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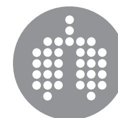

Epoxyeicosatrienoates are the dominant eicosanoids in human lungs upon microbial challenge

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ABSTRACT: Lipoxygenase, cyclo-oxygenase and cytochrome P450 (CYP) products of arachidonic acid (AA) are implicated in pulmonary vasoregulation. The CYP-mediated epoxyeicosatrienoates (EETs) have been described previously as the predominant eicosanoids in human lungs upon stimulation with the Ca^{2+} ionophore A23187. In this study, we challenged perfused human lungs with two microbial agents: *Escherichia coli* haemolysin (ECH) and formyl-methionyl-leucyl-phenylalanine (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 oedema formation. The maximum buffer concentrations of EETs/DHETs surpassed those of LTs plus HETEs and PTs by a factor of four (ECH) or three (AA/fMLP). Dual 5-lipoxygenase/cyclo-oxygenase inhibition caused pronounced reduction of AA/fMLP-induced LT/PT synthesis and oedema 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 oedema formation, suggesting a major contribution of LTs/PTs to vascular leakage and of EETs/DHETs to pressor response.

Consequently, generation of EETs/DHETs is greater than that of LTs plus HETEs and PTs in *ex vivo* perfused human lungs upon microbial challenge suggesting a substantial contribution of these mediators to inflammatory–infectious pulmonary injury.

KEYWORDS: Cyclo-oxygenase, cytochrome P450, eicosanoids, human lung, lipoxygenase, soluble epoxide 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 cyclo-oxygenase (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, ranging from proinflammatory and vasoconstrictive, to anti-inflammatory and vasodilatory properties [1, 4, 5, 11].

LTs are implicated in a variety of vascular abnormalities occurring under conditions of lung inflammation, such as pre- and post-capillary 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 polymorphonuclear neutrophils (PMNs) *in vitro* and in buffer-perfused rabbit lungs, which are known to harbour large quantities of resident neutrophils, monocytes and lymphocytes even after extensive rinsing [14, 15], demonstrated high yields of LT and HETE formation upon simultaneous application of free AA and inflammatory ligands, such as formyl-methionyl-leucyl-phenylalanine (fMLP) or the bacterial toxin *Escherichia coli* haemolysin (ECH) [8, 16, 17]. This abundant mediator generation is a result of substantial AA liberation at sites of

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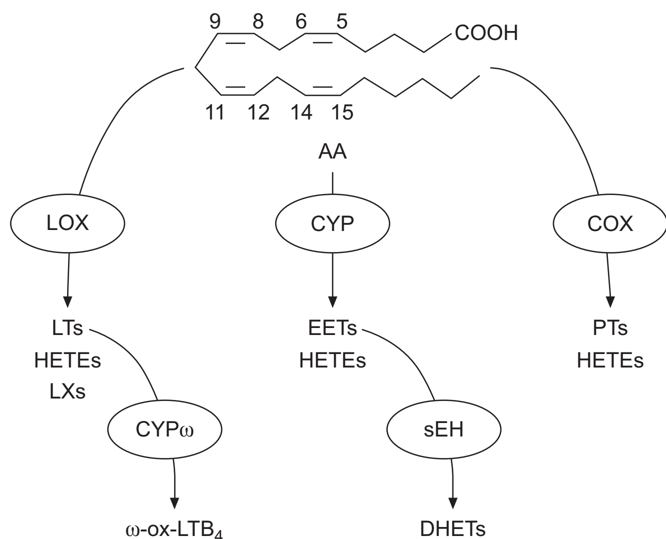

FIGURE 1. The main pathways of the arachindonic acid (AA) cascade. LOX: lipoxygenase; LT: leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4); HETE: hydroxyeicosatetraenoic acid (15-HETE, 11-HETE, 8-HETE, 12-HETE, 9-HETE and 5-HETE); LX: lipoxin (LXA_4 and LXB_4); CYPω: cytochrome P450 ω-oxygenase; ω-ox-LTB_4: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

inflammatory events and leukocyte–endothelial cooperation in LT synthesis, including 1) a transfer of the unstable LT intermediate LTA_4 from PMN feeder cells to acceptor endothelial cells (ECs) with subsequent employment of the enzymatic equipment of the latter for transformation into LTs, and 2) release of free AA from ECs with uptake and processing of this precursor by the adjacent PMNs [3, 16–21].

The ubiquitous, as yet poorly investigated, six HETE regioisomers display varying biological activities, ranging from anti-inflammatory (15-HETE) to proinflammatory (5-HETE) properties, and 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 and PGE_2) functions [1, 5]. TXA_2 and PGI_2 represent a classical example of an antagonistic pair of mediators: vasocontriction and the 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 antiaggregatory 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* metabolisation 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 over a decade ago as chemical correlates of the vasodilatative endothelium-derived hyperpolarising 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 nonphysiological Ca^{2+} ionophore A23187 evoked the enhanced release of a broad array of eicosanoids, with the CYP-mediated EETs being by far the predominant mediators, paralleled by an increase in pulmonary arterial pressure (ΔP_{pa}) and lung weight gain (ΔW).

In the present study, we extended this approach by asking whether the challenge of human lungs with pathophysiologically relevant microbial stimuli *per se*, such as the proteinaceous transmembrane pore-forming exotoxin ECH and the inflammatory and chemotactic bacterial receptor-operated tripeptide fMLP, administered in the presence of exogenous AA availability might elicit a similar eicosanoid profile as A23187 and whether such metabolic responses are linked with vasoconstrictor response and oedema 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 detection (PDAD) and electrospray ionisation (ESI) tandem mass-spectrometric (MS/MS) 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/MS method revealed the release of virtually all the main representatives of the AA cascade in response to both microbial challenges, with the absolute predominance (by a factor of three to four) of the CYP/sEH-mediated EETs/DHETs over the other lipid mediators. This mediator liberation profile was paralleled by a pulmonary arterial vasoconstrictor response, to which the CYP epoxygenase pathway may particularly contribute, and ΔW, which may be mainly a result of LOX and COX activity.

MATERIALS AND METHODS

Materials

AA, 20-carboxy-LTB_4, 20-hydroxy-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, and 15-, 11-, 8-, 12-, 9- and 5-HETE, were purchased from Biomol GmbH (Hamburg, Germany). 14,15-, 11,12-, 8,9-, 5,6-EET, and 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), diethylamine (DEA) and triethylamine were obtained from Sigma Chemie GmbH (Munich, Germany). The 5-LOX inhibitor MK886, the COX inhibitor diclofenac and the CYP epoxygenase inhibitor *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH) were from Cayman. EDTA disodium salt dihydrate, EDTA tetrasodium salt tetrahydrate ($Na_4EDTA \cdot 4H_2O$) and formic acid (FA) were purchased from

E. Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore, Eschborn, Germany). Methanol was purchased from Burdick and 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 purchased from Serag Wiessner (Naila, Germany).

Preparation of ECH

The exotoxin was kindly provided by S. Bhakdi (Institute of Medical Microbiology and Hygiene, Johannes Gutenberg University Mainz, Mainz, Germany) being prepared as previously described [32]. The endotoxin content of the preparation was reduced to ~3 ng LPS per μg protein. The haemolytic titre was assessed directly before use and is expressed in hemolytic units (HU) per mL; 1 HU·mL^{-1} corresponded to ~100 ng protein per mL.

Ex vivo human and rabbit lung perfusion and experimental protocol

The technique of *ex vivo* lung perfusion and ventilation has been described previously by our group [33] and has now been adapted to whole human lungs originating from patients suffering from bronchial carcinoma undergoing lung resection. The study was approved by the University Ethics Committee (Justus Liebig Univesity, Gießen, Germany) 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^{-1}. 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 breaths·min^{-1}; end-expiratory pressure 4 mmHg). 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^{-1} (500 mL total volume). 15 min later, 100 HU ECH (*i.e.* a subcytolitic dose of 0.2 HU·mL^{-1}; n=5) or fMLP (20 μM final concentration in buffer) in the presence of AA (AA/fMLP, n=5) or alone (control for 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 added to the buffer 30 s 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 were collected for eicosanoid analysis 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 that 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 a reference lung for normalisation of the eicosanoid concentrations and ΔW for the other lungs.

The effect of exogenously supplied EET on pressor response and oedema formation was investigated using isolated, perfused and ventilated human (n=2) and rabbit (n=5) lungs employing the aforementioned experimental set-up. Rabbit lungs were perfused as described previously [33], with a flow rate of 150 mL·min^{-1} (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 the time was set to zero.

SPE and CapLC-PDAD-ESI-MS/MS equipment and procedures

Eicosanoids were extracted from lung perfusate by adapting a previous SPE procedure [34] for 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 standards and reference substances for the calculation of the relative retention index (RRI), BHT as an antioxidant, $Na_4EDTA \cdot 4H_2O$, FA and isopropyl alcohol, and were vortexed, centrifuged and subjected to SPE employing octadecylsilane (ODS) cartridges (Chromabond C18ec, 200 mg sorbent mass; Macherey-Nagel, Düren, Germany). The extracted eicosanoids were subjected to CapLC-PDAD-ESI-MS/MS analysis, as described previously [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 (length × internal diameter 250 mm × 300 μm; GROM-SIL 80 ODS-7 pH) from Grom Analytik + HPLC GmbH (Rottenburg-Hailfingen, Germany) employing as the mobile phase a mixture of water/acetonitrile/methanol/FA (130/240/80/0.22, vol/vol/vol/vol; pH 4.30, adjusted with DEA) at a flow rate of 2.7 μL·min^{-1}. The eluted compounds were subjected to dual online spectrophotometric and MS/MS analysis in negative-ion mode. Identity and purity of eluted eicosanoids were triple-checked: chromatographically by isocratic RRI as well as by dual ultraviolet spectrometric and MS/MS analysis.

Statistics

Analytical data, maximal ΔP_{pa} ($\Delta P_{pa,max}$) and maximal ΔW (ΔW_{max}) are expressed as mean±SEM. Statistical evaluation was performed using one-way ANOVA. A p-value <0.05 was 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) summarised as ω-ox-LTB_4, LXA_4 and

LXB_4; 2) CYP epoxygenase-mediated oxiranes 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET; 3) sEH-mediated EET-derived vic-diols 14,15-DHET, 11,12-DHET, 8,9-DHET and 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; and 5) LOX-, CYP- or COX-mediated 15-HETE, 12-HETE, 11-HETE, 9-HETE, 8-HETE and 5-HETE.

The measured eicosanoid levels are absolute values at each time point, reflecting the specific dynamic equilibrium between release and reuptake or 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 and 15-HETE and 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 (data not shown), but some moderate liberation of 15-HETE greater than that of (8-HETE + 12-HETE), 11-HETE, 9-HETE and 5-HETE individually 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 (data 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-fold (fig. 2a). No LTC_4, LTD_4,

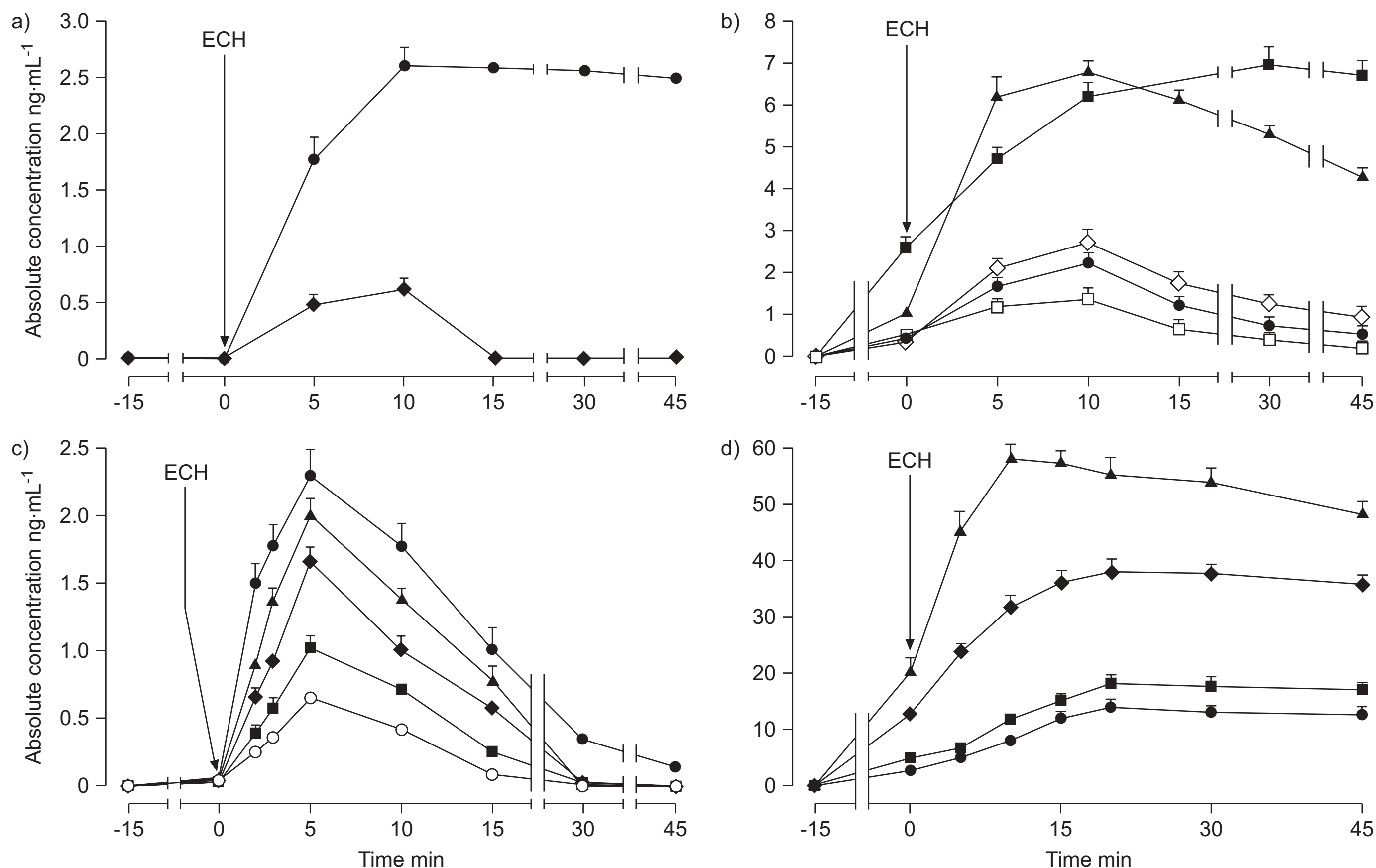

FIGURE 2. Kinetics of a) leukotriene (LT)E_4 (●) and LTB_4 (◆), b) hydroxyeicosatetraenoic acids (HETEs) (■: 15-HETE; ▲: 8- and 12-HETE; ◇: 9-HETE; ●: 11-HETE; □: 5-HETE), c) prostanoids (prostaglandins (PG)E_2 (●), PGI_2 (▲), PGD_2 (◆) and $PGF_{2\alpha}$ (■), and thromboxane (TX)A_2 (○)), and d) epoxyeicosatrienoic (EETs) and dihydroxyeicosatrienoic acids (DHETs) (▲: 11,12-(EET+DHET); ◆: 8,9-(EET+DHET); ■: 14,15-(EET+DHET); ●: 5,6-(EET+DHET)) appearing in the buffer fluid of *ex vivo* isolated, perfused and ventilated human lungs stimulated with *Escherichia coli* haemolysin (ECH). After a baseline period of 15 min, 100 haemolytical units ECH were bolus-injected into the pulmonary artery (time-point 0). 10-mL perfusate samples for eicosanoid analysis were collected from the venous effluent at different time points and subjected to solid-phase extraction followed by capillary liquid chromatography with photodiode array and electrospray ionisation tandem mass-spectrometric detection. The measured eicosanoid levels are absolute concentrations at each time point (n=5; mean±SEM; error bars are missing when falling within the symbol). PGI_2 and TXA_2 concentrations were calculated from the measured 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations, respectively.

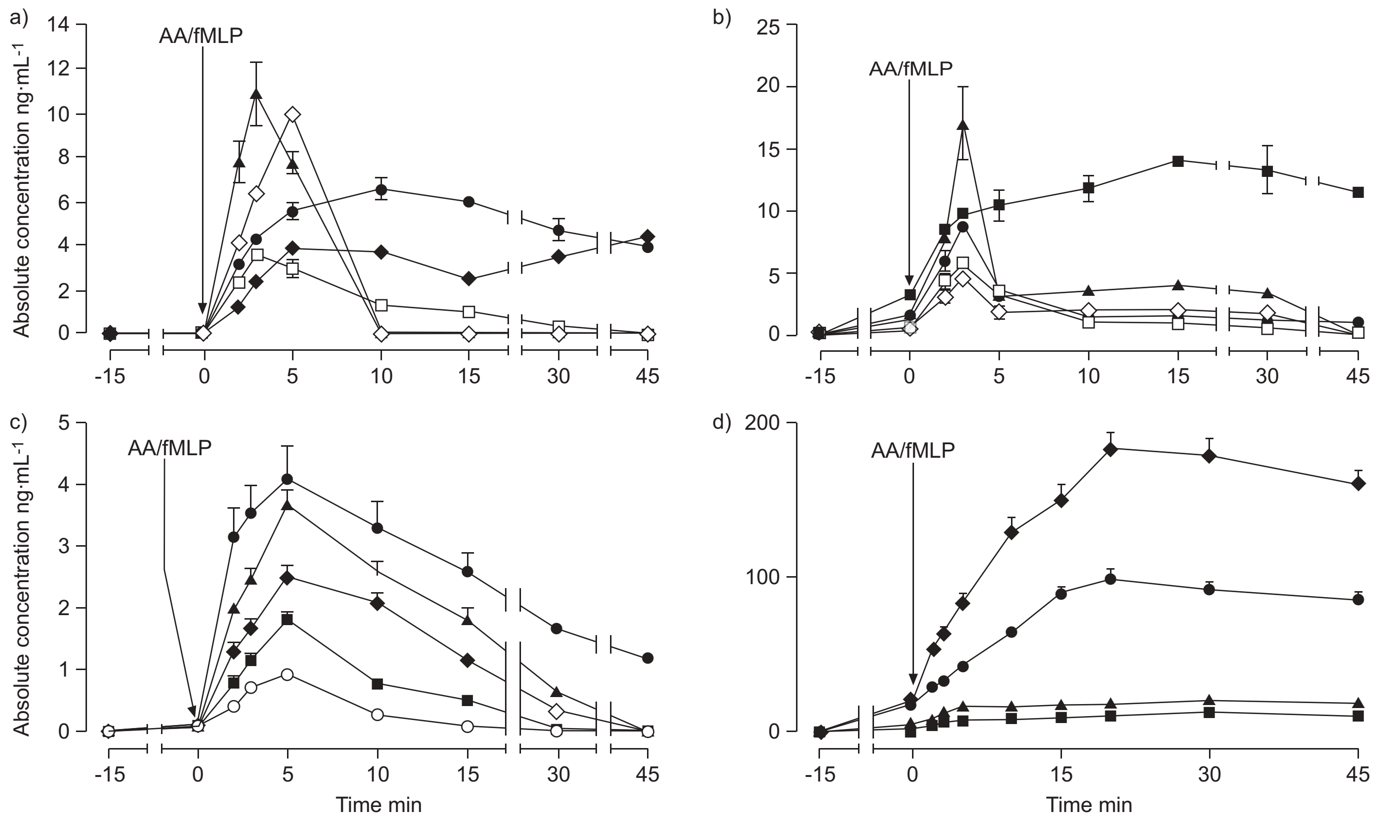

FIGURE 3. Kinetics of a) leukotrienes (LTs; ◆: ω-oxygenase-mediated LTB_4 metabolites; □: LTB_4; ▲: LTC_2; ◇: LTD_4; ●: LTE_4), b) hydroxyeicosatetraenoic acids (HETEs; ■: 15-HETE; ●: 11-HETE; ▲: 8- and 12-HETE; ◇: 9-HETE; □: 5-HETE), c) prostanoids (prostaglandin (PG)I_2 (●), PGE_2 (▲), PGD_2 (◆) and $PGF_{2\alpha}$ (■), and thromboxane (TX)A_2 (○)), and d) epoxyeicosatrienoic (EETs) and dihydroxyeicosatrienoic acids (DHETs) (◆: 8,9-(EET+DHET); ●: 11,12-(EET+DHET); ▲: 5,6-(EET+DHET); ■: 14,15-(EET+DHET)) released in the buffer of isolated, perfused and ventilated human lungs challenged with arachidonic acid/formyl-methionyl-leucyl-phenylalanine (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 0). 10-mL perfusate samples were collected from the venous effluent at different time points and subjected to solid-phase extraction followed by capillary liquid chromatography with photodiode array and electrospray ionisation tandem mass-spectrometric detection. The measured eicosanoid levels are absolute concentrations at each time point (n=5; mean±SEM; error bars are missing when falling within the symbol). PGI_2 and TXA_2 concentrations were calculated from the measured 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations, respectively.

LXA_4 or LXB_4 was detected. Virtually no (<1%) hydrolytic decay products (6t-LTB_4, 6t,12e-LTB_4, 5S,6R-DiHETE or 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- and 12-HETE, paralleled by enhanced liberation and subsequent plateauing of 15-HETE, with c_{max} (6.76 ng·mL^{-1} and 6.95 ng·mL^{-1}, respectively) by far higher than those of 5-, 9- and 11-HETE, which displayed kinetics comparable to 8- and 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 of ~0.5–2.5 ng·mL^{-1}, 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 c_{max} (57.90 ng·mL^{-1} and 37.50 ng·mL^{-1}, respectively) than 14,15-(EET+DHET), 5,6-(EET+DHET) and all other eicosanoid classes. The sum of the quantitatively dominating regioisomers 8,9-(EET+DHET) and 11,12-(EET+DHET) surpassed the sum of the minor regioisomers 5,6-(EET+DHET) and 14,15-(EET+DHET) by a factor of three. The total amount of EETs/DHETs surpassed the sum of LTs, HETEs and PTs four-fold (table 1, fig. 4a).

Eicosanoid release upon challenge with fMLP alone

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

LT and LX liberation after coapplication of AA and fMLP

AA/fMLP provoked the rapid appearance of LTB_4, ω-ox-LTB_4 (20-HO-LTB_4 and 20-COOH-LTB_4) and the cysteinyl-LTs, LT(C,D,E)$_4$, in the perfusate (fig. 3a). In contrast, virtually no (<1%) nonenzymatic LTA_4 hydrolysis products were detected. Of the LTs, LTC_4 and LTD_4 displayed the highest peak values (10.82 ng·mL^{-1} and 9.92 ng·mL^{-1}, respectively; table 1), followed by rapid decline, concomitant with progressive increase and subsequent plateauing of their metabolite LTE_4. In

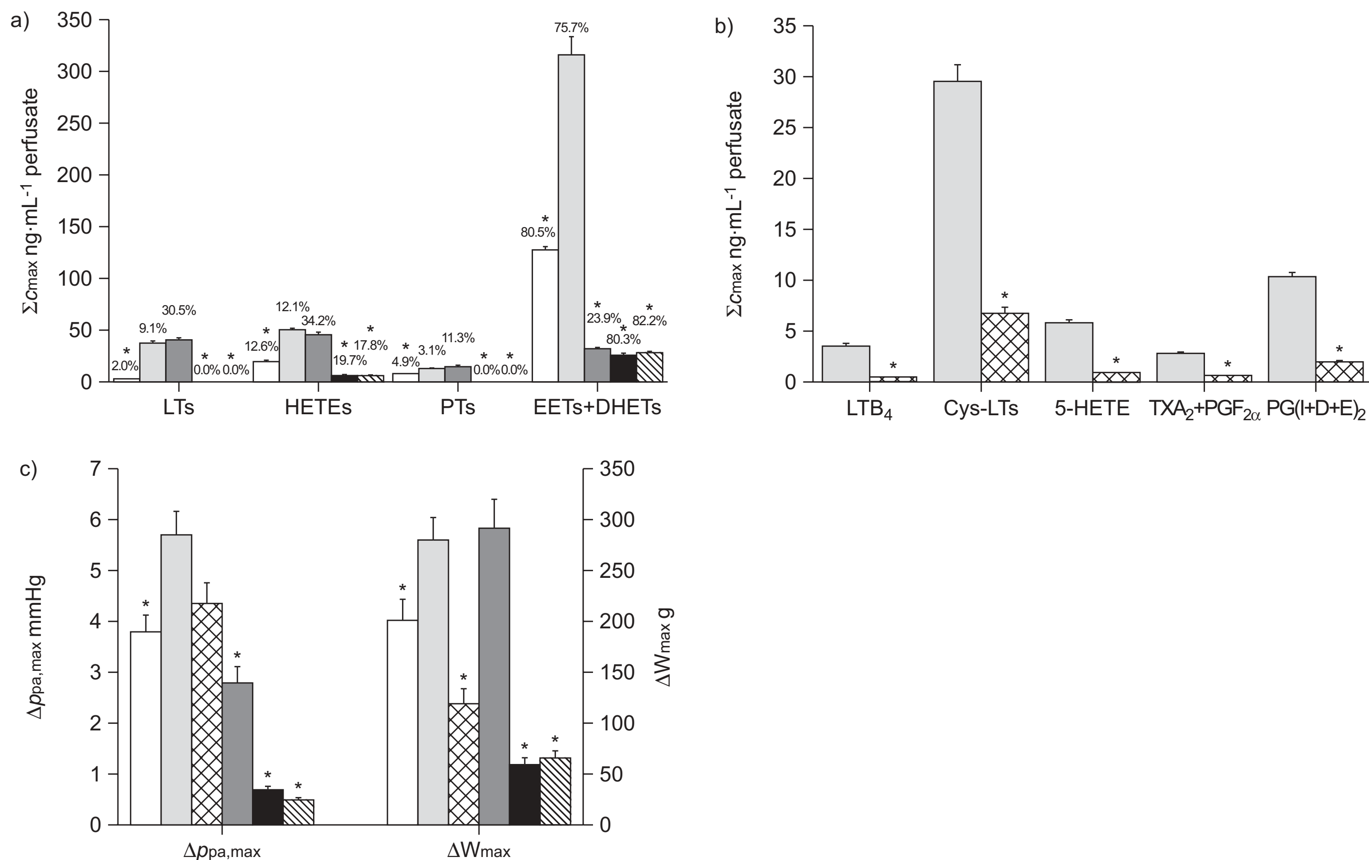

FIGURE 4. Comparison of the a and b) metabolic (eicosanoid generation) and c) pathophysiological (vasoconstriction and oedema formation) response in isolated perfused human lungs under different conditions: challenge with *Escherichia coli* haemolysin (ECH; n=5; □), arachidonic acid/formyl-methionyl-leucyl-phenylalanine (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 epoxygenase inhibitor *N*-methylsulfonyl-6-(2-proargyloxyphenyl)hexanamide (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 (EETs) and dihydroxyeicosatrienoic acids (DHETs) is presented as the sums of the means of the maximal perfusate concentrations (Σc_{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, EETs and DHETs) as % Σc_{max} as compared with 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%. b) The reduction of AA/fMLP-induced synthesis of LTB_4, cysteinyl-LTs, 5-HETE, thromboxane (TX)A_2 plus $PGF_{2\alpha}$, and PGI_2 plus PGD_2, PGE_2 resulting from dual inhibition of LOX and COX by MK886 and diclofenac, respectively. c) Inflammatory pulmonary vasoconstrictor response and endothelial permeability increase/oedema formation are expressed as maximal pulmonary arterial pressure rise ($\Delta P_{pa,max}$) and maximal weight gain (ΔW_{max}), respectively. Data are presented as mean±SEM; error bars are missing when falling within the bar. *: $p<0.05$ *versus* challenge with AA/fMLP.

addition, rapid peaking of LTB_4 was noted (maximum values approximately one-third of those for LTC_4), with subsequent plateauing of its ω-oxidation products. The total amount (measured as by the sum of c_{max} values) of LTs was one order of magnitude above that elicited by application of ECH (table 1). No LXA_4 or 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 and 12-HETE, peaking after 3 min (c_{max} 17.07 ng·mL^{-1}), with subsequent rapid decline. 5-, 9- and 11-HETE displayed kinetics comparable to 8- and 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^{-1}. The total quantity of HETEs was three 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^{-1} and 3.68 ng·mL^{-1}, 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

TABLE 1 Maximal perfusate concentrations (c_{max}) of eicosanoids released from human lungs upon challenge with arachindonic acid (AA) and formyl-methionyl-leucyl-phenylalanine (fMLP) or *Escherichia* coli haemolysin (ECH)

Eicosanoid	c_{max} ng·mL^{-1}	
	ECH	AA/fMLP
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
Σc_{max}	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- and 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
Σc_{max}	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
Σc_{max}	7.74±0.21 (4.89)	13.08±0.40 (3.13)
EETs and 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
Σc_{max}	127.25±3.31 (80.45)	315.99±17.61 (75.71)
Σc_{max}	158.17±3.47 (100.00)	417.36±17.77 (100.00)

Data are presented as mean±SEM or mean±SEM (%). n=5. LT: leukotriene; ω-ox-LTB_4: ω-oxygenase-mediated LTB_4 metabolites; Σc_{max}: sum of c_{max} values; HETE: hydroxyeicosatetraenoic acid; PT: prostanoid; TX: thromboxane; PG: prostaglandin; EET: epoxyeicosatrienoic acid; DHET: dihydroxyeicosatrienoic acid.

far the highest maximal perfusate concentration of all eicosanoids analysed in our study (c_{max} 183.71 ng·mL^{-1}; table 1). 11,12-(EET+DHET) showed a similar profile, reaching half the c_{max} 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) and 11,12-(EET+DHET) surpassed the sum of the minor regioisomers 5,6-(EET+DHET) and 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 three-fold (table 1 and 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 (fig. 4b), while no significant change in the appearance of 8-, 9-, 11-, 12- and 15-HETE and EETs/DHETs was registered (data not shown).

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

Near-constant P_{pa} (9–10 mmHg) 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 (ΔP_{pa} <1 mmHg; fig. 4c). Challenge with both AA/fMLP and ECH provoked a pronounced vascular pressor response with $\Delta P_{pa,max}$ of 5.7±0.47 mmHg and 3.8±0.32 mmHg, respectively, accompanied by marked oedema 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 $\Delta P_{pa,max}$ as compared with AA/fMLP challenge without inhibitors, while blockade of EET/DHET generation with MS-PPOH was paralleled by substantial attenuation of $\Delta P_{pa,max}$ 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 $\Delta P_{pa,max}$ -3.1±0.27 mmHg and 3.4±0.31 mmHg with human and rabbit lungs, respectively, but no oedema formation: ΔW <75 g with human lungs, *i.e.* the same range as in the time-matched baseline control experiments (fig. 4c) and ΔW <1 g with rabbit lungs (data 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) oedema formation. In addition, it was noted that the AA/CYP/sEH-derived EETs/DHETs were, quantitatively, by far the dominant mediator group, apparently substantially contributing to the vasoconstrictor response, but not to the vascular leakage provoked by these agents. However, the LOX/COX-derived LTs/PTs were noted to make a major contribution to endothelial permeability increase, but caused 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, leading to differential effects on the kinetics of eicosanoid biosynthesis, such as the absolute dominance of eicosanoid release upon AA/fMLP coapplication over that upon ECH challenge. 1) The hydrophilic transmembrane pore-forming ECH *per se* enables passive influx of extracellular 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 is not capable *per se* 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 and 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). 1) 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 (characterised 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). 2) In contrast to AA/fMLP, upon ECH challenge, the endogenous precursor AA is provided gradually *via* phospholipase- and, 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 end-product 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 *versus* ECH challenge by a factor of 10, compared with ''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 even after extensive rinsing with buffer fluid, lungs harbour large, ''resident'' intracapillary pools of different leukocytes, but virtually no platelets [14, 15], and that 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 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 hydrolytic decay products of the unstable LT intermediate LTA_4 is indicative of transcellular cooperative LT formation mechanisms. As LOX is restricted to cells of the myeloid lineage [6], transcellular LT synthesis is initialised 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 (PMNs, 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, 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 PMNs, the only cell type equipped with substantial ω-oxygenase activity [5, 50]. In contrast to LOX, the cysteinyl-LT synthesis-mediating glutathione-*S*-transferase leading to the generation of $LT(C,D,E)_4$ occurs both in some myeloid cells, such as mast cells, eosinophils, basophils and monocytes/macrophages, as well as in ECs, but not in PMNs [2, 5, 6, 12]. Consequently, cysteinyl-LTs are generated partly in cells equipped with both LOX and LTC_4-synthase, such as mast cells and macrophages/monocytes [5, 12], but certainly mainly *via* transcellular cooperative synthesis. This includes an LTA_4 shift from leukocyte donor cells to adjacent acceptor ECs followed by rapid conversion of LTA_4 to cysteinyl-LTs [3, 18–21]. 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 proinflammatory 5-HETE was observed (table 1 and figs 2b and 3b).

The vasodilatory PTs PGI_2 (prostacyclin), PGD_2 and PGE_2 predominated 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 and figs 2c and 3c), 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]. Therefore, ECs and resident macrophages, monocytes, lymphocytes and PMNs, known as sites of extraplatelet TXA_2 synthesis [52–54], may serve as

alternative candidates for extraplatelet thromboxane generation. This may explain why even upon coapplication 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 and DHETs in the human lung vasculature upon microbial challenge is intriguing in view of the fact that little is yet known about their vasoregulatory role in the lung. Interestingly, the total amount of the LOX-mediated LTs, the LOX-, COX- or CYP-mediated HETEs, and the COX-mediated PTs, which are well known as mediators of pulmonary vasoconstriction, were dominated by a factor of three to four by that of the EETs/DHETs (fig. 4a, table 1). This absolute dominance of EETs and DHETs over the other lipid mediators of the AA cascade resembles that previously described in human lungs undergoing ΔP_{pa} upon stimulation with A23187 [10] and considering the few available reports that describe the EETs and DHETs as vasoconstrictors in isolated, pressurised rabbit pulmonary arteries [24] and in the hypoxic rabbit and mouse lung [31, 38, 55], the question arises of whether the high EET and DHET amounts may have an impact on vascular pressor response and/or oedema formation in human lungs upon microbial challenge.

Notably, simultaneous inhibition of LOX and COX in the AA/fMLP-challenged human lung with MK886 and diclofenac, respectively, induced a pronounced decrease in LT and PT generation (fig. 4b) paralleled by marked reduction of weight gain (fig. 4c), suggesting a substantial contribution of LTs and PTs to oedema formation. Remarkably, only a limited attenuation of the human lung vascular pressor response to inflammatory challenge was registered (fig. 4c), although the generation of LTB_4, cysteinyl-LTs, 5-HETE, vasoconstrictive PTs (TXA_2 and $PGF_{2\alpha}$) and vasodilatative PTs (PGI_2, PGD_2 and PGE_2) was strongly inhibited (to 12, 23, 15, 21 and 19%, respectively, compared to AA/fMLP-challenge without LOX/COX inhibition; fig. 4b), suggesting a possible contribution of the CYP/sEH-derived EETs and 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 and DHET generation (fig. 4a) accompanied by an attenuation of the pressor response to ~50%, but not of oedema formation (fig. 4c). These results correlate well with those obtained by exogenous application of 11,12-EET to human and rabbit lungs at the same final concentration as the c_{max} of endogenously released 11,12-EET: induction of marked ΔP_{pa}, but no significant ΔW, although 11,12-EET and 8,9-EET, belonged to the quantitatively dominant regioisomers released in the human lung upon microbial challenge; thus, they are a potential prime candidate for causing oedema formation.

These findings are in agreement with reports [57, 58] describing a regiospecific, endothelial permeability-increasing activity of the four EET regioisomers in a concentration range of 3–10 μM, *i.e.* surpassing by far the c_{max} 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 $ng \cdot mL^{-1}$ in table 1), thus suggesting a rather negligible contribution of the EETs to oedema 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 oedema formation.

In conclusion, stimulation of intact human lungs with two microbial agents, ECH and fMLP, elicited pulmonary vasoconstriction and vascular leakage, alongside 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 oedema formation but only limited attenuation of ΔP_{pa}, while selective CYP epoxygenase inhibition induced marked suppression of EET/DHET generation and of the vasoconstrictor response but not attenuation of oedema 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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STATEMENT OF INTEREST

None declared.

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