 μM [3]. This reduced compound is the main metabolite in rats, mice, dogs and monkeys [4]. These results indicate that loxoprofen-Na exerts its pharmacological effects after conversion to the active metabolite (SRS *trans*-OH).

There are some reports that show the existence of arachidonate metabolites in the inflammatory tissue and that some of the metabolites participate in the inflammatory reaction [5, 6]. But few studies have been reported on the effects of anti-inflammatory drugs on tissue PG levels in relation to anti-inflammatory activity. Harada *et al.* [7] reported that PG levels in the pleural exudate increase markedly after the injection of carrageenin into the pleural cavity of rats and that aspirin significantly suppresses the increase.

In the present study, we measured the levels of $PGE_2$, 6-keto-$PGF_{1\alpha}$ and inflammatory markers in the pleurisy model in order to elucidate the relationships between the effects of loxoprofen-Na on PG levels in the inflammatory exudate and on the inflammatory response. Furthermore, we determined the amounts of loxoprofen-Na and the active metabolite (SRS *trans*-OH) in the inflammatory exudate after oral administration of loxoprofen-Na and examined whether the active metabolite had the potency to inhibit *in vitro* $PGE_2$ production of pleural leukocytes at concentrations present in the inflammatory exudate.

## MATERIALS AND METHODS

*Materials.* Loxoprofen-Na and the SRS *trans*-OH

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metabolite were synthesized in our Medicinal Chemistry Research Laboratories [8]. Carrageenin (Viscarin 402) was purchased from Marine Colloids, Springfield, NJ. [$^{14}$C]$PGE_2$ was obtained from the New England Nuclear Corp., Boston, MA. $PGE_2$, indomethacin, f-Met-Leu-Phe and bovine serum albumin were obtained from the Sigma Chemical Co., St. Louis, MO. Dulbecco's modified Eagle's Medium was from the Nissui Pharmaceutical Co., Ltd., Osaka.

*Drug administration.* Loxoprofen-Na and indomethacin were dissolved in 0.5% tragacanth solution and administered orally. The control rats were administered 0.5% tragacanth solution without drugs.

*Induction of pleurisy and sampling of pleural exudate.* Male Wistar rats (7 weeks old) were used throughout the experiments. Pleurisy was induced by the method of Katori *et al.* [9] with a slight modification. Carrageenin suspended in 0.9% saline solution was injected into the right pleural cavity of the rat through a reinforced 25-gauge needle. At given intervals, rats were decapitated under light anesthesia, and 2 mL of phosphate-buffered saline containing 100 units/mL heparin and 100 $\mu$M indomethacin was injected into the pleural cavity. After opening the chest, almost all the fluid was harvested by washing with the buffer, and then centrifuged. The supernatant was frozen at −40° until PGs were extracted.

*Extraction and determination of PGs.* $PGE_2$ and 6-keto-$PGF_{1\alpha}$ in the supernatant were extracted according to the method of Powell [10]. The methyl formate fraction was dried up at 30° under a stream of nitrogen gas, and the residue was resolved in the assay buffer.

$PGE_2$ was radioimmunoassayed with $^{125}$I-labeled $PGE_2$ tyrosylmethylester (New England Nuclear Corp.) and rabbit anti-$PGE_2$ antiserum (Institut Pasteur Production, Paris). The $IC_{50}$ for $PGE_2$ was 1.4 ± 0.1 pg/tube and the detection limit was 0.1 pg/tube. 6-keto-$PGF_{1\alpha}$ was determined with 6-keto-$PGF_{1\alpha}$ radioimmunoassay kits (Amersham, Arlington Heights, IL).

*Protein assay and cell count.* The amount of protein in the inflammatory exudate was determined by the method of Lowry *et al.* [11]. Leukocytes in the cavity were counted microscopically with a Tatai cell counter.

*Determination of the concentration of loxoprofen-Na and the active metabolite.* Loxoprofen-Na (0.2 or 2 mg/kg) was administered orally 2.5 hr after the carrageenin injection, and the pleural exudate and blood were collected 0.5 and 1 hr after the administration of loxoprofen-Na. After the addition of deuterium-labeled internal standard ($d_3$-loxoprofen), the exudate and plasma were acidified to pH 2 with 1 N HCl. Loxoprofen and the SRS *trans*-OH were extracted with benzene. Both compounds were treated with hexafluoroisopropanol and trifluoroacetic anhydride [12]. The obtained derivatives were applied to the gas chromatography–mass spectrometer system (Finnigan MAT 4600) and determined by a selected ion monitoring method.

*$PGE_2$ production of the infiltrated cells* in vitro. Three hours after the carrageenin injection, the cells which infiltrated into the cavity were harvested. The contaminating red cells were destroyed by lowering osmotic pressure. About 90% of the infiltrated cells were identified as polymorphonuclear leukocytes by microscopic observation after Giemsa staining. Cells were suspended in Dulbecco's modified Eagle's medium containing 10 mM HEPES (pH 7.4) and 1% bovine serum albumin. Cells ($5 \times 10^6$ cells/tube) and f-Met-Leu-Phe ($10^{-7}$ M) were incubated with or without drug for 20 min at 37° in a volume of 400 $\mu$L, and then 100 $\mu$L of 500 $\mu$M indomethacin was added into the reaction tube to stop PG production. $PGE_2$ released into the medium was determined by radioimmunoassay.

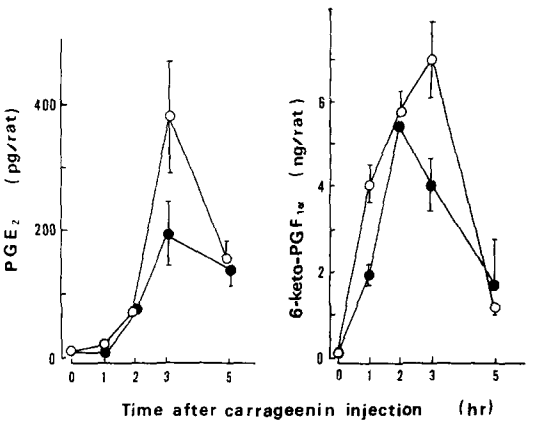


Fig. 1. Time-course of the levels of $PGE_2$ (left panel) and 6-keto-$PGF_{1\alpha}$ (right panel) in the inflammatory exudate after carrageenin injection. PGs were determined by radioimmunoassay as described in Materials and Methods. Key: (○) 1% carrageenin; and (●) 2% carrageenin. Each value is the mean ± SEM of four rats.

## RESULTS

*Time-course of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ levels in the inflammatory exudate.* Figure 1 shows the time-course of the levels of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ in the pleural exudate after the carrageenin injection. When carrageenin was injected in a volume of 0.2 mL, the increase in the levels of both PGs was higher at a concentration of 1% than at 2% carrageenin. At both concentrations of carrageenin, the time-course of the level of $PGE_2$ was similar, while that of 6-keto-$PGF_{1\alpha}$ was different in the peak time (3 hr at 1% and 2 hr at 2%). The increase in the level of 6-keto-$PGF_{1\alpha}$ was observed earlier than that of $PGE_2$. In the subsequent experiments, carrageenin was injected at a concentration of 1% because higher levels of PGs were detected.

*Turnover of $PGE_2$ in the inflammatory exudate.* [$^{14}$C]$PGE_2$ was injected into the pleural cavity 2 hr after the carrageenin injection, and the disappearance of the radioactivity from the cavity was examined. More than 90% of the radioactivity was gone from

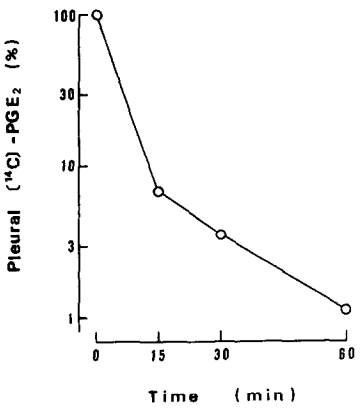


Fig. 2. Disappearance of [$^{14}$C]PGE$_2$ from the pleural cavity. [$^{14}$C]PGE$_2$ (0.2 $\mu$Ci/0.1 mL) was injected into the pleural cavity 2 hr after the carrageenin injection. The pleural exudate was collected with 2 mL of phosphate-buffered saline containing 100 units/mL heparin, and radioactivity was measured. The radioactivity recovered immediately after the [$^{14}$C]PGE$_2$ injection was taken to be 100%. Each value is the mean of four rats.

the cavity within 15 min after the injection (Fig. 2); the half-life was calculated to be about 5 min. This result means that PG levels in the inflammatory exudate represent PG produced in the inflammatory tissue.

*Inhibitory potency of loxoprofen*-Na *on PG production.* The potency of loxoprofen-Na on the inhibition of pleural PG production was determined and compared with indomethacin. When loxoprofen-Na (0.03 to 0.3 mg/kg) was administered orally 2 hr after the carrageenin injection, the peak levels of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ at 1 hr after the administration were dose-dependently suppressed (Table 1). Indomethacin had the same effect but a higher dose was required to inhibit the increase of PG levels. Similar results of the inhibitory potency of these two drugs were also obtained 3 hr after administration (Table 2).

*Time-course of the inhibitory effect of loxoprofen*-Na. Loxoprofen-Na, administered 0.5 hr before the carrageenin injection at a dose of 0.2 mg/kg, reduced the levels of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ up to 5 hr after the carrageenin injection (Fig. 3). One hour after the carrageenin injection, loxoprofen-Na suppressed the PGE$_2$ level to 49% and the 6-keto-PGF$_{1\alpha}$ level to 37% of the control, whereas indomethacin was not effective. Loxoprofen-Na showed a stronger effect than indomethacin at least up to 3 hr after the carrageenin injection. At a dose of 2 mg/kg, both loxoprofen-Na and indomethacin inhibited the increase almost completely up to 5 hr after the carrageenin injection. At this dose, the inhibitory effect of loxoprofen-Na was also stronger than that of indomethacin.

*Effects of loxoprofen*-Na *on protein leakage and leukocyte infiltration.* The protein level and the number of leukocytes in the pleural exudate were measured 3 hr after the carrageenin injection as indexes of the inflammatory reaction. Loxoprofen-Na, administered 30 min before the carrageenin injection at a dose of 2 mg/kg, showed a 55% reduction of protein level and an 81% reduction of the number of leukocytes compared to the control, while indomethacin reduced the values by 16 and 38%, respectively (Table 3). At a dose of 0.2 mg/kg, loxoprofen-Na inhibited protein leakage slightly (17%) but not leukocyte infiltration. Indomethacin at a dose of 0.2 mg/kg was not effective on protein leakage or leukocyte infiltration.

*Concentrations of loxoprofen*-Na *and the active metabolite in the inflammatory exudate.* The concentrations of loxoprofen-Na and the active metabolite (SRS *trans*-OH) in the inflammatory exudate and in plasma 0.5 and 1 hr after oral administration of loxoprofen-Na are shown in Table 4. A significant amount of SRS *trans*-OH was detected in the exudate and in plasma.

*Effect of loxoprofen*-Na *and the active metabolite*

Table 1. Suppressive effects of loxoprofen-Na and indomethacin administered 1 hr before, on the increase of PGs in the inflammatory exudate

| | Dose (mg/kg) | PGE$_2$ (pg/rat) | ID$_{50}$ (mg/kg) | 6-keto-PGF$_{1\alpha}$ (ng/rat) | ID$_{50}$ (mg/kg) |
|---|---|---|---|---|---|
| Expt. 1 | | | | | |
| Control | | 271 ± 60 | | 3.56 ± 0.34 | |
| Loxoprofen-Na | 0.03 | 184 ± 24 | | 2.92 ± 0.18 | |
| | 0.1 | 124 ± 16 | 0.07 | 2.10 ± 0.25 | 0.10 |
| | 0.3 | 25 ± 10 | (0.05–0.09)* | 0.61 ± 0.24 | (0.08–0.14) |
| Expt. 2 | | | | | |
| Control | | 256 ± 60 | | 3.84 ± 0.31 | |
| Indomethacin | 0.03 | 217 ± 42 | | — | |
| | 0.1 | 152 ± 25 | 0.24 | 3.26 ± 0.73 | 0.47 |
| | 0.3 | 120 ± 5 | (0.12–0.62) | 2.31 ± 0.37 | (0.22–1.02) |
| | 1.0 | 75 ± 5 | | 1.25 ± 0.21 | |

Each drug was administered orally 2 hr after the carrageenin injection. The inflammatory exudate was collected 1 hr after drug administration, and both PGs were radioimmunoassayed. Each value is the mean ± SEM of five rats. The ID$_{50}$ was calculated by the method of least squares.

* Values in parentheses indicate the 95% confidence limits.

Table 2. Suppressive effects of loxoprofen-Na and indomethacin administered 3 hr before, on the increase of PGs in the inflammatory exudate

| | Dose (mg/kg) | $PGE_2$ (pg/rat) | $ID_{50}$ (mg/kg) | 6-keto-$PGF_{1\alpha}$ (ng/rat) | $ID_{50}$ (mg/kg) |
|---|---|---|---|---|---|
| Expt. 1 | | | | | |
| Control | | 376 ± 35 | | 4.45 ± 0.54 | |
| Loxoprofen-Na | 0.04 | 295 ± 31 | | 3.47 ± 0.40 | |
| | 0.2 | 122 ± 13 | 0.14 | 1.56 ± 0.19 | 0.16 |
| | 1 | 61 ± 11 | (0.06–0.29)* | 1.08 ± 0.05 | (0.08–0.30) |
| Expt. 2 | | | | | |
| Control | | 406 ± 23 | | 5.47 ± 0.61 | |
| Indomethacin | 0.04 | 357 ± 34 | | 4.38 ± 0.51 | |
| | 0.2 | 234 ± 23 | 0.28 | 3.11 ± 0.24 | 0.27 |
| | 1.0 | 84 ± 11 | (0.18–0.43) | 1.39 ± 0.12 | (0.17–0.41) |

Each drug was administered orally 0.5 hr after the carrageenin injection. The inflammatory exudate was collected 3 hr after drug administration, and both PGs were radioimmunoassayed. Each value is the mean ± SEM of five rats. The $ID_{50}$ was estimated by the least squares test.

* Values in parentheses indicate the 95% confidence limits.

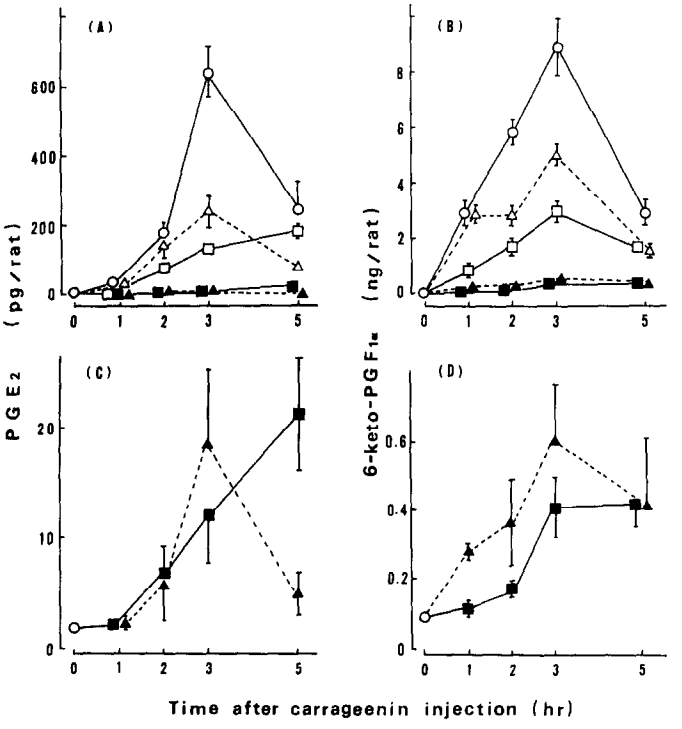


Fig. 3. Time-course of the suppressive effect of loxoprofen-Na and indomethacin on the increase of $PGE_2$ (A and C) and 6-keto-$PGF_{1\alpha}$ (B and D). PG levels after the administration of each drug at a dose of 2 mg/kg were increased (C and D). Each drug was administered orally 30 min before the carrageenin injection. Key: (○) control; (□) loxoprofen-Na, 0.2 mg/kg; (■) loxoprofen-Na, 2 mg/kg; (△) indomethacin, 0.2 mg/kg; and (▲) indomethacin, 2 mg/kg. Each value is the mean ± SEM of five rats.

*on $PGE_2$ production of leukocytes* in vitro. More than 90% of the cells infiltrated into the cavity were leukocytes; thus, PG production in the inflammatory tissue is probably due to leukocytes. We examined the effects of loxoprofen-Na, the active metabolite and indomethacin on $PGE_2$ production *in vitro* with leukocytes that were harvested 3 hr after the carrageenin injection. The harvested leukocytes were capable of producing PGs, but when leukocytes were activated by a chemotactic peptide f-Met-Leu-Phe ($10^{-7}$ M), $PGE_2$ production was enhanced up to 1.6 ng/$10^7$ cells in 20 min. The active metabolite

Table 3. Effects of loxoprofen-Na and indomethacin on the protein level and number of leukocytes in the pleural cavity

| | Dose (mg/kg) | Protein (mg/rat) (% Inh.) | Leukocytes ($\times 10^7$/rat) (% Inh.) |
|---|---|---|---|
| Expt. 1 | | | |
| Control | | 26.1 ± 2.6 | 4.1 ± 0.3 |
| Loxoprofen-Na | 0.2 | 21.7 ± 1.9 (17) | 3.9 ± 0.2 (5) |
| Indomethacin | 0.2 | 27.5 ± 1.1 (−5) | 4.0 ± 0.2 (3) |
| Expt. 2 | | | |
| Control | | 21.4 ± 1.4 | 5.8 ± 0.9 |
| Loxoprofen-Na | 2.0 | 9.7 ± 1.7 (55) | 1.1 ± 0.2 (81) |
| Indomethacin | 2.0 | 18.0 ± 2.2 (16) | 3.6 ± 0.7 (38) |

Drugs were administered orally 0.5 hr before the carrageenin injection. Each value was obtained 3 hr after the carrageenin injection and is the mean ± SEM of five rats. Values in parentheses are the mean per cent inhibition to the control.

Table 4. Concentrations of loxoprofen-Na and the active metabolite (SRS *trans*-OH) in the inflammatory exudate and plasma after oral administration of loxoprofen-Na

| | 0.5-hr Concn (μg/mL) | | 1-hr Concn (μg/mL) | |
|---|---|---|---|---|
| | In exudate | In plasma | In exudate | In plasma |
| Expt. 1 (0.2 mg/kg, p.o.) | | | | |
| Loxoprofen-Na | 0.12 ± 0.02 | 0.12 ± 0.02 | 0.09 ± 0.01 | 0.06 ± 0.01 |
| SRS *trans*-OH | 0.07 ± 0.01 | 0.10 ± 0.01 | 0.05 ± 0.01 | 0.07 ± 0.01 |
| Expt. 2 (2 mg/kg, p.o.) | | | | |
| Loxoprofen-Na | 1.10 ± 0.25 | 1.91 ± 0.23 | 0.67 ± 0.16 | 0.52 ± 0.08 |
| SRS *trans*-OH | 0.41 ± 0.11 | 1.11 ± 0.17 | 0.25 ± 0.06 | 0.51 ± 0.06 |

Loxoprofen-Na was given orally 2 hr after the carrageenin injection. The pleural exudate and plasma were collected 0.5 or 1 hr after the administration of loxoprofen-Na. Each value is the mean ± SEM of four rats.

(the SRS *trans*-OH, 0.01 to 0.3 μM) of loxoprofen-Na inhibited $PGE_2$ production of leukocytes (Fig. 4). The $IC_{50}$ value was 0.08 μM (0.02 μg/mL). This effect was about 20 times stronger than that of the parent compound ($IC_{50}$ = 1.5 μM) and three times stronger than that of indomethacin ($IC_{50}$ = 0.22 μM).

## DISCUSSION

There are several anti-inflammatory agents which have the prodrug property [13]. In general, the concentration of the active forms of these drugs in blood increases slowly after oral administration [14–16]. As for loxoprofen-Na, the active metabolite (SRS *trans*-OH) was detected in plasma and in the inflammatory exudate 30 min after oral administration of loxoprofen-Na. The concentration of the active metabolite in the inflammatory exudate was enough to inhibit $PGE_2$ production of leukocytes *in vitro*. Such a rapid appearance of a significant amount of the active form in the inflammatory tissue explains the potent and immediate effect of loxoprofen-Na.

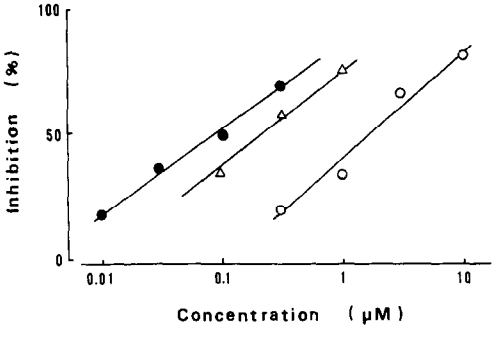


Fig. 4. Inhibitory effects of loxoprofen-Na, the active metabolite (SRS *trans*-OH), and indomethacin on $PGE_2$ production of leukocytes *in vitro*. Key: (○) loxoprofen-Na; (●) SRS *trans*-OH; and (△) indomethacin. Leukocytes were harvested from the pleural cavity 3 hr after the carrageenin injection. Leukocytes, drug and f-Met-Leu-Phe were incubated for 20 min at 37°, and $PGE_2$ released into the medium was radioimmunoassayed. At each drug concentration, the level of $PGE_2$ was determined in four preparations.

In an *in vitro* experiment, the inhibitory effect of the active metabolite of loxoprofen-Na on PG production of leukocytes was about 20 times stronger than that of the parent compound. This result supports the view that loxoprofen-Na is a prodrug. We previously obtained a similar result with 3T6 fibroblast cells which have the ability to convert loxoprofen-Na to the active metabolite [3]. Thus, the active metabolite probably contributes to the suppression of $PGE_2$ production when loxoprofen-Na is incubated with leukocytes. The inhibitory effect of the active metabolite of loxoprofen-Na was about three times as potent as that of indomethacin in leukocytes. This result corresponds to the relative inhibition of PG production in the inflammatory tissue after oral administration of loxoprofen-Na and indomethacin.

In the present study, we showed that the increase in PG levels in the inflammatory tissue was suppressed markedly after oral administration of loxoprofen-Na. The suppressive effect of loxoprofen-Na was observed to be stronger and more rapid than that of indomethacin. The relative potencies of both drugs to inhibit PG production in the inflammatory tissue correlated well with their suppression of protein leakage and leukocyte infiltration. These results are also in good agreement with our previous reports which showed that loxoprofen-Na and indomethacin inhibited carrageenin-induced paw-edema with $ID_{50}$ values of 1.2 and 2.2 mg/kg, respectively [1, 8].

It is well-known that inhibition of PG production is the main anti-inflammatory mechanism of non-steroidal anti-inflammatory drugs, but few reports show the relationship between inhibition of PG production in the inflammatory tissue and suppression of the inflammatory reaction. Loxoprofen-Na, administered at a dose of 0.2 mg/kg, inhibited PG production in the inflammatory tissue by 50% or more throughout 3 hr after carrageenin injection, while the anti-inflammatory effect was not obvious and protein leakage was suppressed only slightly. At a dose of 2 mg/kg, at which PG production was inhibited almost completely, loxoprofen-Na showed a definite anti-inflammatory effect. Similar results were obtained with indomethacin. Thus, the higher dose compared to that which inhibits PG production was necessary to suppress the inflammatory reaction. Almost complete inhibition (90% or more) of PG production in the inflammatory tissue may be essential for the anti-inflammatory effect of non-steroidal anti-inflammatory drugs.

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