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Epoxyeicosatrienoates are the dominant eicosanoids in human lungs upon microbial challenge

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Short Title: Eicosanoids in human lungs

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ABSTRACT: Lipoxygenase, cyclooxygenase, and cytochrome P450 (CYP) products of arachidonic acid (AA) are implicated in pulmonary vasoregulation. The CYP-mediated epoxyeicosatrienoates (EETs) were previously described as the predominant eicosanoids in human lungs upon stimulation with the Ca^{2+} ionophore A23187 (L. Kiss et al., 2000). We now challenged perfused human lungs with two microbial agents - *Escherichia coli* hemolysin (ECH) and formyl-methionyl-leucyl-phenylalanin (fMLP).

Both stimuli elicited pronounced generation of leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), prostanoids (PTs), and EETs/dihydroxyeicosatrienoic acids (DHETs) as assessed by liquid chromatography-mass spectrometry, paralleled by pulmonary artery pressor response and lung edema formation. The maximum buffer concentrations of EETs/DHETs surpassed those of LTs + HETEs + PTs by a factor of four (ECH) or three (AA/fMLP). Dual 5-lipoxygenase/cyclooxygenase inhibition caused pronounced reduction of AA/fMLP-induced LT/PT synthesis and edema formation but only limited attenuation of pulmonary vasoconstriction, while inhibition of CYP epoxygenase clearly attenuated AA/fMLP-induced EET/DHET synthesis and vasoconstriction but not edema formation, thus suggesting a major contribution of LTs/PTs to vascular leakage and of EETs/DHETs to pressor response.

Consequently, generation of EETs/DHETs > (LTs + HETEs +PTs) in *ex-vivo* perfused human lungs upon microbial challenge suggests a substantial contribution of these mediators to inflammatory-infectious pulmonary injury.

KEYWORDS: cyclooxygenase; cytochrome P450; eicosanoids; human lung; lipoxygenase; soluble epoxyde hydrolase.

The lung vasculature is known to be a rich source of lipid mediator biosynthesis from arachidonic acid (AA) via the enzymatic pathways of the AA cascade - lipoxygenase (LOX), cytochrome P450 (CYP), soluble epoxide hydrolase (sEH) and cyclooxygenase (COX) [1-12]. The different eicosanoids encompass the LOX-mediated leukotrienes (LTs) and lipoxins (LXs), the CYP-mediated epoxyeicosatrienoic acids (epoxyeicosatrienoates, EETs), the sEH-mediated dihydroxyeicosatrienoic acids (DHETs), the COX-mediated prostanoids (PTs) (prostaglandins (PGs) and thromboxanes (TXs)) and the *cis-trans* conjugated hydroxyeicosatetraenoic acids (HETEs) mediated by LOX, CYP or COX (overview in Fig. 1). They display a broad array of biological functions, spanning from proinflammatory and vasoconstrictive to antiinflammatory and vasodilatative properties [1, 4, 5, 11].

LTs are implicated in a variety of vascular abnormalities occurring under conditions of lung inflammation, such as pre- and postcapillary vasoconstriction and vascular leakage (cysteinyl-LTs) as well as leukocyte chemotaxis and activation (LTB_4), while HETEs may additionally affect leukocyte kinetics and lung fluid homeostasis [5, 6, 13]. Studies with neutrophils (PMNLs) in vitro and with buffer-perfused rabbit lungs, known to harbor large quantities of resident neutrophils,

monocytes and lymphocytes even after extensive rinsing [14, 15], demonstrated high yield LT and HETE formation upon simultaneous application of free AA and inflammatory ligands such as fMLP or the bacterial toxin *E. coli* hemolysin [8, 16, 17]. This abundant mediator generation is a result of substantial AA liberation at sites of inflammatory events and leukocyte-endothelial cooperation in LT synthesis, including i) a transfer of the unstable LT intermediate LTA_4 from PMNL feeder cells to acceptor endothelial cells (ECs) with subsequent employment of the enzymatic equipment of the latter for transformation into LTs, and ii) release of free AA from ECs with uptake and processing of this precursor by the adjacent PMNLs [3, 16-21].

The ubiquitous, as yet poorly investigated six HETE regioisomers display varying biological activities reaching from anti(15-HETE)- to proinflammatory (5-HETE) properties, including chemotaxis (5-HETE, 11-HETE and 12-HETE) and regulation of the enzymatic pathways of the AA cascade [1, 5, 13, 22, 23].

The physiological properties of the well-investigated PTs reach from vasoconstrictive (TXA_2, $PGF_{2\alpha}$) to vasodilatative (PGI_2, PGD_2, PGE_2) functions [1, 5]. TXA_2 and PGI_2 represent a classical example of an antagonistic pair of mediators: vasocontriction and the thereof resulting mismatch between pulmonary perfusion and ventilation provoked by TXA_2 is counteracted by prostacyclin (PGI_2). Likewise, the adhesive and aggregatory actions of TXA_2 are counteracted by the anti-adhesive and anti-aggregatory PGI_2.

Among the AA-derived lipid mediators, the CYP-mediated EETs and their metabolites, the sEH-mediated DHETs, with reportedly contradictory vasoregulative functions are of particular interest. They are described as both vasodilators and vasoconstrictors acting by themselves, via metabolization by COX or by inducing the expression of COX and thus the enhanced production of prostanoids [11, 24-26]. In contrast to the systemic cardiovascular compartment, where EETs/DHETs were proposed and discussed since over a decade as chemical correlates of the vasodilatative endothelium-derived hyperpolarizing factor (EDHF) [27-30], the role of these CYP/sEH-mediated autacoids in the pulmonary circulation is poorly investigated. Interestingly, first reports describe them as both vasoconstrictors and vasodilators [11, 24] and, as previously described by our group [10], inflammatory challenge of *human* lungs with the non-physiological Ca^{2+} ionophore A23187 evoked the enhanced release of a broad array of eicosanoids, with the CYP-mediated EETs being the by far predominant mediators, paralleled by pulmonary arterial pressure rise (ΔPAP) and lung weight gain (ΔW).

We now extended this approach by asking whether the challenge of human lungs with pathophysiologically relevant microbial stimuli, like the proteinaceous transmembrane pore-forming exotoxin *Escherichia coli* hemolysin (ECH) per se and the inflammatory and chemotactic bacterial receptor-operated tripeptide formyl-methionyl-leucyl-phenylalanin (fMLP) administered in the presence of exogenous AA availability, might elicit a similar eicosanoid profile as A23187, and whether such metabolic response is linked with vasoconstrictor response and edema formation in the human pulmonary compartment. Studies were performed with isolated perfused and ventilated intact human lungs, employing a combined technique of multistep solid-phase extraction

(SPE) of buffer fluid followed by isocratic capillary liquid chromatography (CapLC) with dual online photodiode array (PDA) and electrospray ionization (ESI) tandem mass-spectrometric (MS^2) detection for simultaneous assessment of all LOX-, COX-, CYP-, and sEH-mediated representatives of the AA cascade [31]. Comprehensive eicosanoid profiling with this CapLC-PDAD-ESI-MS^2 method revealed the release of virtually all main representatives of the AA cascade in response to both microbial challenges, with the absolute predominance (by a factor of 3-4) of the CYP/sEH-mediated EETs/DHETs over the other lipid mediators. This mediator liberation profile was paralleled by an pulmonary arterial vasoconstrictor response, to which the CYP epoxygenase pathway may particularly contribute, and lung weight gain, which may be mainly a result of LOX and COX activity.

MATERIALS AND METHODS

Materials

AA, 20-COOH-LTB_4, 20-HO-LTB_4, LTC_4, LTD_4, LTE_4, PGB_1, 6t-LTB_4, 6t,12e-LTB_4, LTB_4, LXA_4, LXB_4, 5S,6R-DiHETE, 5S,6S-DiHETE, 15-, 11-, 8-, 12-, 9-, and 5-HETE, were purchased from Biomol GmbH (Hamburg, Germany). 14,15-, 11,12-, 8,9-, 5,6-EET, 14,15-, 11,12-, 8,9-, 5,6-DHET and 5,6-DHET δ-lactone were supplied by Biomol and by Cayman Chemical Company (Ann Arbor, MI, USA). 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, TXB_2 and stearidonic acid (SDA) were obtained from Cayman. fMLP, butylated hydroxytoluene (BHT), diethyl amine (DEA) and tri-ethyl amine (TEA) were obtained from Sigma Chemie GmbH (Munich, Germany). The 5-LOX inhibitor MK886, the COX inhibitor diclofenac and the CYP epoxygenase inhibitor N-methyl-sulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH) were from Cayman. Ethylenediamine-tetraacetic acid disodium salt dihydrate (EDTA $Na_2 \bullet 2H_2O$), tetrasodium salt tetrahydrate (EDTA $Na_4 \bullet 4H_2O$) and formic acid (FA) were purchased from E. Merck (Darmstadt, Germany). Water was purified with a MILLI-Q system (Millipore, Eschborn, Germany). Methanol was delivered by Burdick & Jackson (Muskegon, MI, USA), acetonitrile was purchased from J. T. Baker (Deventer, The Netherlands) and isopropyl alcohol was obtained from Fluka AG (Buchs, Switzerland). KREBS-HENSELEIT-buffer (KHB) was delivered by Serag Wiessner (Naila, Germany).

Preparation of ECH

The exotoxin was kindly provided by Dr. Sucharit Bhakdi, Mainz, being prepared as previously described [32]. The endotoxin content of the preparation was reduced to ~3 ng LPS/μg protein. The hemolytic titer was assessed directly before use and is expressed in hemolytic units (HU) per milliliter; 1 HU/mL corresponded to ~100 ng protein/mL.

Ex-vivo human and rabbit lung perfusion and experimental protocol

The technique of ex-vivo lung perfusion and ventilation has been described by our group [33] and was now adapted to whole human lungs originating from patients suffering from bronchial carcinoma and undergoing lung resection. The study was approved by the University Ethics Committee, and informed consent was obtained from each patient prior to surgery. The vascular bed of the resected lungs was immediately and extensively rinsed with KHB. The carcinoma area was clamped, for washout of blood the perfusate was initially not recirculated. The flow was slowly

increased to 400 mL/min. Then lungs were placed in a temperature-equilibrated housing chamber at 37°C, freely suspended from a force transducer for monitoring of lung weight, and ventilated with 5.3% CO_2, 21% O_2, 73.7% N_2 (tidal volume, 500 mL; frequency, 12 min^{-1}; endexpiratory pressure, 4 mm Hg). After a steady state period of 20-30 min, the perfusate was exchanged and the lungs were recirculatingly perfused with a pulsatile flow rate of 600 mL/min (500 mL total volume). 15 min later, 100 hemolytical units (HU, i.e., a sub-cytolitic dose of 0.2 HU/mL) of ECH per se (n=5) or fMLP (20 μM final buffer concentration) in the presence of AA (AA/fMLP, n=5) or alone (control to the AA/fMPL experiments, n=2) was bolus-injected into the pulmonary artery and time was set to zero. AA (10 μM final concentration) was admixed to the buffer 30 sec before bolus injection of fMLP. Time-matched baseline control experiments (n=2) were performed without application of ECH or (AA/)fMLP. In experiments with AA/fMLP challenge under conditions of simultaneous inhibition of 5-LOX and COX (10 μM MK886 and 30 μM diclofenac, respectively, n=2) or inhibition of CYP epoxygenase (10 μM MS-PPOH, n=2), the respective inhibitors were added immediately after starting the recirculating perfusion. 10-mL perfusate samples for eicosanoid analysis were collected from the venous effluent 15 min before (-15 min), immediately prior to (0 min) as well as 2, 3, 5, 10, 15, 20 30, and 45 min after AA/fMLP or ECH challenge. Eicosanoid concentration results were corrected for the progressive mediator concentrating effect resulting from the gradual reduction of the total perfusate volume due to removal of 10-mL samples, by considering the total amount of each eicosanoid measured at each time point as contained in the initial total perfusate volume of 500 mL. In addition, eicosanoid release levels and weight gain were corrected for differences in original lung weights by using one of the lungs as reference lung for normalization of the eicosanoid concentrations and increases in lung weight for the other lungs.

The effect of exogenously supplied EET on pressor response and edema formation was investigated with isolated perfused and ventilated human (n=2) and rabbit (n=5) lungs employing the above described experimental set-up. Rabbit lungs were perfused, as described [33], with a flow rate of 150 mL/min (250 mL total volume) and ventilated with the same gas mixture as human lungs. With both human and rabbit lungs, after the initial 15-min steady state period, 11,12-EET (0.3 μM final concentration) was bolus-injected into the pulmonary artery and time was set to zero.

SPE and CapLC-PDAD-ESI-MS^2 equipment and procedures

Eicosanoids were extracted from lung perfusate by adapting a previous SPE procedure [34] to the extraction of all representative mediators of the AA cascade. Briefly, after collection, all samples were supplemented with constant amounts of PGB_1 and SDA as internal standard (IS) and reference substances for the calculation of the relative retention index (RRI), BHT as antioxidant, EDTA $Na_4 \bullet 4H_2O$, FA and isopropyl alcohol, vortexed, centrifuged and subjected to SPE employing ODS cartridges (CHROMABOND C18ec, 200 mg sorbent mass; Macherey-Nagel, Düren, Germany). The extracted eicosanoids were subjected to CapLC-PDAD-ESI-MS^2 analysis, as described [31], employing a 1100 Series capillary LC unit (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) interfaced with an Esquire 3000+ ion trap mass spectrometer

(Bruker Daltonik GmbH, Bremen, Germany). Separation of autacoids was performed on capillary columns (250 mm x 300 µm = L x i.d.; GROM-SIL 80 ODS-7 pH) from Grom Analytik + HPLC GmbH (Rottenburg-Hailfingen, Germany) employing as mobile phase a mixture of water/acetonitrile/methanol/FA (130:240:80:0.22, v/v/v/v; pH 4.30, adjusted with DEA) at a flow rate of 2.7 µl/min. The eluting compounds were subjected to dual online spectrophotometric and tandem mass-spectrometric analysis in the negative ion mode. Identity and purity of eluting eicosanoids were triple checked: chromatographically by isocratic RRI as well as by dual UV-spectrometric and MS^2 analysis.

Statistics

Analytical data, maximal pulmonary arterial pressure rise (ΔPAP_{max}) and maximal lung weight gain (ΔW_{max}) are expressed as mean ± SEM. Statistical evaluation was performed using one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Overview of lipid mediator generation

Both challenge with ECH and combined application of the precursor fatty acid (AA) and fMLP resulted in the liberation of all representative AA-derived LOX, CYP, sEH, and COX products in the perfused human lungs. The following eicosanoid groups of the AA cascade (Fig. 1) were analysed:

1.) LOX-mediated LTs and LXs: LTB_4, cysteinyl-LTs (LTC_4, LTD_4, LTE_4), ω-oxidation metabolites of LTB_4 (20-HO-LTB_4 and 20-COOH-LTB_4) summarized as ω-ox-LTB_4, LXA_4 and LXB_4;

2.) CYP epoxygenase-mediated oxiranes 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET;

3.) sEH-mediated EET-derived vic-diols 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET;

4.) COX-mediated PTs: 6-keto-$PGF_{1\alpha}$ as an indicator of the unstable prostacyclin (PGI_2), PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB_2 as an indicator of the unstable TXA_2;

5.) LOX-, CYP- or COX- mediated 15-HETE, 12-HETE, 11-HETE, 9-HETE, 8-HETE, 5-HETE.

The measured eicosanoid levels are absolute values at each time point, reflecting the specific dynamic equilibrium between release and re-uptake/breakdown of each mediator and thus, depending on the velocity of the elimination process, they more or less accurately reflect actual liberation. According to the rate of removal after reaching maximal concentration, two types of patterns were observed:

1) Avid elimination (generation/release clearly dominated by re-uptake/breakdown) with levels tending to zero for $LT(B,C,D)_4$, PTs, (5-,8-,9-,12-)HETE, and

2) Minimal elimination (balance between generation/release and re-uptake/breakdown) leading to virtually plateauing levels for LTE_4, ω-ox-LTB_4, 15-HETE, EETs/DHETs.

Mediator release under baseline conditions

In the initial 15-min baseline periods, i.e., in the absence of ECH or AA/fMLP administration, no baseline release of LTs (Figs. 2a and 3a, respectively) and LXs (not shown), but some moderate

liberation of 15-HETE > (8-HETE + 12-HETE), 11-HETE, 9-HETE, 5-HETE was detected (Figs. 2b and 3b). Virtually no baseline release of PTs was registered (Figs. 2c and 3c). Substantial baseline liberation of 8,9-(EET+DHET) and 11,12-(EET+DHET) was noted, in contrast to only moderate release of 5,6-(EET+DHET) and 14,15-(EET+DHET) (Figs. 2d and 3d). In the time-matched baseline controls without inflammatory challenge, moderate liberation of HETEs, more substantial appearance of EETs/DHETs but no LT, PT (Fig. 4a), and LX (not shown) release was registered.

LT and LX release upon ECH challenge

Administration of ECH provoked rapid release of LTB_4, peaking after 10 min, and enhanced liberation with subsequent plateauing of LTE_4. The maximum perfusate level of LTE_4 surpassed that of LTB_4 four times (Fig. 2a). No LTC_4, LTD_4, LXA_4 or LXB_4 was detected. Virtually no (< 1%) hydrolytic decay products (6t-LTB_4, 6t,12e-LTB_4, 5S,6R-DiHETE, 5S,6S-DiHETE) of the unstable LT intermediate LTA_4 were detected.

HETE liberation after ECH challenge

After moderate baseline release, ECH induced peaking kinetics of 8+12-HETE, paralleled by enhanced liberation and subsequent plateauing of 15-HETE, with maximal perfusate concentrations (c_{max} = 6.76 ng/mL and c_{max} = 6.95 ng/mL, respectively) by far higher than those of 5-, 9- and 11-HETE, which displayed kinetics comparable to 8+12-HETE (Fig. 2b).

PT formation elicited by administration of ECH

Administration of ECH provoked the liberation of all PTs with comparable kinetics, at concentrations between ~ 0.5 and ~ 2.5 ng/mL, peaking after 5 min (Fig. 2c).

EET/DHET generation provoked by ECH challenge

ECH elicited the release of all EETs/DHETs, with a rank order of 11,12-(EET+DHET) > 8,9-(EET+DHET) > 14,15-(EET+DHET) > 5,6-(EET+DHET) (Fig. 2d). 11,12-(EET+DHET) and 8,9-(EET+DHET) displayed rapidly increasing liberation profiles reaching by far higher maximal concentrations (c_{max} = 57.90 ng/mL and c_{max} = 37.50 ng/mL, respectively) than 14,15-(EET+DHET) and 5,6-(EET+DHET), and all other eicosanoid classes. The sum of the quantitatively dominating regioisomers 8,9-(EET+DHET) + 11,12-(EET+DHET) surpassed the sum of the minor regioisomers 5,6-(EET+DHET) + 14,15-(EET+DHET) by a factor of 3.0. The total amount of EETs/DHETs surpassed the sum of LTs, HETEs and PTs fourfold (Table 1, Fig. 4a).

Eicosanoid release upon challenge with fMLP alone

In the experiments with fMLP alone, moderate liberation of HETEs, more substantial appearance of EETs/DHETs but no LT, PT (Fig. 4a), and LX (not shown) release was registered.

LT and LX liberation after co-application of AA and fMLP

AA/fMLP provoked the rapid appearance of LTB_4, ω-ox-LTB_4 (20-HO-LTB_4 + 20-COOH-LTB_4) and the cysteinyl-LTs $LT(C,D,E)_4$ in the perfusate (Fig. 3a). In contrast, virtually no (< 1%) non-enzymatic LTA_4 hydrolysis products were detected. Within the total number of LTs, LTC_4 and LTD_4 displayed the highest peak values (10.82 ng/mL and 9.92 ng/mL, respectively, see Table 1),

followed by rapid decline, concomitant with progressive increase and subsequent plateauing of their metabolite LTE_4. In addition, rapid peaking of LTB_4 was noted (maximum values ~ 1/3 as compared to LTC_4) with subsequent plateauing of its ω-oxidation products. The total amount (as measured by $\Sigma\, c_{max}$) of LTs was one order of magnitude above that elicited by application of ECH (Table 1). No LXA_4/LXB_4 was detected.

HETE release elicited by administration of AA/fMLP

Following baseline release, AA/fMLP provoked enhanced release of all HETEs. The highest perfusate concentration was noted for 8-HETE + 12-HETE, peaking after 3 min (c_{max} = 17.07 ng/mL), with subsequent rapid decline. 5-, 9- and 11-HETE displayed kinetics comparable to 8+12-HETE, however, with lower peak values (Fig. 3b). In contrast, enhanced liberation of 15-HETE resulted in markedly increased plateau levels reaching a maximum concentration of 14.06 ng/mL. The total quantity of HETEs was 3 times higher than in ECH-challenged lungs (Table 1).

PT generation after AA/fMLP challenge

Upon exposure to AA/fMLP, PTs showed generation profiles similar to those in ECH-challenged lungs: rapid peaking appearance followed by sustained decline of PGI_2 (measured as the longer-lived 6-keto-$PGF_{1\alpha}$), PGD_2, PGE_2, $PGF_{2\alpha}$, and TXA_2 (measured as the longer-lived TXB_2) was registered (Fig. 3c). The highest peak values were noted for PGI_2 and PGE_2 (4.11 ng/mL and 3.68 ng/mL, respectively). The total PT quantity was 1.8 times higher than upon ECH challenge.

EET/DHET formation elicited by AA/fMLP challenge

AA/fMLP challenge provoked dramatically enhanced and progressive 8,9-(EET+DHET) release (Fig. 3d), displaying by far the highest maximal perfusate concentration of all eicosanoids analyzed in our study (c_{max} = 183.71 ng/mL; Table 1). 11,12-(EET+DHET) showed a similar profile, reaching half the maximal concentration of 8,9-(EET+DHET). In addition, AA/fMLP induced some minor liberation of 14,15-(EET+DHET) and 5,6-(EET+DHET). The sum of the quantitatively major regioisomers 8,9-(EET+DHET) + 11,12-(EET+DHET) surpassed the sum of the minor regioisomers 5,6-(EET+DHET) + 14,15-(EET+DHET) by a factor of 8.5. The total quantity of the EETs/DHETs surpassed the total sum of all LTs, HETEs and PTs threefold (Table 1, Fig. 4a). Notably, the 8,9-(EET+DHET) > 11,12-(EET+DHET) predominance found in response to AA/fMLP stimulation differed from the 11,12-(EET+DHET) > 8,9-(EET+DHET) predominance in response to ECH challenge. The overall amount of EETs/DHETs post AA/fMLP was 2.6 times higher than post ECH.

Eicosanoid release upon AA/fMLP challenge after previous CYP, LOX and COX inhibition

AA/fMLP challenge of human lungs after previous administration of the CYP epoxygenase inhibitor MS-PPOH resulted in a dramatic attenuation of EET/DHET release, while the liberation of the other eicosanoid groups - LTs, HETEs, and PTs - was not significantly affected (Fig. 4a).

Dual inhibition of 5-LOX and COX in AA/fMLP-challenged human lungs with MK886 and diclofenac, respectively, resulted in marked decrease in LT/5-HETE and PT generation (inset in Fig. 4a), while no significant change in the appearance of 8-, 9-, 11-, 12-, and 15-HETE and

EETs/DHETs was registered (not shown).

Pulmonary vascular response provoked by ECH, AA/fMLP and EET challenge

Near constant PAP (9-10 mm Hg) was noted in the initial 15-min baseline periods as well as in the time-matched baseline control experiments and in the control experiments with fMLP alone ($\Delta PAP < 1$ mm Hg, Fig. 4b). Challenge with both AA/fMLP and ECH provoked a pronounced vascular presssor response with ΔPAP_{max} of 5.7 ± 0.47 mm Hg and 3.8 ± 0.32 mm Hg, respectively, accompanied by marked edema formation resulting in a ΔW_{max} of 280.1 ± 22.1 g and 201.1 ± 21.1 g, respectively. In experiments with AA/fMLP-challenged human lungs, simultaneous inhibition of LT and PT synthesis with MK886 and diclofenac, respectively, resulted in marked reduction of ΔW_{max} but only limited attenuation of ΔPAP_{max} as compared to AA/fMLP challenge without inhibitors, while blockade of EET/DHET generation with MS-PPOH was paralleled by substantial attenuation of ΔPAP_{max} but not of ΔW_{max}.

Application of exogenous 11,12-EET to human (n=2) and rabbit (n =5) lungs in approximately the same concentration as that of the endogenously released 11,12-EET (0.3 μM), provoked marked ΔPAP_{max} - 3.1 ± 0.27 mm Hg and 3.4 ± 0.31 mm Hg with human and rabbit lungs, respectively - but no edema formation: $\Delta W < 75$ g with human lungs, i.e., the same range as in the time-matched baseline control experiments (Fig. 4b), and $\Delta W < 1$ g with rabbit lungs (not shown).

DISCUSSION

Previous investigations performed with *non-human* lungs of different species under various pathophysiological conditions, including challenge with A23187, ECH and fMLP [2, 3, 5, 8, 9, 18, 19, 31, 35-42], revealed the generation of *individual* eicosanoids/eicosanoid groups (TXA_2, PGI_2, LTs or HETEs), paralleled by pressor response and increased endothelial permeability. Intriguingly, challenge of *human* lungs with A23187 elicited the generation of *all* main LOX- and CYP-mediated eicosanoids dominated by the CYP-mediated EETs and paralleled by lung injury [10]. In extension to these findings, the present work demonstrates that exposure of perfused *human* lungs to two different, naturally occuring bacterial agents - ECH and fMLP – induces (1) the generation of virtually *all* main lipid mediators of the AA cascade – LOX-mediated LTs, COX-mediated PTs, CYP/sEH-mediated EETs/DHETs, and HETEs mediated by LOX, COX or CYP – paralleled by (2) vasoconstriction and (3) edema formation. In addition, it was (4) noted that the AA/CYP/sEH-derived EETs/DHETs were the quantitatively by far dominant mediator group, apparently substantially contributing to the vasoconstrictor response, but not to the vascular leakage provoked by these agents. On the other hand, (5) the LOX/COX-derived LTs/PTs were noted to have a major contribution to endothelial permeability increase, but causing only limited pulmonary vasoconstriction.

The two bacterial agents employed in the present study initiate eicosanoid generation via different mechanisms, which impose different requirements for *endo-/exogenous* free precursor AA availability, thus having differential effects on the kinetics of eicosanoid biosynthesis, like the absolute dominance of eicosanoid release upon AA/fMLP co-application over that upon ECH challenge:

1) The hydrophilic transmembrane pore-forming ECH *per se* enables passive influx of extra-

cellular Ca^{2+} and thus induces *gradual* phospholipase-dependent release of *endogenous* free precursor AA from cell membrane lipid pools [17, 39] by starting from zero and in *limited* amounts, which are readily converted by COX, LOX and CYP to equally *limited* amounts of eicosanoids.

2) In contrast to ECH, the receptor-operated ligand fMLP *per se* is not capable of major activation of *endogenous* AA-releasing phospholipases. Thus its capability to initiate the AA cascade is essentially dependent on the availability of *exogenous* free precursor AA [16, 43]. As the *whole* amount of *exogenously* supplied free AA is available *at once, before* initiation of the AA cascade by bolus-injection of fMLP, in this case the precursor is converted undelayed and quantitatively to eicosanoids, yielding higher maximal buffer concentrations than upon ECH challenge.

These different action mechanisms/AA availabilities may also explain the apparent lack of the LTC_4 and LTD_4 release patterns upon ECH challenge (Fig. 2a) in contrast to the abundant liberation profiles of all cysteinyl-LTs upon co-application of AA/fMLP (Fig. 3a):

a) In the case of AA/fMLP co-application, due to the availability of the *entire exogenously supplied* precursor AA amount from the *very beginning*, not only the stable accumulating endproduct (characterized by progressive increasing and subsequent plateauing kinetics) LTE_4, but also the short-lived intermediates (illustrated by fast peaking kinetics) LTC_4 and LTD_4 were generated in considerable and thus well detectable amounts (Fig. 3a).

b) In contrast to AA/fMLP, upon ECH challenge, the *endogenous* precursor AA is provided *gradually* via phospholipase- und thus time-dependent hydrolytic release from membrane lipids. Under these conditions of *limited* precursor availability, the fast peaking intermediates LTC_4 and LTD_4 are rapidly further converted at much lower, not detectable concentrations, finally resulting in the stable accumulating endproduct LTE_4 present at higher and thus detectable concentrations (Fig. 2a). Of all eicosanoid classes, the LTs displayed the most pronounced dominance upon AA/fMLP vs. ECH challenge, namely by a factor of ten as compared to “only” 3, 1.8, and 2.6 for HETEs, PTs, and EETs/DHETs, respectively. This may be explained by an additional amplification of the increase in LT generation due to *exogenously* supplied AA by multiple transcellular cooperative biosynthesis mechanisms typical for LTs, comprising a broad array of cell types, as described in the following:

1) The monitored LT profiles are compatible with the finding that:

a) Even after extensive rinsing with buffer fluid, lungs harbor large ”resident” intracapillary pools of different leukocytes, but virtually no platelets [14, 15], and that

b) The various resting pulmonary leukocyte populations - intravascular, interstitial, and alveolar macrophages [44, 45], lung mast cells [46], intravascular granulocytes [3, 18], monocytes [47] and lymphocytes [48] - are known to be involved in the biosynthesis of 5-LOX-derived lipid mediators.

2) The strong potency of the human lung vasculature for LT release in response to microbial challenge together with the lack (< 1%) of the hydrolytic decay products of the unstable LT intermediate LTA_4 is indicative of transcellular cooperative LT formation mechanisms:

a) As 5-LOX is restricted to cells of the myeloid lineage [6], transcellular LT synthesis is initialized in leukocytes, where LTA_4 is in part rapidly converted by LTA_4 hydrolase to the chemotactic LTB_4. The ”surplus” LTA_4, is released from *donor* leukocytes (neutrophils,

monocytes, macrophages etc.) into the extracellular space, from where it is avidly taken up by other *acceptor* leukocytes, e.g., lymphocytes [49] or adjacent pulmonary *acceptor* ECs and converted to LTs, thus completely escaping extracellular degradation to the LTA_4 decay products. The rapid conversion of LTB_4 to the accumulating ω-ox-LTB_4 (Fig. 3a) suggests that LTB_4 generation occured mainly in PMNLs, the only cell type equipped with substantial ω-oxygenase activity [5, 50].

b) In contrast to 5-LOX, the cysteinyl-LT synthesis-mediating glutathion-S-transferase and thus the generation of $LT(C,D,E)_4$ occurs both in some myeloid cells, like mast cells, eosinophils, basophils, and monocytes/macrophages, as well as in ECs, but not in neutrophils. [2, 5, 6, 12]. Consequently, cysteinyl-LTs are generated partly in cells equipped with both 5-LOX and LTC_4-synthase, such as mast cells and macrophages/monocytes [5, 12], but certainly mainly via transcellular cooperative synthesis, including LTA_4 shift from leukocyte donor cells to adjacent acceptor ECs followed by rapid conversion of LTA_4 to cysteinyl-LTs [3, 18-21].

c) Transcellular cooperation in LT biosynthesis includes not only a shift of LTA_4 from leukocyte donor to vascular acceptor cells with subsequent generation of LTs, but also release of free AA from activated ECs followed by uptake and processing of this precursor by adjacent leukocytes [16, 17].

The vasoregulatory and pro-/anti-inflammatory role of the HETEs in the pulmonary vasculature is largely unknown. Interestingly, continuous abundant liberation upon microbial challenge of the anti-inflammatory 15-HETE, previously isolated from human lung [5, 51], was noted. In contrast, only minor liberation of the pro-inflammatory 5-HETE was observed (Table 1, Figs. 2b and 3b).

The vasodilatative PTs PGI_2 (prostacyclin), PGD_2 and PGE_2 dominated quantitatively over the vasoconstrictive PTs TXA_2 and $PGF_{2\alpha}$ (the levels of the strong vasoconstrictor TXA_2 were 3.2-4.4 times lower than those of the potent vasodilator PGI_2) (Table 1, Figs. 2c and 3c) thus suggesting a rather moderate contribution of the vasoconstrictive PTs to the pressor response in the human lung. Although platelets are known as the main source of TXA_2 biosynthesis, in contrast to resident leukocytes, the pool of marginated platelets in blood-free washed and perfused lungs is negligible [14]. Thus, ECs and resident macrophages, monocytes, lymphocytes, and PMNLs, known as sites of extraplatelet TXA_2 synthesis [52, 53, 54], may serve as alternative candidates for extraplatelet thromboxane generation. This may explain why even upon co-application of AA/fMLP, i.e. availability of *exogenous* precursor AA, TXA_2 is the quantitatively absolute minor mediator (Table 1).

The release of large amounts of CYP/sEH-mediated EETs/DHETs in the human lung vasculature upon microbial challenge is intriguing in view of the fact that little is yet known about their vasoregulative role in the lung. Interestingly, the total amount of the 5-LOX-mediated LTs, the LOX-, COX-, or CYP-mediated HETEs and the COX-mediated PTs, already well-known as mediators of pulmonary vasoconstriction, were dominated by a factor of 3-4 by that of the EETs/DHETs (Fig. 4a, Table 1). Taking into account that this absolute dominance of EETs/DHETs over the other lipid mediators of the AA cascade resembles that previously described in human lungs undergoing pulmonary artery pressure rise upon stimulation with A23187 [10] and

considering the few as yet available reports which describe the EETs/DHETs as vasoconstrictors in isolated pressurized rabbit pulmonary arteries [24] and in the hypoxic rabbit and mouse lung [31, 38, 55], the question arises, if the high EET/DHET amounts may have an impact on vascular pressor response and/or edema formation in human lungs upon microbial challenge.

Notably, simultaneous inhibition of 5-LOX and COX in the AA/fMLP-challenged human lung with MK886 and diclofenac, respectively, induced pronounced decrease in LT and PT generation (inset in Fig. 4a) paralleled by marked reduction of weight gain (Fig. 4b), thus suggesting a substantial contribution of LTs and PTs to edema formation. Remarkably, only a limited attenuation of the human lung vascular pressor response to inflammatory challenge was registered (Fig. 4b), although the generation of LTB_4, cysteinyl-LTs, 5-HETE, vasoconstrictive PTs (TXA_2+$PGF_{2\alpha}$) and vasodilatative PTs (PGI_2+PGD_2+PGE_2) was strongly inhibited (to 12%, 23%, 15%, 21%, and 19%, respectively, as compared to AA/fMLP-challenge without 5-LOX/COX inhibition; see inset in Fig. 4a), thus suggesting a possible contribution of the CYP/sEH-derived EETs/DHETs to pulmonary vasoconstriction.

Indeed, application of the selective [56] CYP epoxygenase inhibitor MS-PPOH prior to fMLP/AA challenge, though only undertaken in two experiments due to the shortage of human lungs available for the perfusion experiments, resulted in a considerable reduction of EET/DHET generation (Fig. 4a) accompanied by an attenuation of the pressor response to ~ 50% but not of edema formation (Fig. 4b). These results well correlate with the results obtained by exogenous application of 11,12-EET to human and rabbit lungs at the same final concentration as the maximal concentration of *endogenously* released 11,12-EET: Induction of marked ΔPAP, but no significant ΔW was registered, although 11,12-EET, along with 8,9-EET, belonged to the quantitatively by far dominant regioisomers released in the human lung upon microbial challenge, thus being a potential prime candidate possibly causing edema formation.

These findings are in agreement with first reports [57, 58] describing a regiospecific endothelial permeability-increasing activity of the four EET regioisomers in a concentration range of 3-10μM, i.e., by far surpassing the maximal concentrations of the EETs released in human lungs upon challenge with AA/fMLP (0.04 μM/14,15-(EET+DHET)-0.56 μM/8,9-(EET+DHET), concentrations in μM were converted from the respective values in ng/mL in Table 1), thus suggesting a rather negligible contribution of the EETs to edema formation under these conditions.

Altogether, these data indicate that EETs generated in human lungs under conditions of challenge with bacterial agents elicit pronounced pressor response but no edema formation.

In conclusion, stimulation of intact human lungs with two microbial agents – ECH and fMLP – elicited pulmonary vasoconstriction and vascular leakage, alongside with the liberation of substantial amounts of all representative mediators of the AA cascade (LTs, HETEs, PTs, and EETs/DHETs) into the perfusate. In response to both stimuli, the CYP/sEH-mediated EETs/DHETs clearly dominated over the sum of the other eicosanoid classes. Dual inhibition of LOX/COX in the AA/fMLP-challenged human lung caused pronounced reduction of LT/PT biosynthesis and edema formation but only limited attenuation of pulmonary arterial pressure rise, while selective CYP epoxygenase inhibition induced marked suppression of EET/DHET generation and of the vaso-

constrictor response but not attenuation of edema formation, in accordance with exogenous EET application, which caused pulmonary arterial pressure rise but no lung weight gain. Next to well-known LOX- and COX-mediated pulmonary vascular abnormalities, a major contribution of the CYP/sEH-mediated EETs/DHETs to the development of pulmonary pressor response may thus be expected.

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FIGURE LEGENDS

FIGURE 1. The main pathways of the AA cascade: CYP, LOX, and COX. AA = arachidonic acid; CYP = cytochrome P450; CYPω = cytochrome P450 ω-oxygenase; LOX = lipoxygenase; COX = cyclooxygenase; sEH = soluble epoxide hydrolase; EETs = epoxyeicosatrienoic acids (14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET); DHETs = dihydroxyeicosatrienoic acids (14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, 5,6-DHET δ lactone); HETEs = hydroxyeicosatetraenoic acids (15-HETE, 11-HETE, 8-HETE, 12-HETE, 9-HETE, 5-HETE); LXs = lipoxins (LXA_4, LXB_4); LTs = leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4, ω–ox–LTB_4 = ω–oxygenase-mediated LTB_4 metabolites (20-HO-LTB_4, 20-COOH-LTB_4); PTs = prostanoids (TXs = thromboxanes (TXA_2, TXB_2), PGs = prostaglandins (PGI_2, 6-keto-$PGF_{1\alpha}$, PGE_2, PGD_2, $PGF_{2\alpha}$).

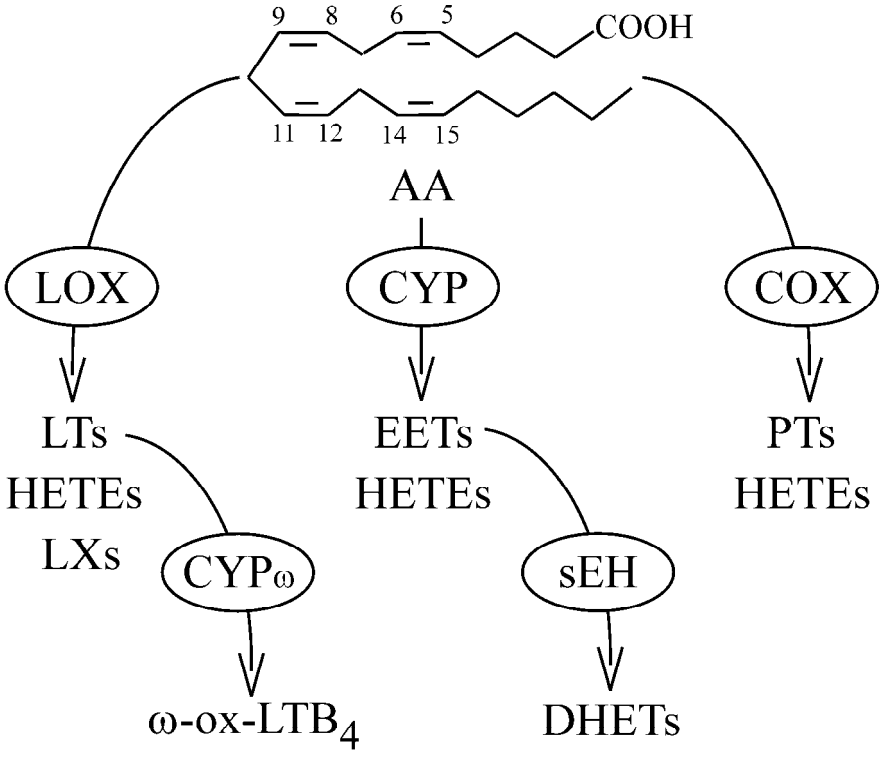

Figure 1

FIGURE 2. Kinetics of a) leukotrienes (LTs), b) hydroxyeicosatetraenoic acids (HETEs), c) prostanoids (PTs = prostaglandins (PGs) and thromboxanes (TXs)), and d) epoxyeicosatrienoic + dihydroxyeicosatrienoic acids (EETs + DHETs) appearing in the buffer fluid of isolated *ex-vivo* perfused and ventilated human lungs stimulated with *E. coli* hemolysin (ECH). After a baseline period of 15 min, 100 hemolytical units (HU) of ECH were bolus-injected into the pulmonary artery (time point zero). 10-mL perfusate samples for eicosanoid analysis were collected from the venous effluent at different time points and subjected to solid-phase extraction (SPE) followed by capillary liquid chromatography with photodiode array and electrospray ionization tandem mass-spectrometric detection (CapLC-PDAD-ESI-MS^2). The measured eicosanoid levels are absolute concentrations at each time point (n=5; mean ± SEM; error bars are missing when falling into the symbol). * PGI_2 and TXA_2 concentrations were calculated from the measured 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations, respectively.

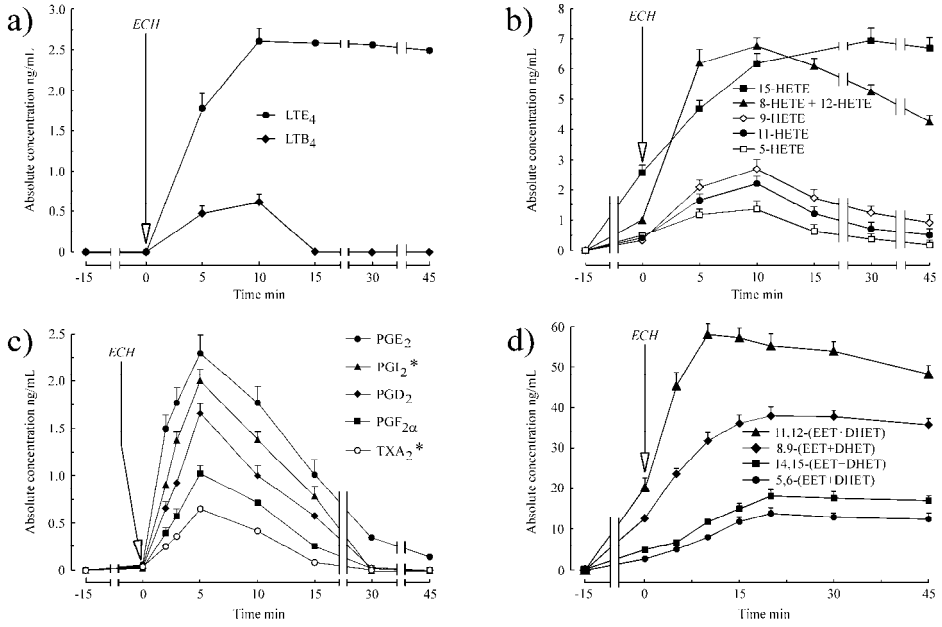

Figure 2

FIGURE 3. Kinetics of a) leukotrienes (LTs), b) hydroxyeicosatetraenoic acids (HETEs), c) prostanoids (PTs = prostaglandins (PGs) and thromboxanes (TXs)), and d) epoxyeicosatrienoic + dihydroxyeicosatrienoic acids (EETs + DHETs) released in the buffer of isolated perfused and ventilated human lungs challenged with arachidonic acid/formyl-methionyl-leucyl-phenylalanin (AA/fMLP). After a 15-min baseline period, lungs were challenged with AA and fMLP (20 µM and 10 µM final concentration, respectively; time point zero). 10-mL perfusate samples were collected from the venous effluent at different time points and subjected to solid-phase extraction (SPE) followed by capillary liquid chromatography with photodiode array and electrospray ionization tandem mass-spectrometric detection (CapLC-PDAD-ESI-MS^2). The measured eicosanoid levels are absolute concentrations at each time point (n=5; mean ± SEM; error bars are missing when falling into the symbol). * PGI_2 and TXA_2 concentrations were calculated from the measured 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations, respectively.

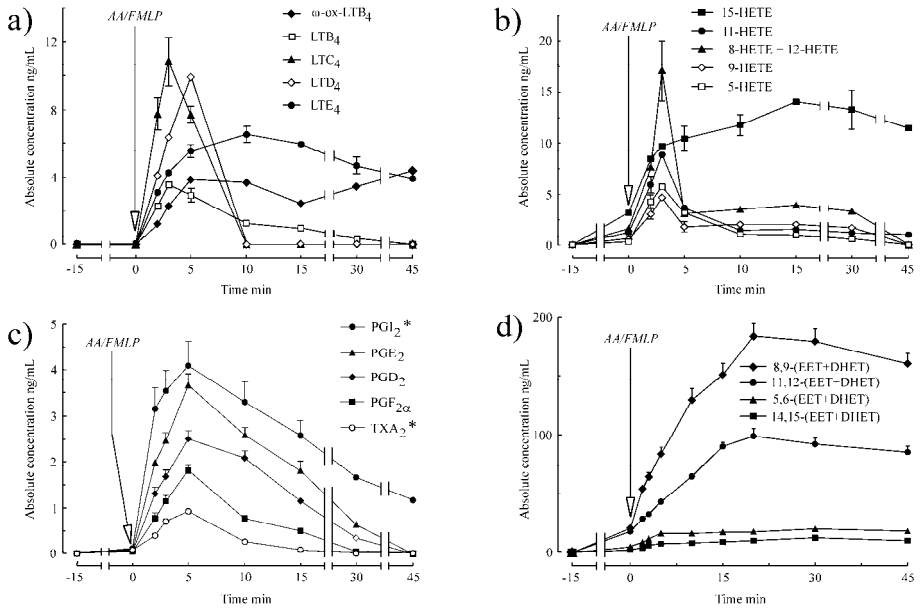

Figure 3

FIGURE 4. Comparison of the a) metabolic (eicosanoid generation) and b) pathophysiological (vasoconstriction and edema formation) response in isolated perfused human lungs under different conditions: challenge with *E. coli* hemolysin (ECH) (n=5), arachidonic acid/formyl-methionyl-leucyl-phenylalanin (AA/fMLP) (n=5), AA/fMLP in the presence of both the lipoxygenase (LOX) inhibitor MK886 and the cyclooxygenase (COX) inhibitor diclofenac (n=2), AA/fMLP in the presence of the selective cytochrome P450 (CYP) epoxygenase inhibitor MS-PPOH (n=2), fMLP alone (n=2), and time-matched baseline control (n=2). a) The generation of the four main lipid mediator classes of the AA cascade – leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), prostanoids (PTs), and epoxyeicosatrienoic + dihydroxyeicosatrienoic acids (EETs+DHETs) - is displayed and represented by the sums of the means of the maximal perfusate concentrations ($\Sigma\ c_{max}$) of the eicosanoids belonging to the respective class. The values reported on top of the bars represent the relative amount of each eicosanoid class (LTs, HETEs, PTs, and EETs+DHETs) in terms of percentages of the respective sum of maximal concentrations as compared to the total amount of eicosanoids generated under the same experimental conditions (challenge with ECH, AA/fMLP, MS-PPOH+AA/fMLP, fMLP alone, or time-matched baseline control, respectively). The sum of percentages under the same experimental conditions amounts to 100%. The inset displays the reduction of AA/fMLP-induced synthesis of leukotriene B_4 (LTB_4), cysteinyl-leukotrienes (cys-LTs), 5-hydroxyeicosatetraenoic acid (5-HETE), thromboxane A_2 plus prostaglandin $F_{2\alpha}$ (TXA_2+$PGF_{2\alpha}$), and prostaglandin I_2 plus prostaglandin D_2 plus prostaglandin E_2 ($PG(I+D+E)_2$) resulting from dual inhibition of 5-LOX and COX by MK886 and diclofenac, respectively. b) Inflammatory pulmonary vasoconstrictor response and endothelial permeability increase/edema formation are expressed as maximal pulmonary arterial pressure rise (ΔPAP_{max}) and maximal weight gain (ΔW_{max}), respectively. Mean ± SEM; error bars are missing when falling into the symbol. * $P<0.05$ versus challenge with AA/fMLP.

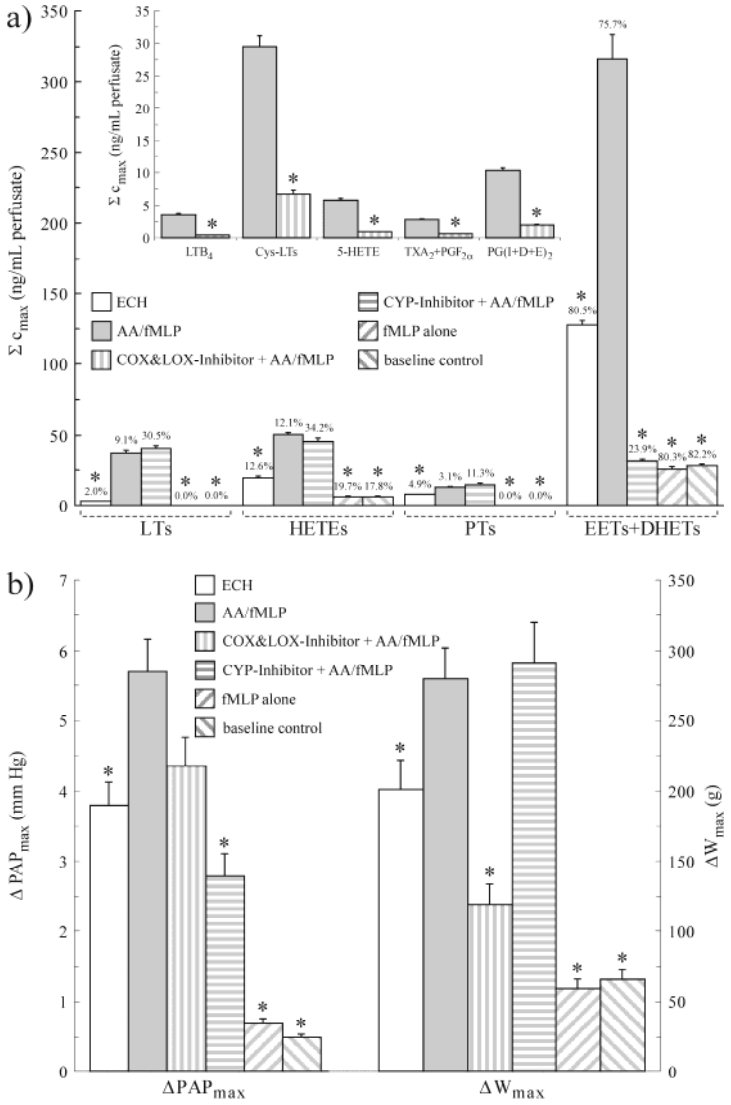
a)
Σ c_max (ng/mL perfusate)
ECH
AA/fMLP
COX&LOX-Inhibitor + AA/fMLP
CYP-Inhibitor + AA/fMLP
fMLP alone
baseline control
LTB4
Cys-LTs
5-HETE
TXA2+PGF2α
PG(I+D+E)2
LTs
HETEs
PTs
EETs+DHETs
b)
ΔPAP_max (mm Hg)
ΔW_max (g)
ΔPAP_max
ΔW_max

Figure 4

TABLE 1 **Maximal perfusate concentrations of eicosanoids released from human lungs upon challenge with AA and fMLP or ECH (n=5)**

Eicosanoid group	Eicosanoid	ECH		AA + fMLP	
		c_{max} (ng/mL)	Σc_{max} (%)	c_{max} (ng/mL)	Σc_{max} (%)
LTs	ω-ox-LTB_4	---		4.80 ± 0.29	
	LTB_4	0.61 ± 0.06		3.50 ± 0.18	
	LTC_4	---		10.80 ± 1.21	
	LTD_4	---		9.92 ± 0.86	
	LTE_4	2.59 ± 0,17		8.80 ± 0.75	
	Σ c_{max} [LTs]	**3.20 ± 0.18**	**2.02**	**37.82 ± 1.70**	**9.06**
HETEs	15-HETE	6.95 ± 0.82		14.06 ± 1.01	
	11-HETE	2.21 ± 0.48		8.90 ± 0.61	
	8-HETE + 12-HETE	6.76 ± 0.11		17.07 ± 0.98	
	9-HETE	2.70 ± 0.21		4.68 ± 0.27	
	5-HETE	1.36 ± 0.19		5.76 ± 0.34	
	Σ c_{max} [HETEs]	**19.98 ± 1.00**	**12.63**	**50.47 ± 1.59**	**12.09**
PTs	TXA_2	0.65 ± 0.05		0.93 ± 0.061	
	PGI_2	2.11 ± 0.09		4.11 ± 0.27	
	PGD_2	1.66 ± 0.13		2.54 ± 0.18	
	PGE_2	2,30 ± 0.11		3,68 ± 0.19	
	$PGF_{2\alpha}$	1.02 ± 0.08		1.82 ± 0.12	
	Σ c_{max} [PTs]	**7.74 ± 0.21**	**4.89**	**13.08 ± 0.40**	**3.13**
EETs + DHETs	14,15-(EET+DHET)	18.05 ± 1.4		12.66 ± 0.84	
	11,12-(EET+DHET)	57.90 ± 0.7		99.16 ± 9.38	
	8,9-(EET+DHET)	37.50 ± 2.9		183.71 ± 14.82	
	5,6-(EET+DHET)	13.80 ± 0.3		20.46 ± 1.39	
	Σ c_{max} [EETs+DHETs]	**127.25 ± 3.31**	**80.45**	**315.99 ± 17.61**	**75.71**
	Σ c_{max} [Eicosanoids]	**158.17 ± 3.47**	**100.00**	**417.36 ± 17.77**	**100.00**

Data are presented as mean ± SEM. AA: arachidonic acid; c_{max}: maximal concentration; Σ c_{max}: sum of maximal concentrations; ECH: *Escherichia coli* hemolysin; EET: epoxyeicosatrienoic acid; DHET: dihydroxyeicosatrienoic acid; fMLP: formyl-methionyl-leucyl-phenylalanine; HETE: hydroxyeicosatetraenoic acid; LT: leukotriene; PT: prostanoid.