Important Role of Peptide Leukotrienes (p-LTs) in the Resting Tonus of Isolated Human Bronchi

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ABSTRACT—The quality of the resting tonus in isolated human bronchi was investigated using a peptide leukotriene (p-LT) antagonist, a 5-lipoxygenase inhibitor and others. (E)-2,2-Diethyl-3'-[2-[2-(4-isopropyl)thiazoyl]ethenyl]succinanilic acid sodium salt (MCI-826), a newly synthesized compound that is a highly selective antagonist to LTD₄ and LTE₄, markedly relaxed the isolated human bronchi at low concentrations. A selective and competitive arachidonate 5-lipoxygenase inhibitor, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861), also potently lowered the tonus. In addition, a large amount of spontaneously formed p-LTs was detected in the isolated human bronchial tissue as well as the lung parenchymal tissue. The isolated human bronchi responded to indomethacin treatment with contractions and the acceleration of p-LT formation. Atropine, an anticholinergic; mepyramine, an antihistaminic; and OKY-046, a thromboxane synthetase inhibitor, all showed no effect on the resting tonus. Taking into consideration the high responsiveness of the human airway smooth muscle to p-LTs and the present results, which were different from those on isolated guinea pig tracheas, it is strongly suggested that the spontaneously formed p-LTs largely participate in the resting tonus of the majority of isolated human bronchi.

Keywords: Leukotriene, Bronchi, Resting tonus, Antagonist, 5-Lipoxygenase

MATERIALS AND METHODS

Reagents

Reagents used and their sources were: (E)-2,2-diethyl-3'-[2-[2-(4-isopropyl)thiazoyl]ethenyl]succinanilic acid sodium salt (MCI-826, donated from Mitsubishi Kasei, Tokyo); 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861, donated from Takeda Chem. Ind., Osaka); dextran (Nacalai Tesque, Kyoto); (E)-3-[4-(imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride (OKY-046, donated from Kissei Pharm., Matsumoto); atropine sulfate and gelatin (Merck, Darmstadt, FRG); prostaglandin (PG) B₁ (Funakoshi, Tokyo); dimethyl sulfoxide (DMSO), acetylcholine chloride (ACh), charcoal, histamine dihydrochloride, leukotriene (LT) C₄, LTD₄ and LTE₄ (Wako Pure Chem., Osaka); radioactive [14,15-³H(N)]-LTC₄ (1.45 TBq/mmol), -LTD₄ (1.48 TBq/mmol) and -LTE₄ (1.48 TBq/mmol) (New England Nuclear, Boston, MA, USA); C-18 reversed phase Amprep column (Amersham, Buckinghamshire, UK); liquid scintillation cocktail (Atomlight, New England Nuclear); mepyramine maleate and l-isopro-
terenol bitartrate (Sigma Chem., St. Louis, MO, USA).

The ethanol used was obtained by distillation of tax-
free ethanol in laboratory glassware at a bp. 78.0–
78.5°C.

Before use, atropine, ACh, OKY-046 and mepyramine
were dissolved in physiologic saline; PGB1 and indometh-
acin, in methanol; MCI-826, in distilled water; and AA-
861, in DMSO.

Rabbit anti-LTC4 plasma, which was obtained in
our laboratory by immunization with LTC4-conjugated
Ascaris suum extracts and which possessed highly cross
reactivity to LTD4 99% and LTE4 34%, was used follow-
ing a 700-fold dilution for radioimmunoassay (RIA).

Animals

Hartley, male guinea pigs weighing 550–1,120 g were
purchased from Japan SLC, Inc., Hamamatsu.

Human lungs

Macroscopically normal human lung portions were ob-
tained at the time of surgical resection for carcinoma and
used for experiments as soon as possible.

Preparation of isolated tracheal segments from guinea
pigs

Guinea pigs were killed by bleeding from the femoral
artery following a blow on the head. The trachea was split
longitudinally through the ventral cartilage and cut into
segments, each of which was two-cartilage-rings-wide (5).

Preparation of isolated human bronchi

Spiral strips of human bronchi, about 2 mm in width
× 2 cm in length, were prepared from the portion less
than 3 mm in inner diameter, as previously reported (4).

Preparation of human and guinea pig lung fragments

Human and guinea pig lung parenchymas were frag-
mented into pieces of 1 × 1 × 2–3 and 0.5 × 0.5 × 0.5 mm
in size, respectively, with a McIlwain tissue chopper (6).

Measurements of the tonus of the isolated human bronchi

The isolated human bronchial preparation (loading
weight: 300 mg) was suspended in a 5-ml organ bath, and
examinations were carried out under similar conditions to
the previous report (4). In brief, after almost constant con-
tractions by repeated applications of 7 × 10–6 M ACh
were observed as an indication that the sensitivity had
become stable, the respective drugs were added and their
influences were observed: MCI-826 at 2.5 × 10–8 M for
30 min, AA-861 at 10–6 M for 50 min, and atropine,
mepyramine and OKY-046 at 10–6 M for 20 min. Follow-
ing these treatments, the respective preparations were fur-
ther treated with 10–6 M isoproterenol for 10 min for the
maximum relaxation. Most researchers have used an
isometric transducer to measure of isolated airway tissue
movements. However, we have employed an isotonic
transducer instead for the following reasons: 1. Airway
smooth muscles constrict or relax under a limited extent
of weak, negative intrapleural pressure in vivo. 2. Be-
cause of the functional properties of the isometric trans-
ducer, it is inherently difficult to measure exactly the relax-
ation of isolated airway smooth muscles with it.

Assays of p-LTs formed spontaneously in the bronchial,
tracheal and/or lung parenchymal tissues

Incubation: The segments of human bronchi and lung
parenchymas, and of guinea pig tracheas and lung paren-
chymas were suspended with Tyrode's solution and incu-
bated at 37°C for 1 hr (10 ml/g wet tissue). After the incu-
bation, the tissue was immersed in dry ice–acetone-
chilled ethanol (4 ml/g wet tissue) and homogenized with
a phycotron (Niti-on, Chiba) at 15,000 r.p.m. for 20 sec
preceded by addition of distilled water (1 ml/g wet tis-
sue).

Purification and radioimmunoassay (RIA): To the sam-
ples of homogenate were added radiolabelled [3H]-LTC4,
-LTD4 and -LTE4 (each 33 Bq) in order to calculate the
respective overall recoveries of p-LTs and gelatin solution
(final conc. of 70 µg/ml) to improve the recovery from
the subsequent step of C-18 reversed phase Amprep
column chromatography. Following addition of 100 ng/g
wet tissue of PGB1 as an internal standard and centrifuga-
tion (11,000 × g, 20 min, 4°C), the resultant supernatant
was concentrated to 1 ml/specimen under reduced pres-
sure. Then, the p-LTs in the specimen were successively
purified by C-18 reversed phase Amprep column chro-
matography and HPLC as described elsewhere (7). The
respective p-LTs in the fractions from HPLC were esti-
mated by RIA (7) and expressed as pmole/g wet tissue.

Assays of p-LTs formed in and released from isolated
human bronchial strips in the presence and absence of
indomethacin

The isolated human bronchial strips suspended in
5-ml organ baths as described above were treated with
or without 3 × 10–6 M indomethacin for 40 min at 37°C.
After the treatment, both of the tissue and the medium
(Tyrode's solution) were recovered from the bath. The
assay of p-LTs in the tissue was carried out following the
same procedures described above and that in the medium
was also executed following the same procedures, but
without the homogenization and centrifugation steps.
RESULTS

Effects on the resting tonus of the human bronchi

Effect of the p-LT antagonist, MCI-826, on the resting tonus of the isolated human bronchi is illustrated in Fig. 1.

The compound at a concentration as low as $2.5 \times 10^{-8}$ M did not demonstrate obvious effects within a few min after its addition, but thereafter, it caused a substantial time-dependent relaxation that reached approx. 90% of the maximum at 30 min.

The resting tonus of the human bronchus was also greatly reduced by the treatment with $10^{-6}$ M AA-861, a selective and competitive inhibitor of arachidonate 5-lipoxygenase (8, 9) (Fig. 2). On the other hand, atropine, an anticholinergic; mepyramine, an antihistaminic; and OKY-046, a thromboxane synthetase inhibitor, little affected the tonus (Fig. 2).

Amounts of p-LTs in the human and guinea pig pulmonary tissues

Table 1 represents the amounts of the respective p-LTs formed spontaneously in the fragmented pulmonary tissues of the human and guinea pig.

In the human bronchi, all kinds of p-LTs, particularly LTD₄ and LTE₄, were found in noticeable quantities. The levels of LTD₄ and LTE₄ reached 2 and 4.5 times, respectively, as much as that of LTC₄. Human lung parenchymas also produced respectable amounts of p-LTs, each of which was greater in quantity than that of the human bronchi. On the other hand, although detectable amounts of p-LTs were found in both the guinea pig tracheal and lung parenchymal tissues, their p-LTs in the respective tissues were in only trivial amounts. Compared with the human pulmonary tissues in terms of the amount of p-LTs, the amounts formed in the trachea and lung parenchyma

| Species and tissue (n) | Amount of p-LTs (pmole/g tissue) |
|-----------------------|---------------------------------|
|                       | LTC₄ | LTD₄ | LTE₄ | total (as p-LTs) |
| Human                 |      |      |      |                  |
| bronchus (6)          | 0.45±0.11 | 1.07±0.53 | 2.11±0.60 | 4.20±1.47 |
| lung parenchyma (7)   | 1.05±0.03 | 1.21±0.33 | 7.50±1.28 | 9.99±2.04 |
| Guinea pig            |      |      |      |                  |
| trachea (4)           | 0.04±0.004 | 0.16±0.09 | 0.07±0.02 | 0.17±0.01 |
| lung parenchyma (4)   | 0.22±0.02 | 0.33±0.05 | 0.15±0.04 | 0.72±0.13 |

Values represent the mean±S.E. of the number of experiments shown in parentheses. Guinea pig tracheas (approximately 200 mg/animal) and lung parenchymas (500 mg/animal) from 20 and 5 animals, respectively, were used in each experiment for p-LT estimation.
of guinea pigs were approx. 1/25 and 1/14 of those in the human bronchus and lung parenchymas, respectively.

**Effects of indomethacin on the resting tonus of the isolated human bronchi and the formation of p-LTs in the tissue**

By the treatment with $3 \times 10^{-6}$ M indomethacin, the isolated human bronchi resulted in a time-dependent and considerable contraction that reached a plateau level at 35 min after the drug addition, followed by a slight and transient relaxation (Fig. 3).

After a 40-min treatment with $3 \times 10^{-6}$ M indomethacin, the suspended human bronchi and media were collected and their p-LT levels were estimated. Greater amounts of p-LTs by the treatment with indomethacin were found in the tissue and medium than those in the controls without the drug treatment (Table 2).

![Fig. 3. Effects of indomethacin on the resting tonus of isolated human bronchi. Indomethacin was added at the final concentration of $3 \times 10^{-6}$ M. Each point represents the mean ±S.E. of 10 experiments.](image)

**DISCUSSION**

The present experiments indicate a high probability that the resting tonus of isolated human bronchi is largely maintained by spontaneously formed p-LTs. Conclusive proof for this is found from the following experimental results: First, the newly synthesized compound MCI-826, a potent and highly selective antagonist of p-LTs (4), markedly lowered the resting tonus at a concentration as low as $2.5 \times 10^{-8}$ M. Differently from FPL 55712, which is used as a worldwide standardized p-LT antagonist (10), MCI-826 does not have some of the intrinsic drawbacks that have been obstacles to studies in the p-LT field. That is, the compound shows highly potent antagonistic activities against LTD₄ and LTE₄ (pA₂ values: LTD₄, 8.7; LTE₄, 9.5, assayed by isolated guinea pig tracheas), despite producing no substantial inhibition against LTC₄ (4) and having little or no antagonistic effect on the contractions by other agonists (4). Second, AA-861, a selective and competitive arachidonate 5-lipoxygenase inhibitor (8, 9), also potently relaxed the smooth muscle. Third, the careful purification and RIA revealed that large quantities of p-LTs were spontaneously formed in the isolated human bronchial tissues, which were at levels more than 20 times those in the isolated guinea pig tracheal tissue. In addition to these results, the important role of p-LTs in the resting tonus is further backed by the extremely high responsiveness of isolated human bronchi to these arachidonate lipoxygenase metabolites (1).

In the isolated guinea pig tracheas, arachidonate cyclooxygenase metabolite(s) has been considered to be a principal mediator responsible for maintaining the resting tonus since indomethacin, an arachidonate cyclooxygenase inhibitor, and an antagonist of contractile cyclooxygenase metabolites (TP-receptor antagonist) potently lower the resting tonus (11–14). Differently from isolated human bronchi, it is clear that p-LTs do not participate in the resting tonus of the isolated guinea pig tracheal tissue. Because the preparation is not relaxed at all by the treatment with MCI-826 even at high concentrations (4), although considerable relaxation is observed with FPL 55712, a relatively non-specific p-LT antagonist (4), and because the tissue did not spontaneously form sufficient amounts of p-LTs to be responsible for the resting tonus as shown in the present results.

In contrast to isolated guinea pig tracheas, the isolated human bronchi was contracted by the treatment with indomethacin. The reason for this is likely to be further facilitated formation of arachidonate lipoxygenase metabolites (especially p-LTs in this case) by the drug acting to block the arachidonate cascade to cyclooxygenase pathway, as demonstrated by the results shown in Table 2.

**Table 2. Effect of indomethacin on spontaneous formation and release of p-LTs from the human bronchi**

|                      | Amount of p-LTs (pmole/g wet tissue) |
|----------------------|--------------------------------------|
|                      | tissue  | medium | total    |
| Control              | 2.9±1.21 | 3.5±0.72 | 6.4±1.91 |
| Indomethacin-treated | 5.1±1.27 | 8.3±1.15* | 13.4±1.27* |

Human bronchi suspended in a 5-mL Magnus bath were treated with or without $3 \times 10^{-6}$ M of indomethacin for 40 min. After the treatment, both the tissue and the medium (Tyrode's solution) were recovered from the bath and processed as described in the Materials and Methods for the purification and estimation of p-LTs. Values represent means±S.E. of 5 experiments. *: statistically significant difference from the control at P<0.05 (unpaired t-test).
chi, the greater part of LTC₄ can exist in the cells themselves, in which the LT is formed, because the LTC₄ released from the cells is considered to be very rapidly converted to LTD₄ by γ-glutamyltranspeptidase, an enzyme abundantly located in the membrane surfaces of a variety of cell types (15, 16).

It is not yet identified which cell spontaneously forms p-LTs, which contribute to the resting tonus of the isolated human bronchi, but at least mast cells must be involved in this because a large amount of LTC₄ is formed in the cell during in vitro anaphylaxis (17). Consequently, LTD₄ and LTE₄ detected in the human bronchi may be distributed into the interstices of the tissue at high concentrations, and some of each may contribute to the retention of the resting tonus through the continuous stimulation of p-LT receptors on the bronchial smooth muscle cells.

Quite recently, we found that there were a few isolated human bronchial specimens in which the resting tonus was only slightly affected by a p-LT antagonist, but in contrast, was greatly lowered by a selective TP-receptor antagonist which antagonizes the contractions of isolated human bronchi induced by not only TXA₂ but also PGF₂α, PGD₂, 9α,11β-PGF₂α and others (unpublished data, S. Kohno et al.). This observation suggests that the resting tonus of a limited number of specimens is mainly maintained by a contractile prostanoid(s) via the arachidonate cyclooxygenase pathway.

In the bioassays of contractile agonists and/or their antagonists using isolated human airway preparations, the resting tonus frequently hampers an accurate analyses of their levels. The pretreatments of the tissue with a selective p-LT antagonist and/or TP receptor antagonist may lead to more clear results to determine if these agonists and/or antagonists are neither directly nor indirectly associated with arachidonate cyclooxygenase metabolites.

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