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RELEASE OF LEUKOTRIENES INTO THE PERFUSATE OF CALCIUM-IONOPHORE STIMULATED RABBIT LUNGS

INFLUENCE OF 5-LIPOXYGENASE INHIBITORS*

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Abstract—Arachidonic acid and the calcium ionophore A23187 are known to provoke a pulmonary artery pressor response, edema formation and release of thromboxane B2 (TxB2) and 6-keto prostaglandin-F1α (6-keto PGF1α) into the recirculating perfusion fluid of isolated blood-free perfused rabbit lungs. Here we investigated the release of leukotrienes (LTs) by repetitive 0.1 μM A23187 challenge in the presence or absence of cyclooxygenase and 5-lipoxygenase inhibitors. RP-HPLC analysis of perfusion fluid extracts persistently showed peaks with retention times of authentic LTC4, -D4, -E4 and -B4. Fractionated RP-HPLC eluate subjected to radioimmunoassay (RIA) with LTC4 and LTB4 antibodies showed two major peaks of immunoreactivity corresponding to those compounds and minor immunoreactivity with LTD4 and LTE4 in accordance with the stated cross-reactivities of the LTC4 antibody. Good correlation for both LTB4 and LTC4 levels measured by RP-HPLC versus RIA of collected HPLC peaks was found. Five to ten min after A23187 challenge, LTC4, -D4 and -B4 levels ranged from 800 to 1600 pg/ml perfusate. LTC4 reached a maximum level at 20 min whereas LTB4 slightly increased over a 35 min period. Upon repeated A23187 challenge, interrupted by rinsing phases with fresh perfusion fluid, the LT release was reproducible several times with increasing reaction strength. This performed in presence of increasing concentration of the 5-lipoxygenase inhibitors AA-861 or U-60,257 caused a dose-dependent inhibition of the release of all LTs with an IC_{50} of approximately 10^{-8} to 10^{-7} M and 10^{-6} M, respectively. Cyclooxygenase inhibition with acetylsalicylic acid at doses completely suppressing the A23187 induced pressor response did not inhibit the peptidoleukotriene release and only slightly depressed LTB4 release. Conclusion: using a rapid and sensitive extraction and RP-HPLC method isolated lungs are found to release nanomolar amounts of LTs into the perfusate upon repetitive A23187 challenge, suppressed by 5-lipoxygenase inhibition.

Slow reacting substance of anaphylaxis (SRS-A) has long been known to be generated in the lung, for example upon antigen challenge and after application of the calcium ionophore A23187, and to exert long lasting contractions of smooth muscles in this organ, whereby especially its bronchoconstrictor properties have been investigated (for reviews see Piper [1, 2], Leitch [3] and Lewis and Austen [4]). Since the discovery of its chemical constitution as the sulfidopeptide leukotrienes (LT)§ LTC4, LTD4 and LTE4, potent microvascular effects have been demonstrated, especially the initiation of plasma leakage in the postcapillary venules of skin and hamster cheek pouch. An effecter of the microvascular accumulation of leukocytes in these tissues, the dihydroxyeicosatetraenoic acid LTB4 was recognised as a potent chemotactic and chemokinetic agent. In the pulmonary vascular bed the sulfidopeptide leukotrienes have been shown to exert strong vasoconstrictive potency, however partially mediated by induction of the vasoconstrictive cyclooxygenase products such as the endoperoxides, thromboxane A2 (TxA2) and prostaglandin F2α [5–9]. The actions of LTB4 in the pulmonary vasculature have not been investigated and there is still controversy whether arachidonic acid (AA) lipoxygenase products, either exogenously added as the sulfidopeptide leukotrienes or presumed to be endogenously formed after application of AA or A23187, may alter the permeability characteristics in the pulmonary vascular bed [5, 10–14]. In chopped or enzymatically dispersed lung tissue several groups were able to demonstrate the formation of LTC4, LTD4 or LTE4 after challenge with antigen, A23187 or platelet activating factor [15–20], whereas LTB4 was found to be the predominant leukotriene released from alveolar macrophages *in vitro* [21–23]. It has also been possible to demonstrate the release of LTC4 and LTD4 into the collected effluent of antigen or platelet activating factor stimulated isolated lungs [24, 25].

In the present study we demonstrate that the sulfidopeptide leukotrienes LTC4, LTD4 and LTE4 as

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§ Abbreviations used: AA, arachidonic acid; ASA, acetylsalicylic acid; HETE, hydroxyeicosatetraenoic acid; KHB, Krebs Henseleit bicarbonate buffer; LT, leukotriene; PG, prostaglandin; RIA, radioimmunoassay; RP-HPLC, reverse phase high performance liquid chromatography; Tx, thromboxane; ww, wet weight.

well as LTB4 are released into the recirculating blood-free perfusion fluid of A23187 stimulated isolated rabbit lungs, perfused and ventilated under physiologically stable conditions. The release of all leukotrienes (LTs) can be reproduced several times upon repeated application of ionophore in the same isolated lung and can be blocked in a dose-dependent manner by the 5-lipoxygenase inhibitors AA-861 (IC_{50} approximately 10^{-8} to 10^{-7} M) and U-60,257 (IC_{50} approx. 10^{-6} M). These substances will therefore be valuable tools for further elucidation of the biological significance of the sulfidopeptide leukotrienes and LTB4 in the pulmonary vascular bed.

MATERIALS AND METHODS

Materials

The leukotrienes B4, C4, D4 and E4 as well as 20-OH LTB4 were generous gifts from Dr J. Rokach, Merck Frosst (Dorval, Canada). Additional LTC4 as well as TxB2 were graciously supplied by Ono Pharmaceutical (Osaka, Japan). The lipoxygenase inhibitors AA-861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone) and U-60,257 (6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$ PGI1) were gifts from Dr M. Nishikawa, Takeda Chemical Industries (Osaka, Japan) and Dr M. Bach, Upjohn Co. (Kalamazoo, U.S.A.), respectively. The following chemicals were purchased: Aspisol® (D,L-lysin-mono-acetylsalicylate/glycin; 9:1, w/w), Bayer AG (Leverkusen, F.R.G.); calcium ionophore A23187, Calbiochem GmbH (Frankfurt, F.R.G.); 6-keto PGF1α, Sigma GmbH (München, F.R.G.); rabbit anti-6-keto PGF1α and anti-TxB2, Paesel GmbH (Frankfurt, F.R.G.); HPLC grade methanol and chloroform, Burdick & Jackson (Muskegon, U.S.A.). All other analytical grade biochemicals were obtained from Merck AG (Darmstadt, F.R.G.).

Anti-LTB4 was received from Dr J. Salmon, Wellcome Research (Beckenham, U.K.). Materials for the LTC4 radioimmunoassay along with (14,15-^{3}H(N)) LTC4 (34.0 Ci/mmol specific activity), (14,15-^{3}H(N)) LTB4 (52.0 Ci/mmol), (5,8,9,11,12,14,15-^{3}H(N)) 6-keto PGF1α (120–180 Ci/mmol) and (5,6,8,9,11,12,14,15-^{3}H(N)) TxB2 (100–150 Ci/mmol) were purchased from New England Nuclear GmbH (Dreieich, F.R.G.).

Methods

Model of isolated rabbit lungs. The model has been previously described [12, 26]. Briefly, rabbits of either sex (bodyweight 2.3–2.8 kg) were deeply anaesthetized with 60–90 mg pentobarbital-sodium/kg bodyweight and were anticoagulated with 1000 U/kg bodyweight heparin. The isolated lungs, suspended freely from a force transducer in a chamber warmed to 38°, were ventilated with 4% CO_2, 17% O_2 and 79% N_2 (frequency, 45 strokes/min; tidal volume, 30 ml) and were perfused with Krebs Henseleit bicarbonate buffer (KHB) in a recirculating system (circulating volume, 125 ml) with a pulsatile flow of 100 ml/min. By the alternate use of two different perfusion systems it was possible to perform numerous perfusion phases in the same isolated lung, each with fresh perfusion fluid with or without the addition of A23187 and/or inhibitors. The physical parameters of perfusion and ventilation pressures and the weight of the isolated lung were registered continuously by pressure and force transducers. Due to the constant perfusion flow, alterations of perfusion pressure are interchangeable with alterations of vascular resistance. Only those lungs were selected that after a steady state period of at least 30 min were completely blanched and showed no spontaneous edema formation or changes of ventilation or perfusion pressure. Random light microscopical examination of these lungs revealed no adherence of erythrocytes, platelets or leukocytes to the vascular wall.

RIA of TxB2 and 6-keto PGF1α. TxA2 and PGI2 were assayed serologically from the KHB as their stable hydrolysis products TxB2 and 6-keto PGF1α. Each mixture contained a 0.1 ml sample or 5–500 pg of an authentic standard, 0.1 ml of diluted antiserum (cross-reactivity with other prostaglandins each <0.05%), tritium labelled TxB2 or 6-keto PGF1α and 10 mM phosphate buffer (pH 7.4) containing 0.1% gelatine to a total volume of 0.5 ml. Antibody–antigen complexes were separated from free antigen by adding dextran coated charcoal suspension. The limit of detection was 5 pg for TxB2 and 10 pg for 6-keto PGF1α and 50% binding occurred at 50 pg for TxB2 and 170 pg for 6-keto PGF1α.

Extraction and recovery of leukotrienes. Samples (20 ml) of the recirculating KHB from the isolated lungs were placed on an ice bath before extraction within 1 hr. Each sample was then applied to a six ml octadecyl solid phase extraction column (Analytichem, Harbor City, U.S.A.) which had been preconditioned with two column volumes each of heptane, methanol and ice-cold water. The columns were then successively washed with three column volumes of ice-cold water and twice with 0.5 ml ice-cold chloroform. Leukotrienes were eluted by two successive washes with 0.5 ml ice-cold methanol and placed under a nitrogen stream to remove a light boiling methanol–water–chloroform azeotrope before *in vacuo* evaporation. Samples were reconstituted in 50 or 75 μl methanol and spun before injecting 25 μl into the HPLC unit.

There was better recovery of the peptidoleukotrienes from columns that had been used once previously as compared to those in their initial use: probes containing 75, 50 or 25 ng each of LTC4, LTB4, LTD4 and LTE4 in 20 ml of KHB were extracted as described using columns that were new or that had been used once beforehand. The methanol concentrates were analyzed by HPLC in which the individual peaks eluting with LT retention times were quantified by comparing their integrated absorbance at 280 nm to a three-point external calibration curve for each substance. For fresh columns the recovery of LTB4 was 71.1 ± 1.9% (mean ± S.E.M., N = 9); LTC4, 20.6 ± 0.5% (N = 6); LTD4, 39.9 ± 1.7% (N = 6) and LTE4, 35.2 ± 1.2% (N = 6). The recoveries using columns which were used once beforehand were: LTB4, 77.7 ± 2.2% (N = 13); LTC4, 39.9 ± 2.5% (N = 11); LTD4, 58.4 ± 3.2% (N = 13) and LTE4, 60.7 ± 2.3% (N = 13). For the experiments with the isolated lungs, a stock of columns used previously in

recovery experiments was taken. After their second use they were discarded.

Extraction of blank KHB as well as KHB recirculated in the perfusion apparatus in absence of lung (control phase in each isolated lung experiment) showed no peaks of absorbance at 280 nm with LT retention times using fresh as well as once-used extraction columns.

RP-HPLC. The method used was adapated from that of Mathews *et al.* [27]. The HPLC unit consisted of a M600 pump (Gynkothek, München, F.R.G.) operated at 1.3 ml/min, a model 7125 injection valve (Rheodyne, Cotati CA, U.S.A.) with a 50 μl injection loop and a Spectroflow 773 variable wavelength ultraviolet spectrophotometer (Kratos, Karlsruhe, F.R.G.) set at 280 nm. Peak areas were quantitated using a System I integrator (Spectra-Physics, Darmstadt, F.R.G.). The column (25 × 0.46 cm) was packed with ODS-Hypersil 5 μm particles (Shandon, Runcorn, U.K.) using a slurry packing procedure. A 3.3 × 0.46 cm guard column dry packed with Vydac 201-RP (Macherey-Nagel, Düren, F.R.G.) was fitted and the entire column shortly rinsed with water in order to remove traces of methanol before equilibration overnight with 0.5% disodium EDTA in water/methanol, 9:1 (v/v) [28]. The column was again rinsed with water before equilibration with methanol/water/acetic acid 72:28:0.16 (v/v) adjusted to pH 4.9. All solvent mixtures were filtered with a Gelman HT-450 membrane filter and degassed *in vacuo* before use.

As a further verification of our HPLC methodology, RIAs for LTC4 and LTB4 were performed in eluate fractions from each extract originating from the isolated lung experiments. Eluates for the time period 0.5 min before to 0.5 min after the current LTC4 and LTB4 standard retention times were collected, dried under reduced pressure and stored at −20° until RIA. The average recovery for this step was 43 ± 4% for LTC4 and 80 ± 2% for LTB4 (mean ± S.E.M., N = 3 each).

RIAs for LTB4 and LTC4. LTB4 RIA of evaporated HPLC eluates was based upon the procedure of Salmon [29]. Samples were reconstituted in assay buffer (50 mM Tris, pH 8.6, plus 0.1% gelatin) and rabbit anti-LTB4 serum at a 1:750 dilution was added, along with 15,000 dpm of tritiated LTB4. LTB4 was detectable on its radioligand binding curve over the range from 16 to 1000 pg with 50% binding at 120 pg. A mixture of 5(*S*),12(*R*)- and 5(*S*),12(*S*)-6,8,10-*trans*-14-*cis*-dihydroxyeicosatetraenoic acid (HETE) diastereomers (6-*trans* LTB4s) were reported to exhibit 3.3% cross-reactivity in the assay; 5(*S*),12(*S*)-6,10-*trans*,8,14-*cis*-diHETE, 0.14%; 12-HETE enantiomers, 2.0%; the C-6 sulfidopeptide LTs and 5-HETE enantiomers less than 0.03% [30].

LTC4 RIA of HPLC eluates was performed as per instructions provided by the commercially available kit. Relative cross-reactivities of the antibody are: 5(*R*),6(*R*)-LTC4 100%, 11-*trans*-LTD4 60.5%, LTD4 55.3%, LTE4 8.6% and LTB4 0.006%. The detection range of LTC4 was 25–1600 pg and 50% displacement of bound radioligand occurred at 300 pg.

Protocol of stimulation and inhibition. After the steady state period the perfusion fluid was exchanged and after 2 min A23187 dissolved in 50 μl DMSO was injected into the pulmonary artery to give a final concentration of 0.1 μM in the recirculating perfusion fluid. Ten min after A23187 injection the KHB was exchanged, followed by a 10 min rinsing phase with fresh buffer. The cycle of stimulation phase and rinsing phase was repeated up to five times (i.e. four repetitions after the initial stimulation) within the same isolated lung. At 1, 3, 5 and 10 min after A23187 injection, 1 ml buffer fluid was removed for measurement of TxB2 and 6-keto PGF1α. For leukotriene determination, 20 ml KHB was removed at 5 and 10 min. Where applied, inhibitors were admixed to each KHB perfusate in the order of increasing concentration subsequent to the initial stimulation with A23187 (e.g. first rinsing phase and first repetition of stimulation in presence of 10^{-8} M AA-861, second rinsing phase and stimulus repetition in presence of 10^{-7} M AA-861 and so on). The vehicles used were ethanol for AA-861 (up to 130 μl), 40 mM Tris buffer (up to 100 μl) for U-60,257 and water for ASA (up to 100 μl). They had been shown to exert neither influence on perfusion pressure, nor release of cyclooxygenase products or leukotrienes. In control lungs the cycles of perfusion phases were repeated without application of A23187.

Statistics. Simple regressions and two-way analysis of variance for unbalanced data were performed.

Table 1. Release of TxB2, 6-keto PGF1α and leukotrienes after A23187 challenge*

Metabolite (pg/ml)	Time: 1 min	3 min	5 min	10 min
TxB2 (N = 16)†	931 ± 72	806 ± 64	734 ± 65	
6-keto PGF1α (N = 16)†		2027 ± 177	2033 ± 157	2074 ± 144
LTB4 (N = 15)‡			1243 ± 124	1369 ± 143
LTC4 (N = 11)§			1486 ± 322	1610 ± 382
LTD4 (N = 9)‡			800 ± 103	922 ± 144

* The table gives the concentrations of TxB2, 6-keto PGF1α and the LTs B4, C4 and D4 in the recirculating perfusion fluid at different times after an initial stimulation with 0.1 μM A23187 in different isolated lungs (mean ± S.E.M.).

† TxB2 and 6-keto PGF1α were detected by RIA of the perfusion fluid.

‡ LTB4 and LTD4 were calculated from the peak areas in the HPLC chromatograms of the perfusate extracts.

§ LTC4 was calculated from the RIA of collected eluate of HPLC LTC4-peaks. The values are corrected for the LTC4 loss due to the peak collection protocol.

RESULTS

Pulmonary artery pressure response and release of TXB2 and 6-keto PGFlα

As known from previous experiments the calcium-ionophore A23187 provokes a pulmonary artery pressor response with a pressure peak between the first and the third minute after A23187 injection, which is reversible by rinsing the lung with fresh perfusion fluid [7, 12, 26]. The height of the pressure peak after the initial application of the ionophore in the present experiments was 36.8 ± 2.6 mm Hg (mean ± S.E.M., N = 18). The perfusate levels of radioimmunologically detected TxB2 (maximum concentration reached within the first 3 min) [7] and 6-keto PGFLα as given in Table 1, correspond to those found in the isolated lung model in previous studies [7, 26]. Both the pressor response and the release of the cyclooxygenase products can be repeated with increasing reaction strength in the same isolated lung (Fig. 3). In absence of A23187 there was no change of perfusion pressure and the levels of TxB2 and 6-keto PGFlα ranged below 100 and 200 pg/ml, respectively (measured in three control lungs with 5, 5 and 7 perfusion phases).

Release of leukotrienes into perfusion fluid

Following A23187 application, the HPLC analysis of perfusion fluid extracts persistently showed absorbance (280 nm) peaks with retention times of standard LTC4, -B4, -E4 and -D4, as typically depicted in Fig. 1. The material with LTC4 retention time was only partially resolved from a preceding unknown peak. As calculated from peak area the levels of LTs ranged between 800 and 1600 pg/ml perfusion fluid at five and ten min after the calcium ionophore application (Table 1). Additional evidence to support the identity of the peaks with retention times of standard LTC4 and LTB4 was obtained in one experiment where the entire HPLC eluate from 1 to 24 min was collected in 15 sec fractions and RIAs with anti-LTC4 and anti-LTB4 were performed with each fraction (Fig. 2). The major peaks of immunoreactivity in each assay corresponded to the retention times of LTC4 and LTB4, respectively. According to the stated cross-reactivities of the LTC4 antibody, there was additional immunoreactivity found in the fractions corresponding to the retention times of LTE4 and LTD4. From all other isolated lung experiments, where LTB4 and LTC4 peaks were systematically collected, a linear correlation between the HPLC measured concentrations and the RIA values of the collected peak fractions of $r = 0.97$ (LTB4) and $r = 0.92$ (LTC4) was found (Fig. 4). The slopes of the regression lines were 0.79 for LTB4 and 0.30 for LTC4, which is comparable to the recoveries of 0.80 and 0.43 of these LTs in the peak collection protocol (see Methods).

The HPLC chromatograms as well as the anti-LTB4 assay showed two small peaks at 10.6 and 11.5 min (Fig. 2), which likely correspond to the 6-*trans* LTB4 diastereomeric pair. This was further supported by experiments with a bulk of stimulated human granulocytes (data not shown), in which HPLC of the extracted supernatants delivered enough material with identical retention times to perform ultraviolet spectra. The two peaks were collected separately and both their spectra showed an absorption maximum at 268 nm with shoulders at 258 and 280 nm, characteristic for the 6-*trans*-LTB4s [30, 31]. These experiments also revealed that material co-eluting with LTE4 showed the typical LTE4 ultraviolet pattern with an absorption maximum at 280 nm and shoulders at 270 and 291 nm [32], although an absorption band at 231 nm was the largest in the spectrum. This suggested the coelution of an unknown substance, possibly containing a diene chromophore, with LTE4 in the HPLC system used.

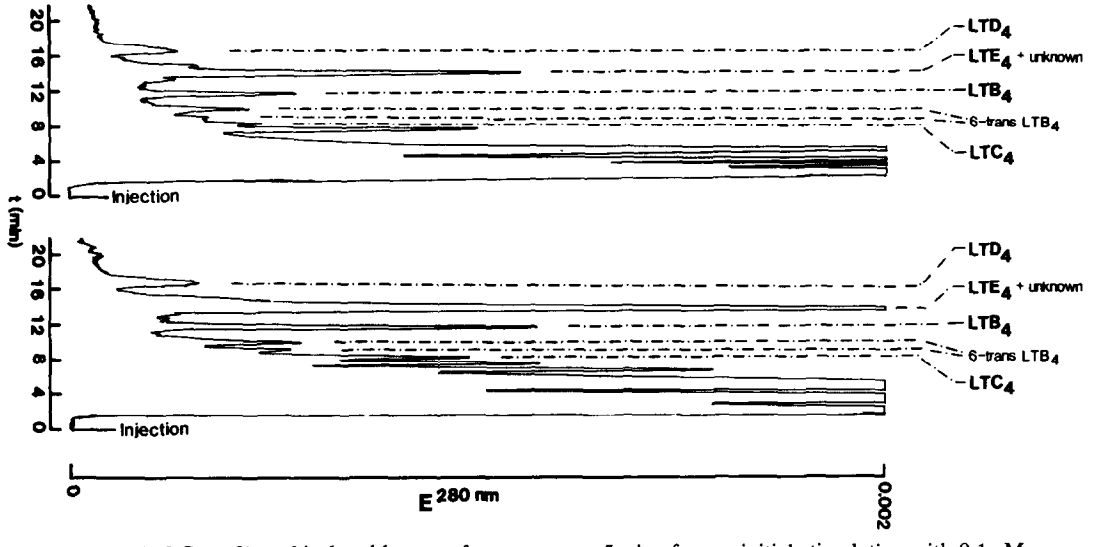

Fig. 1. HPLC profiles of isolated lung perfusate extracts 5 min after an initial stimulation with 0.1 μM A23187 (lower panel) and 5 min after a repeated A23187 challenge in presence of 10^{-8} M AA-861 in the same lung (upper panel). The elution times of standard LTs are indicated in boldface. The argument for the identity of the two small peaks between LTC4 and LTB4 representing the 6-*trans* LTB4 diastereomeric pair as well as the possible co-elution of an unknown substance with LTE4 is given in the text.

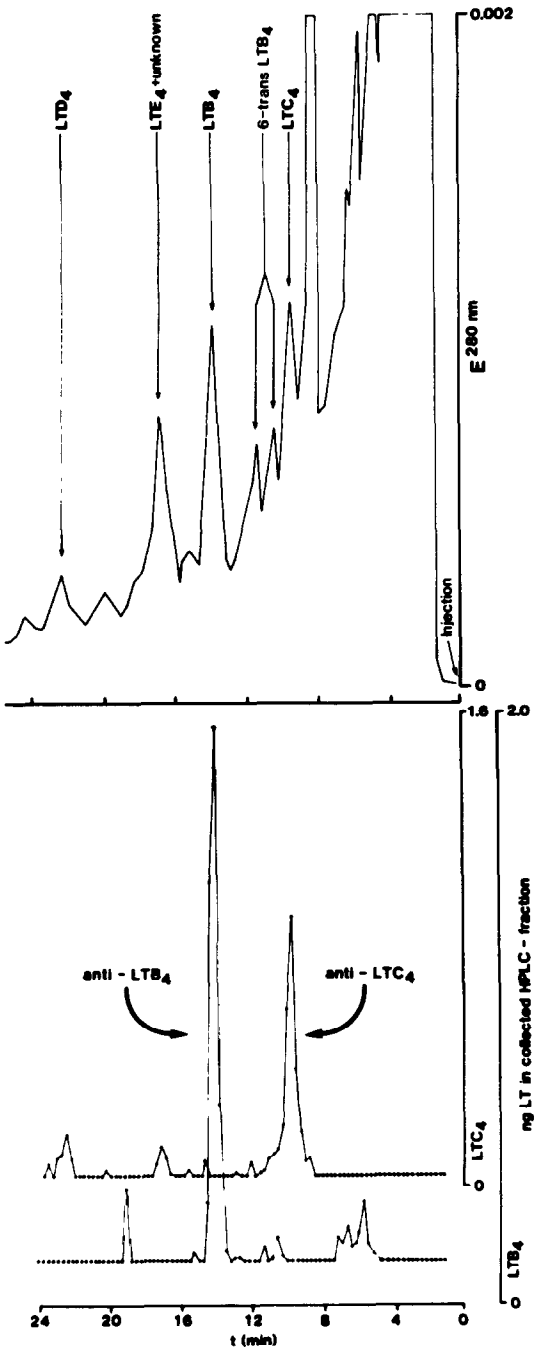

Fig. 2. Upper panel: HPLC profile of a lung perfusate extract 5 min after a challenge with 0.1 μM A23187. The retention times of LT standards, as well as the suggested identification of 6-*trans* LTB4 isomers, are indicated on the top of the panel. In comparison to Fig. 1, the time scale has been expanded 3:1 and the retention times of all LTs were shifted to higher values due to the use of a HPLC mobile phase with slightly higher water content. Lower panel: Immunoreactivity of the HPLC eluate corresponding to the above depicted chromatogram with LTB4 and LTC4 antibodies. The eluate was collected in 15 sec fractions between 1 and 24 min, evaporated under reduced pressure and reconstituted in 250 μl RIA buffer, of which separate 100 μl aliquots were taken for serological determination of the LTs. The concentration of LT is expressed in ng per eluate fraction.

For this reason the peak areas with LTE4 retention times from lung perfusate extracts were not quantified in the present study (except as a basis of comparison in two kinetic experiments, Fig. 5). Immunoreactive material found with anti-LTB4 between 4.9 and 7.5 min as well as a single peak fraction at 19.0 min was not identified. The polar material cannot be 20-OH-LTB4, since this metabolite has a retention time of 4.5 min. The large front consisting of polar compounds in the chromatograms of lung perfusate extracts excluded the detection of 20-OH-LTB4. In all perfusate extracts of stimulated as well as non-stimulated lungs, but not of perfusate circulated in absence of lung, there was an additional large peak eluting shortly before LTC4 (full scale peak at 8.7 min, Fig. 2), which had no immunoreactivity with either anti-LTC4 or anti-LTB4 and which was not further investigated.

In two time course experiments, 5 ml samples were collected every 5 min up to 35 min after a solitary stimulation with 0.1 μM A23187, revealing a rapid formation of both LTB4 and LTC4 within the initial 5 min, with a subsequent slight further increase of the circulating LTB4 concentration and a downward trend of LTC4 concentration after having reached a maximum value at 20 min (Fig. 5). The possibility of an unknown substance co-eluting with LTE4 in HPLC not excluded, the material coeluting with LTE4 retention time showed a much higher level compared to LTC4 and LTB4 and it continued to rise in concentration throughout the 35 min period (depicted as "LTE4" in Fig. 5).

Upon repetition of the 0.1 μM A23187 stimulation subsequent to rinsing phases with fresh KHB, release of leukotrienes into the recirculating perfusion fluid could be reproduced with increasing concentration as depicted for the first to fourth repetition of A23187 challenge in Fig. 3. The maximal release of the peptidoleukotrienes LTC4 and LTD4 occurred after the third stimulation (second repetition), whereby the increase in LTB4 synthesis was more subdued, reaching a maximum of 140% of its concentration after initial stimulation upon the second stimulation (first repetition).

In control experiments without application of A23187, the levels of LTB4, -C4, -D4 and of the material coeluting with LTE4 ranged below the HPLC detection limit. Collection of HPLC eluate corresponding to LTB4 and LTC4 standard retention times with performance of RIA detected 21 ± 4 pg LTB4 and 25 ± 7 pg LTC4 per ml KHB (mean ± S.E.M. of 5, 5 and 7 perfusion phases of three different isolated lungs, measured 12 min after exchanging perfusion fluid).

Effect of cyclooxygenase inhibitor acetylsalicylic acid

Application of water soluble ASA in two experiments caused a dose-dependent inhibition of the release of the cyclooxygenase products TxB2 and 6-keto PGF1α as well as a reduction of the pulmonary artery pressor response (Fig. 6a). The release of LTC4 and LTD4 was not inhibited, whereas the LTB4 level was slightly depressed to 70% of the initial value upon the third repetition of A23187 challenge in presence of 10^{-4} M ASA.

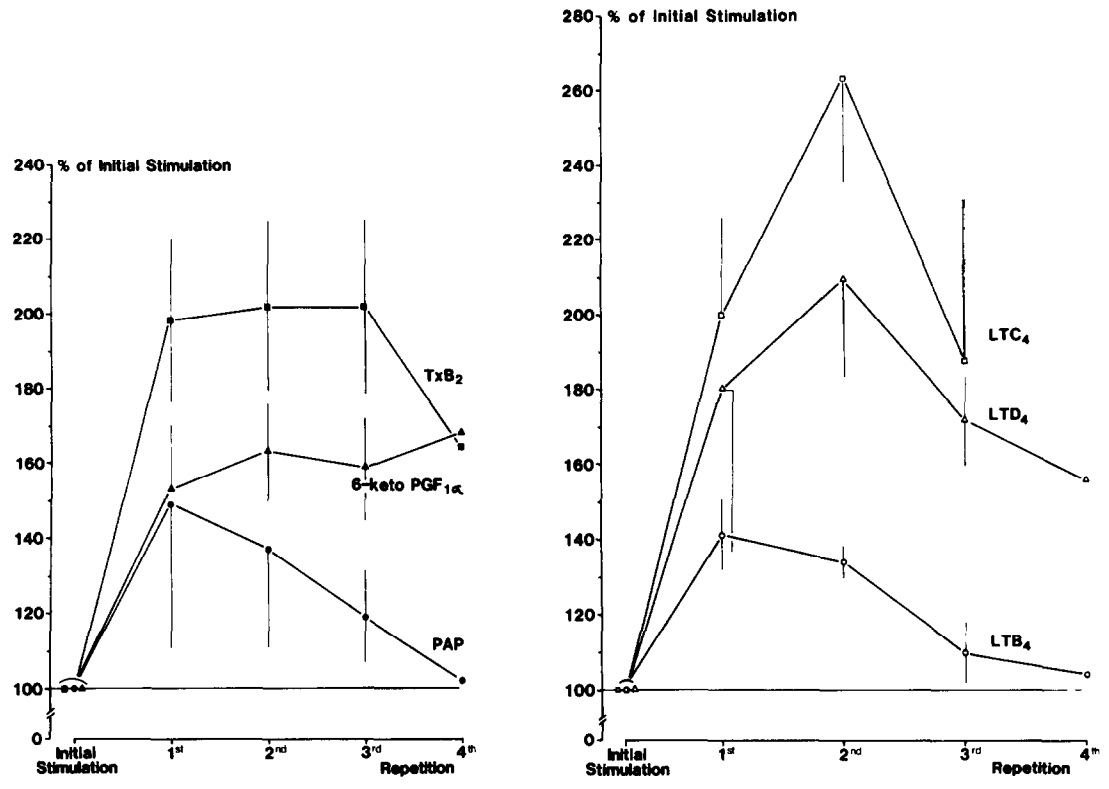

Fig. 3. Pulmonary artery pressor (PAP) response and release of TXB2 and 6-keto PGFlα (left panel) as well as release of LTs C4, D4 and B4 (right panel) provoked by the first to fourth repetition of the A23187 challenge (0.1 μM) in relation to the initial A23187 stimulation in the same isolated lung. For calculation of the relative values the height of the pulmonary artery pressure peak as well as the maximum concentrations of TxB2, 6-keto PGFlα or LTs are taken. The absolute values of the initial A23187 challenge are given in Table 1. The values of LTD4 and LTB4 are derived from their HPLC areas, whereas the LTC4 values are calculated from the RIA of the collected LTC4 peaks. The figures show means ± S.E.M. of 4 independent experiments (first to third repetition) or two independent experiments (fourth repetition, except for LTC4).

Dose-dependent inhibition of leukotriene release by 5-lipoxygenase inhibition

The 5-lipoxygenase inhibitor AA-861 caused a dose-dependent inhibition of the release of all LTs (Fig. 6b): compared to the first repetition of A23187 challenge in the control experiments (Fig. 3), the concentration of 10^{-8} to 10^{-7} M AA-861 caused an inhibition of LT release of about 50%, and in presence of 10^{-5} M AA-861 all LT values ranged below 5% of the initial value. The release of TxB2 and 6-keto PGFLα was only slightly depressed by the highest AA-861 dose, whereas the pulmonary artery pressor response is markedly reduced in this concentration. A similar inhibition profile was achieved with U-60,257, albeit at higher concentration. Figure 6c shows the dose-dependent suppression of LT release with approximately 50% inhibition at 10^{-6} M (compared to the first repetition in Fig. 3) without influence on the release of either TxB2 or 6-keto PGFlα but with significant suppression of the pulmonary artery pressor response at 10^{-5} M U-60,257. At this concentration the LT release ranged between 15 and 40% of the initial values.

DISCUSSION

There are several lines of evidence to support the finding that peptidoleukotrienes as well as LTB4 are released into the recirculating perfusion fluid upon A23187 challenge of isolated rabbit lungs: (1) In all such experiments the HPLC profile of lung perfusate extracts showed peaks with the retention times of standard LTC4, -D4 and -E4 as well as LTB4. (2) The major peaks of immunoreactivity of the fractionated HPLC eluate with anti-LTC4 and anti-LTB4 are superimposeable with the LTC4 and LTB4 peaks of the corresponding chromatogram. According to the stated cross-reactivities of the LTC4 antibody, additional immunoreactive material was detected in the fractions collected from the peaks having LTD4 and LTE4 retention times. (3) There is a good correlation between the amounts of LTC4 and LTB4 calculated from their HPLC areas and the amounts detected in the HPLC eluate fraction by RIA. (4) Inhibition of the cyclooxygenase pathway of arachidonate metabolism did not suppress peptidoleukotriene release, although it did reduce LTB4 production. (5) Release of all LTs was suppressed through 5-lipoxygenase inhibition by two structurally unrelated agents, AA-861 and U-60,257. Additionally there is a good reason to look upon the two minor peaks eluting before LTB4 as the 6-*trans* LTB4 diastereomeric pair, as they are also "recognised" to a minor degree by the LTB4 RIA, compatible with

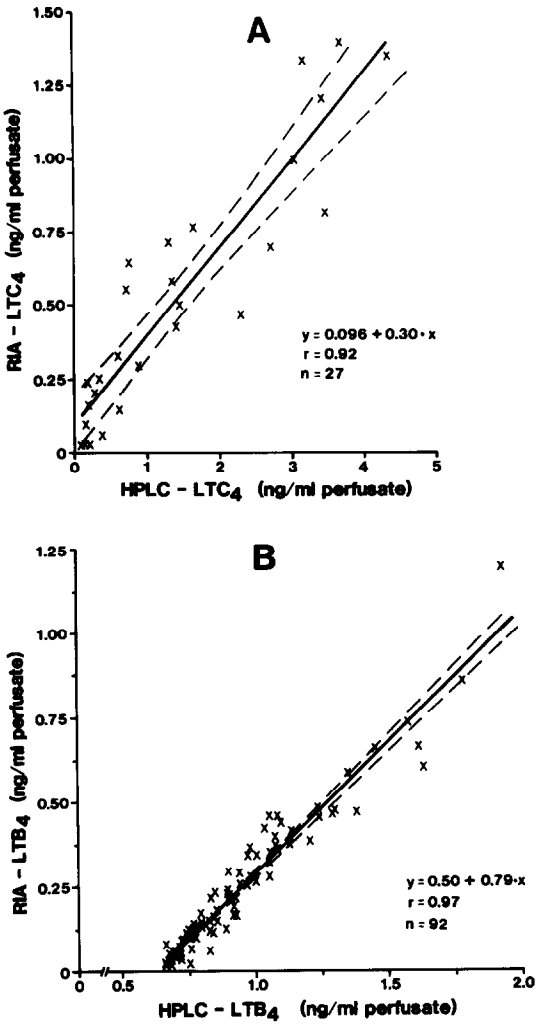

Fig. 4. Linear regression analyses between HPLC measured perfusate LTC4 (A) and LTB4 (B) concentrations and those values determined radioimmunologically from the corresponding HPLC peak eluates with LTC4- and LTB4-antibodies. The individual values represent probes originating from various perfusion phases of A23187 challenged lungs with and without application of inhibitors. For LTC4, HPLC quantification was not possible in all experiments due to insufficient resolution from an unknown peak eluting shortly beforehand (off-scale peak at 8.7 min in Fig. 2). Therefore only data from 8 isolated lung experiments with good peak resolution are included. LTB4 values from 18 experiments are included. The slopes of the linear regression curves, 0.79 for LTB4 and 0.30 for LTC4, are comparable to the recoveries of 0.80 and 0.43 of these LTs in the applied peak collection procedure (see Methods).

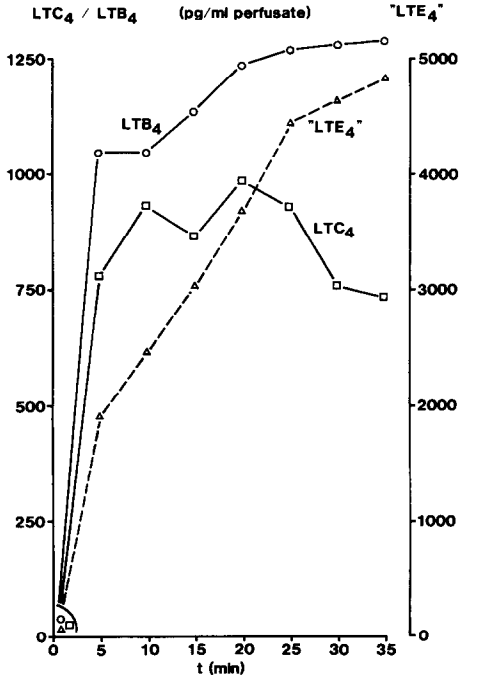

Fig. 5. Time course of LT formation in the recirculating perfusion fluid after a solitary stimulation with 0.1 μM A23187 without exchange of perfusion fluid over 35 min (mean of two independent experiments). LTB4 concentration was derived from its HPLC peak area, whereas LTC4 concentration was calculated from the RIA of the collected peak eluate, corrected for the LTC4 loss in the peak collection protocol. LTD4 was beneath the HPLC detection limit due to the 5 ml collection volume used, necessary in terms of the restrictive minimum perfusion volume and the number of samples taken within the single stimulation phase. The "LTE4" curve (note separate scale to the right) represents all HPLC peak material coeluting with standard LTE4, which possibly includes a co-eluting unknown substance (see Results).

the stated cross-reactivity of the antibody used and as they have identical retention times as material collected from A23187 stimulated human granulocytes, which revealed a typical ultraviolet spectrum. Conclusive evidence, however, would demand an isolation of the material originating from the isolated lung and comparison with the chemical properties of authentic reference compounds.

The concentrations of the peptidoleukotrienes and of LTB4 released into the perfusion fluid of the A23187 challenged isolated rabbit lungs are in the same range as those of TxB2 and 6-keto PGF1α. Calculated on the basis of wet lung weight (ww), the average amount measured 10 min after 0.1 μM A23187 challenge was 290 ng/g ww LTC4 and 250 ng/g ww LTB4. Though not exactly quantifiable due to coelution with unknown material, the amounts of LTE4 appear to range at even higher levels. In two previous studies with detection of LTD4 [24] as well as LTC4 and LTD4 [25] in the collected effluent of isolated lungs, no quantifiable values were given. Experiments with chopped lung tissue with either A23187 or antigen challenge are reported to deliver less than 2.5 ng LTD4/g ww [17], 13–50 ng/g ww of each peptidoleukotriene as well as 108–188 ng/g ww of LTB4 [16], 130 ng/g ww LT-like material measured by bioassay [15] and about 600–2000 ng/g ww of peptidoleukotrienes measured together with a group specific RIA [18].

Compared to this wide range of LT release from chopped lung *in vitro*, the amounts of LTs released into the perfusion fluid of the A23187 challenged isolated rabbit lungs are impressively high. This is especially true, as the A23187 concentration

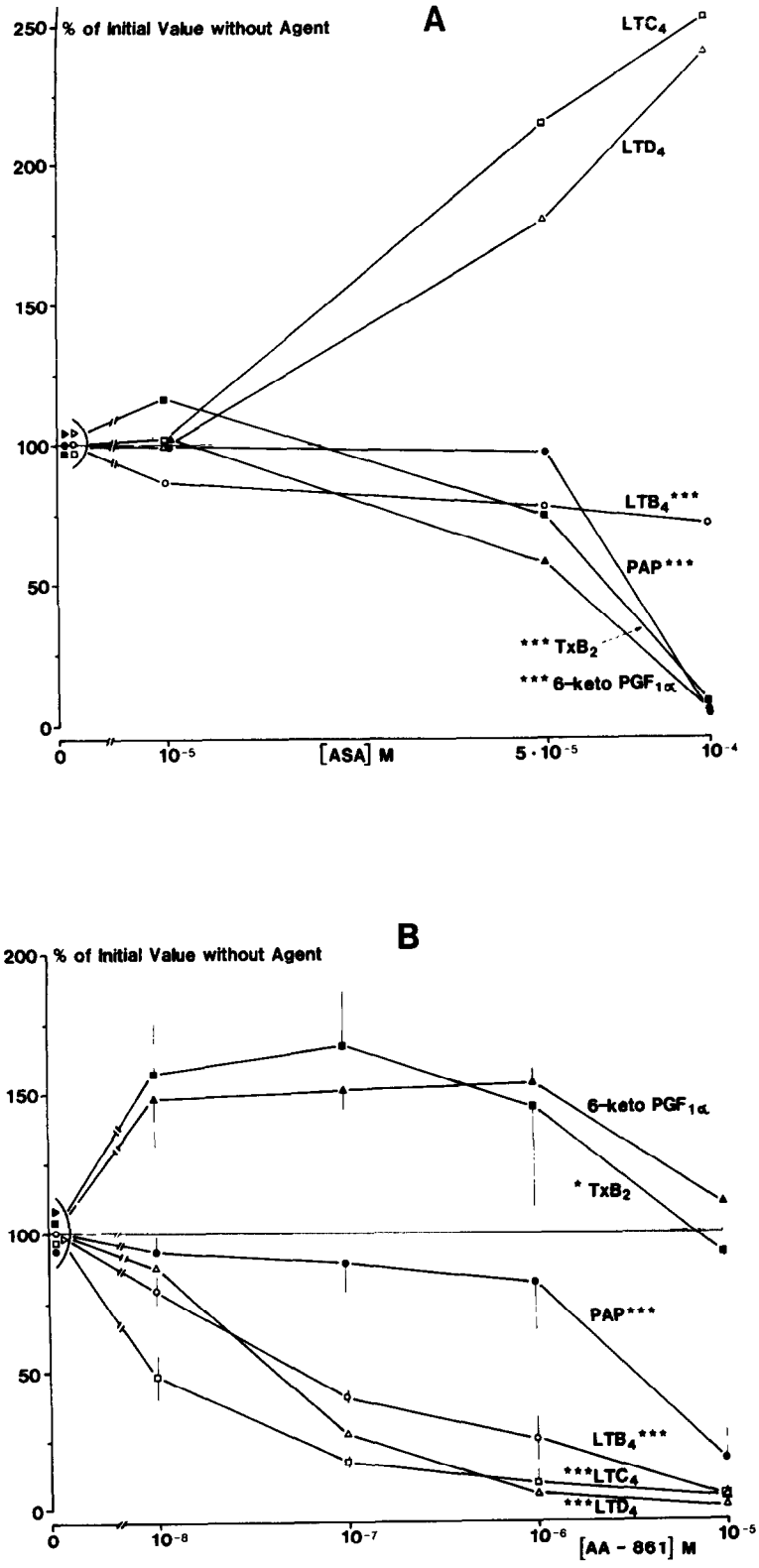

Fig. 6 (*continued*).

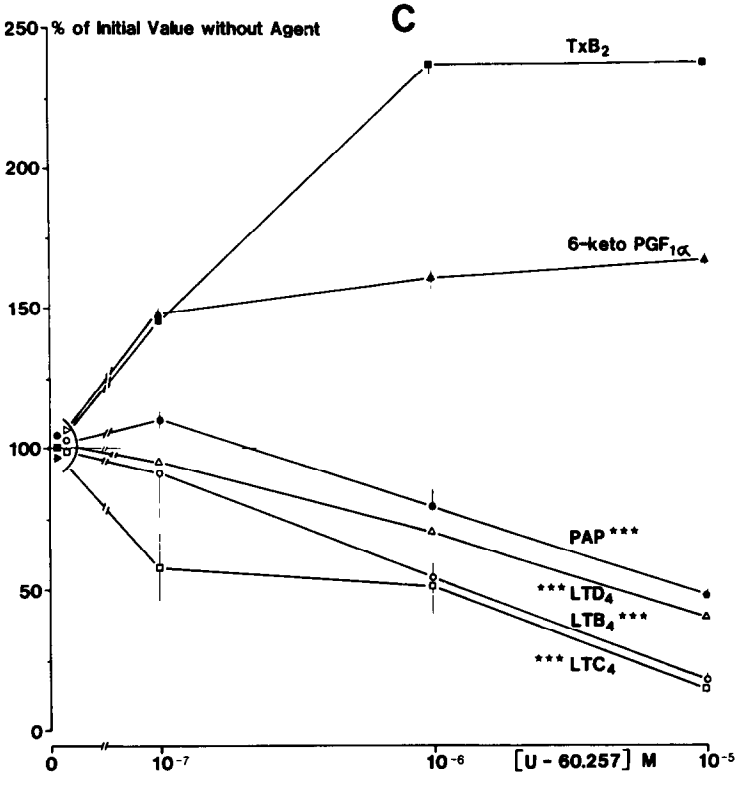

Fig. 6. Pulmonary artery pressor (PAP) response and release of TxB2 and 6-keto PGFlα as well as LTC4, -D4 and -B4 provoked by the first to third or fourth repetition of A23187 challenge in presence of rising concentrations of (A) ASA, (B) AA-861 and (C) U-60,257. For calculation of the values, relative to the initial stimulation in the same isolated lung in absence of inhibitor, the height of the pulmonary artery pressure peak as well as the maximum levels of TxB2, 6-keto PGFlα and LTs are taken. The values of LTD4 and LTB4 are derived from the HPLC areas, whereas the LTC4 values are calculated from the RIA of the collected LTC4 peaks. The values represent the mean (±S.E.M.) of four (AA-861 and U-60,257) or two (ASA) independent experiments. Concerning LTD4 only two experiments with AA-861 and two with U-60,257 delivered enough LTD4 material to allow the calculation of peak areas in presence of increasing inhibitor concentrations. The curves for each parameter in presence of the different inhibitors were compared to the values provoked by repeated A23187 challenge in absence of inhibitors (Fig. 3) by two-way analysis of variance: * $P < 0.05$; *** $P < 0.001$.

(0.1 μM) ranged more than one order of magnitude below that in the previously reported studies and as the LT release could be reproduced several times within the same isolated lung after 10 min rinsing phases, which suggests that the A23187 dosage used was still submaximal. The rate of LTC4- and LTB4-release after injection of the stimulus was very rapid in the present study, levelling off already 5 min after application, whereas in studies with minced pulmonary tissue maximum peptido-LT production was found to occur between 45–60 min of incubation with 2–6 μM A23187 [18].

The cellular origin of the AA metabolites detected in the perfusion fluid of the isolated lung model has not been established. Endothelial cells are very likely candidates for the noted PG12 release. As the lungs are completely free of sticking granulocytes and sticking thrombocytes due to the many-fold perfusion phases before initial A23187 challenge, lung parenchymal cells such as macrophages [21–23] or mast cells [33] have to be considered as the cellular origin of TxA2, sulfopeptide leukotrienes and LTB4. Besides being a site for LT production, the lung is known to metabolize sulfidopeptide leukotrienes: LTC4 is metabolized via LTD4 to LTE4 in lung homogenate [17, 34], and in isolated rat lungs [35], whereas LTE4 and LTB4 are recovered unaltered. In the experiments with isolated rat lungs the main metabolite of radiolabelled LTC4 admixed to the perfusion fluid for 10 min was LTE4 (24% of radioactivity) with additional detection LTD4 (7%) and LTC4 sulfoxide (6%) [35]. This is in accordance to the present study, in which a plateau with slight further increase of LTB4, a slight decrease of LTC4 after a maximum at 20 min and a steady increase of material presumed to contain LTE4 over 35 min was found.

Due to the reproducibility of the A23187 challenged LT release in the same isolated lung, the detection of these AA metabolites in the recirculating perfusion fluid offers itself for pharmacological studies of inhibitors. In this study the

5-lipoxygenase inhibitor AA-861 [36] dose-dependently suppressed the release of all LTs with 50% inhibition in the concentration range of 10^{-8} to 10^{-7} M with nearly complete inhibition at 10^{-5} M. This is in accordance with studies showing the reduction of biologically detected SRS-A release from guinea pig lungs by AA-861, however with 50% inhibition between 10^{-6} M to 3×10^{-5} M [37, 38]. The structurally unrelated 5-lipoxygenase inhibitor U-60,257 [39], reported to suppress the LT release from chopped lung of two human asthmatics at 10^{-4} M [16], showed the same inhibitory profile as AA-861 in the present study with approximately 50% inhibition at 10^{-6} M.

TxA2 has been shown to be the major vasoconstrictive cyclooxygenase product from isolated rat, guinea pig and rabbit lungs [7, 26, 40–43]. Studies with cyclooxygenase inhibitors (as with ASA in this study) and thromboxane synthetase inhibitors [7, 12, 26] as well as with the thromboxane receptor antagonist BM 13.177,* gave strong evidence that the A23187 induced pulmonary artery pressor response is predominantly mediated via TxA2, which could be mimicked by the stable thromboxane analogue U-46619 [26]. Additionally, exogenously applied sulfidopeptide leukotrienes have been found to increase pulmonary vascular resistance [6, 8, 44]. In rabbit [6] and sheep [43] lungs, in contrast to rat lungs [8], this effect appears to be again mediated via the generation of cyclooxygenase products. In the present study both 5-lipoxygenase inhibitors reduced the pulmonary artery pressor response after A23187 challenge to 48% (U-60-257) and 17% (AA-861) at their maximum concentration of 10 M. This inhibitory effect might again give evidence of complex synergistic mechanisms of LTs and AA-cyclooxygenase products in rising pulmonary vascular resistance, among which LT induced thromboxane generation may be one aspect. Further elucidation of this interaction, using tools such as PG-, Tx- and LT-antagonists, will be necessary.

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