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RESEARCH PAPER

A pirinixic acid derivative (LP105) inhibits murine 5-lipoxygenase activity and attenuates vascular remodelling in a murine model of aortic aneurysm

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BACKGROUND AND PURPOSE

Arachidonic acid derivatives play a central role in inflammation processes. Arachidonic acid is metabolized by several enzymes, particularly cyclooxygenases (COX), 5-lipoxygenase (5-LOX) and microsomal prostaglandin E-synthase-1 (mPGES-1) to pro-inflammatory mediators.

EXPERIMENTAL APPROACH

We determined the effect of LP105, a pirinixic acid derivative which acts as inhibitor of 5-LOX, COX and mPGES-1, on aortic aneurysm development in mice and on 5-LOX activity in murine monocytes.

KEY RESULTS

In a monocyte cell line (RAW264.7), LP105 inhibited 5-LOX in whole cells (IC_{50}: 1–3 μM) and in supernatants (IC_{50}: ~10 μM). Oral administration of LP105 to mice resulted in therapeutic tissue and plasma levels. Aortic aneurysms were induced in $ApoE^{-/-}$ mice by angiotensin II (AngII) and LP105 (5 mg·day^{-1} per animal) was co-administered to a subgroup. Compared with animals receiving AngII alone, the LP105+AngII group showed a lower heart rate, a trend towards reduced heart to body weight ratio but similar hypertensive responses. AngII alone significantly increased aortic weight and diameter but co-treatment with LP105+AngII prevented these changes. LC/MS-MS studies revealed increased 15-hydroxytetraenoic acid (15-HETE) and 14,15-epoxyeicosatrienoic acid (14,15-EET) plasma levels in LP105-treated animals. In the murine kidney, mRNAs of EET-generating or metabolizing enzymes and of 5-LOX and 15-LOX were unaffected by LP105. LP105 also did not inhibit the EET-metabolizing soluble epoxide hydrolase.

CONCLUSIONS AND IMPLICATIONS

LP105 was a potent inhibitor of monocyte 5-LOX and reduced AngII-induced vascular remodelling in mice. A shift of arachidonic acid metabolism to the protective EET pathway may contribute to the beneficial effects of LP105.

Abbreviations

5-LOX, 5-lipoxygenase; AngII, angiotensin II: COX, cyclooxygenase; EET, epoxyeicosatrienoic acid

Introduction

Macrophages are important elements of the innate immune system. They are involved in phagocytosis of opsonized particles and debris, have direct microbiocidal properties, contribute to antigen presentation and release proteases into the extracellular space. Macrophages are also a rich source of cytokines and inflammatory mediators. Inappropriate activation of these cells can be harmful for the organism. In the cardiovascular system, activation of macrophages by oxidized

low-density lipoprotein (LDL) promotes arteriosclerosis (Tabas, 2010) and the release of proteases from macrophages during vascular inflammation can promote aneurysm formation and plaque destabilization (Rizas *et al.*, 2009).

Derivatives of arachidonic acid are well established signalling lipids generated and released from activated macrophages. Some of these eicosanoids, the 5-lipoxygenase (5-LOX) metabolite leukotriene (LT)B_4, the cysteinyl-containing LTC_4, D_4 and E_4, as well as the microsomal prostaglandin E_2 synthase-1 (mPGES-1) product prostaglandin (PG)E_2 can act as potent pro-inflammatory mediators (Radmark *et al.*, 2007; Wymann and Schneiter, 2008). Signalling lipids can however also be anti-inflammatory. Thus, the cytochrome-P450 epoxygenase products of arachidonic acid, the epoxyeicosatrienoic acids (EETs) (Fleming, 2007) potently inhibit activation of the transcription factor nuclear factor κB. For several lipid mediators particularly the ability to activate peroxisome proliferator-activated receptors (PPARs) has been linked to anti-inflammatory actions (Devchand *et al.*, 1996), and also for several drugs, a pleiotropic activation of PPARs has been shown to contribute to their anti-inflammatory effects (Lehmann *et al.*, 1997).

The observation that pro-inflammatory arachidonic acid derivatives are formed by several pathways, such as cyclooxygenases (COX) and 5-LOX, suggested that simultaneous inhibition of the enzymes involved in these processes could be a more effective approach to the treatment of inflammatory diseases (Schmelzer *et al.*, 2006; Liu *et al.*, 2010). Among such compounds, particularly those acting as dual 5-LOX and mPGES-1 inhibitors are of special interest considering the pro-inflammatory synergy of the two systems in disease conditions such as septicaemia and cardiovascular disease.

We recently reported the synthesis of a pirinixic acid derivative, termed LP105, which potently inhibits 5-LOX with an IC_{50} of 1.5 μM (Werz *et al.*, 2008), but which also acts as an inhibitor for COX-1 (IC_{50} approx. 5–8 μM) and mPGES-1 (IC_{50} 2.6 μM) (Koeberle *et al.*, 2008). Moreover, LP105 activates the PPARα pathway with an EC_{50} of 11 μM and the PPARγ pathway with an EC_{50} of 7.5 (Popescu *et al.*, 2007).

In the present study we sought to determine whether LP105 provides beneficial effects *in vivo*. Because the compound interferes with the development of conditions associated with macrophage-mediated inflammation, we tested it in a model of aortic aneurysm, induced in $ApoE^{-/-}$ mice treated with angiotensin II. We observed that LP105 attenuated disease development and this effect was accompanied by reduced formation of 5-LOX products but an increased formation of protective arachidonic acid derivatives, in particular the EETs.

Methods

Animal experiment

All animal care and experimental procedures were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol.

Animals were maintained under a 12 h/12 h day–night cycle. Standard chow was fed to the animals (Ssniff, Soest, Germany; 87.7% dry substance, 19% protein, 3.3% fat, 4.9% crude fibre, 6.4% crude ash, 54.1% N-free extractives, 36.5% starch, 4.7% sugar; gross energy 16.3 $MJ \cdot kg^{-1}$). All animals received drinking water *ad libitum*. For pharmacokinetic studies, C57/BL6 mice and Wistar Kyoto rats were purchased from Charles Rivers (Deisenhofen, Germany).

For aortic aneurysm studies, C57/BL6-$ApoE^{-/-}$ (F10) breeding pairs ($ApoE^{-/-}$) were purchased from Taconic (Ejby, Denmark). Aneurysm experiments were performed exclusively in male $ApoE^{-/-}$ mice starting at an age of 6 months. The 5-LOX inhibitor LP105 was administered to the animals via feed pellets at a concentration of 1 $mg \cdot g^{-1}$ chow.

Determination of 5-LOX product formation in intact cells and vascular segments

RAW 264.7 cells (mouse monocyte-macrophage) were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI, Dulbecco's modified Eagle's medium contained 50 $\mu g \cdot mL^{-1}$ streptomycin, 50 $IU \cdot mL^{-1}$ penicillin and 10% fetal calf serum.

For determination of 5-LOX product formation in intact cells, 1.5×10^7 RAW cells were resuspended in 1 mL Phosphate buffered saline containing glucose (1 $mg \cdot mg^{-1}$) and calcium chloride (1 $mmol \cdot L^{-1}$) buffer. After pre-incubation with the test compounds at 37°C for 15 min, 5 $\mu mol \cdot L^{-1}$ A23187 was added together with 20 $\mu mol \cdot L^{-1}$ arachidonic acid. After 10 min at 37°C, the reaction was stopped by addition of 1 mL ice-cold methanol and 5-LOX metabolites (5-hydroxyeicosatetraenoic acid (5-HETE), LTB_4, *trans*-LTB_4 and epi-*trans* LTB_4) formed were extracted and analysed by HPLC as described previously (Werz and Steinhilber, 1996).

Vascular 5-LOX activity was determined in rat aortic segments. These were incubated in MCDB131 medium (200 μL, 37°C) in the presence or absence of LP105 for 60 min and subsequently stimulated with ionomycin (10 $\mu mol \cdot L^{-1}$) for 10 min. The reaction was stopped by the addition of methanol (200 μL). Lipids were extracted and measured by LC-MS/MS as described previously (Revermann *et al.*, 2009).

For the determination of 5-LOX activity in S100 cell supernatants, RAW 264.7 cells were resuspended in phosphate-buffered saline (PBS) containing 1 mM EDTA, and sonicated (3 × 10 s) at 4°C. Homogenates were then centrifuged at 100 000× *g* for 70 min at 4°C. Aliquots of S100 supernatants, corresponding to 2×10^7 cells, were diluted with ice-cold PBS containing 1 $mmol \cdot L^{-1}$ EDTA, and 1 $mmol \cdot L^{-1}$ ATP. After incubation with the test compounds for 10 min at 4°C, samples were pre-warmed for 30 s at 37°C and 2 $mmol \cdot L^{-1}$ $CaCl_2$ and 20 $\mu mol \cdot L^{-1}$ arachidonic acid was added to start 5-LOX product formation. The reaction was stopped after 10 min by the addition of 1 mL ice-cold methanol and 5-LOX products formed were analysed by HPLC as described for intact cells (Werz and Steinhilber, 1996).

Pharmacokinetic studies

All pharmacokinetic studies were performed in C57/BL6 mice.

LP105 in drinking water. LP105 (0.1 mM and 1 mM final concentration) was dissolved in 2-(hydroxypropyl)-β-

cyclodextrin (1.125% final concentration) and water, sonicated for 10 min and administered to the animals *ad libitum*. Five days after treatment start, animals were killed and blood samples were collected 1 and 9 h after fasting start.

LP105 subcutaneously injected. LP105 (0.1 mM final concentration) was dissolved in ethanol and diluted in a 0.5% albumin solution (1%). A single subcutaneous injection (200 µL injection volume) was applied interscapularly. Animals were killed and blood samples were collected 1, 4, 8, 16, 24 and 48 h after injection.

LP105 in feed pellets. LP105 was pelletted into standard chow resulting in a final concentration of 1 mg·g^{-1} chow. Mean food intake was 5 g·chow day^{-1} per mouse. Two days after the onset of feeding, animals were killed and blood samples were collected 0, 7 and 12 h after fasting start.

LP105 in corn oil given by gavage. LP105 was powdered and corn oil (Sigma, Munich, Germany) was added to give a final concentration of 5 mg mL^{-1} or 50 mg·mL^{-1}. One hundred microlitres of the corn oil/LP105 suspension were administered to the mice in a single gavage. Animals were killed and blood samples were collected 2, 4, 8, 12 and 16 h after gavage.

LP105 in polyethylene glycol given by gavage. LP105 (final concentration 5 mg·mL^{-1}) was dissolved in polyethylene glycol (PEG400, RT Chemikalien, Essen, Germany) and administered to the mice in a single gavage. Animals were killed and blood samples were collected 2, 4 and 8 h after gavage.

LP105 plasma and tissue level and protein binding determination. HPLC analysis was developed and carried out in a Varian ProStar instrument (Varian, Walnut Creek, USA) with ultraviolet detection at 255 nm. Seventy per cent acetonitrile (Phase A) and 30% phosphate buffer pH 2.1 (Phase B) at a flow rate of 1 mL·min^{-1} were used as mobile phases. Solvents were degassed by an online degasser of the ProStar System. The column used was an Inertsil ODS-2 5 µm, 250 × 4 mm protected by an Inertsil ODS-2 guard column 5 µm 10 × 4 mm. Each 50 µL of the samples was injected using the autosampler of the ProStar system. According to International Conference of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Q2A and Q2B the method was validated and all results fulfil the required guideline criteria. Calibration curves from 0.1 to 50 µg·mL^{-1} were used for quantification via the external standard method. All calibration curves and quality controls were prepared freshly. R^2 of the calibration curves were >0.998 and the biases of the quality control samples were <15%. limit of detection and limit of quantification were found to be 50 ng·mL^{-1} (in methanol) and 100 ng·mL^{-1} (in methanol) respectively.

For quantification, plasma samples were prepared as follows. In a 1.5 mL Eppendorf vial, 550 µL H_2O, 150 µL plasma and 300 µL methanol were mixed carefully. For quantification of cardiac tissue concentrations, the heart was powdered in liquid nitrogen, resuspended in 700 µL water and 300 µL methanol and subsequently cleared by centrifugation. Eight hundred and fifty microlitres of the mixture were put on an Extrelut® column and afterwards extracted with 6 mL diethyl ether. The diethyl ether was evaporated with nitrogen until dryness and the residue was dissolved in 200 µL methanol and injected into the HPLC system.

The plasma protein binding of LP105 was determined using an *in vitro* method. Human serum albumin was added to a 0.067 M KH_2PO_4, Na_2HPO_4·(pH 7.4) buffer to a concentration of 41.8 mg·mL^{-1}. This protein concentration reflects the plasma level *in vivo*. The final LP105 concentrations were 0.3, 1, 3, 10 and 30 µg·mL^{-1}.

Aliquots (2 mL) of protein-enriched buffer fortified with LP105 were placed in ultracentrifugation tubes and allowed to equilibrate at a shaking water bath for at least 2 h at room temperature. The tubes were placed in a 70.1Ti rotor and centrifuged in a Beckman Optima LE-80 K centrifuge for 15 h at 37°C and 150 000× *g*. After centrifugation 300 µL aliquots of supernatant were carefully removed from the upper first mL which is protein-free and subjected to HPLC analysis.

Aneurysm induction

ApoE$^{-/-}$ mice were given a 4 week infusion of AngII (1.44 mg·kg^{-1}·body weight^{-1}·day^{-1}) via subcutaneously implanted osmotic minipumps. After 4 weeks, mice were killed; the surrounding tissue of each aorta was dissected and aortas were photographed and weighed. Aneurysm severity was classified according to Daugherty *et al.* (2001) by observers unaware of the treatments.

Systolic blood pressure and heart rate measurement

Systolic blood pressure was determined every day, beginning 1 week before implantation of the minipumps and continuing for the duration of the study, using a computerized tail-cuff system (BP-2000, Visitech Systems, Inc., Apex, NC). To avoid procedure-induced anxiety, mice were initially accustomed to the instrument for five consecutive days before the actual recorded measurements. Moreover, the first 10 of 20 blood pressure values recorded at each session were disregarded, and the remaining 10 values were averaged and used for analysis. A minimum number of three successful individual measurements per day and per group were required for data inclusion in statistical analysis.

Blood sample analyses

Blood samples were collected by transcardial puncture with non-filled (for aneurysm study) or with heparin-coated syringes (for pharmacokinetic studies). LOX and soluble epoxide hydrolase (sEH) substrates and metabolites (5-HETE, LTB_4, *trans*-LTB_4, epi-*trans* LTB_4, 15-HETE, 14,15-EET, 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), 5,6-EET, 5,6-DHET, 11,12-DHET, 8,9-DHET) were extracted and analysed by HPLC and LC-MS/MS as described previously (Revermann *et al.*, 2009).

sEH activity assay

The sEH activity was assayed using a previously described method (Barbosa-Sicard *et al.*, 2009) with minor modifications in murine liver microsomes. Reactions were performed at 37°C for 20 min in a total volume of 100 µL of potassium phosphate buffer (100 mmol·L^{-1}, K_2HPO_4/KH_2PO_4, pH 7.2), containing 5 µg of protein. Reactions (37°C) were started by the addition of 14,15-EET (10 µmol·L^{-1}) and were stopped by

putting the reaction on ice and adding ethyl acetate. Experiments were performed in the absence and presence of the solvent [1% dimethyl sulphoxide (DMSO)], sEH inhibitor 1-adamantyl-3-cyclohexylurea (ACU; 10 μmol·L^{-1}) and the 5-LOX inhibitor LP105 (10 μmol·L^{-1}).

PCR analysis

Organs were removed, shock frozen and powdered in liquid nitrogen. RNA was isolated using RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). cDNA synthesis was performed applying SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers, semiquantitative real-time PCR with ABsolute QPCR SYBR Green Mix and ROX as reference dye (Thermo Scientific) in Mx4000 (Stratagene) with appropriate primers. Relative expressions of target genes were normalized to eukaryotic translation elongation factor 2 (EEF2), analysed by delta-delta-Ct method and given as percentage compared to control experiments. PCRs for cytochrome p450 (CYP)2C genes were done without SYBR Green but with fluorescent labelled probes. Primer and probe sequences: ALOX5: forward: 5′-CGGCTTCCCTTT GAGTATT GATGC-3′; reverse: 5′-CAGGAACTGGTAGCCAAACATGAG-3′. ALOX15: forward: 5′-GACACTTGGTGGCTGAGGTCTT TG-3′; reverse: 5′-GCTCCAGCTTGCT TGAGAAGATCC-3′. PLA2G4A: forward: 5′-GTTCTACGTGCCACCAAAGTAACC-3′; reverse: 5′-TCCATGACGTAGTTGGCATCCATC-3′. CYP2C38: forward: 5′-CCCA CTCCTTTCCCGATTAT-3′; reverse: 5′-CAGAAAACTCCTCCCCATGA-3′; probe: 5′-CY5-ATCAGAGCTTCCTTCACTGCTTCATACCCA-3′. CYP2C40: forward: 5′-AGGT CCAGCGGTACATTGAC-3′; reverse: 5′-CA CAAATCCGTTTTCCTGCT-3′; probe: 5′-FAM-TTCATCCTCA AGGGAACACAGGTAA-3′. CYP2C44: forward: 5′-CAAAAAG GC TTGGTGGTGTT-3′; reverse: 5′-CCACAGATGGCCAAA TTCTC-3′; probe: 5′-FAM-TTACATCGACTGTTTCCTCAGC AAGAT-3′. EEF2: forward: 5′-GACATCACCAAG GGTGT GCAG-3′; reverse: 5′-GCGGTCAGCACACTGGCATA-3′. SCNN1G: forward: 5′-ATGCTTCCAAACGAAGATGG-3′; reverse: 5′-AGTTGGGGTGTTGCTGGTAG-3′. CPT1A: forward: 5′-CTCAGTGGGAGCGACTCTTCA-3′; reverse: 5′-GGCCTCT GTGGTACACGACAA-3′; FATP: forward: 5′-CGTGAAGGCTT TGACCCCCGT-3′; reserve: 5′-ACAAATGCGGGCATGGAC TCTCTC-3′.

Data and statistical analysis

All values are mean SEM. Statistical analysis was performed using analysis of variance (ANOVA) followed by Fisher's Least Significant Difference *post hoc* test, unpaired *t*-test and Mann–Whitney test. A *P*-value of less than 0.05 was considered as statistically significant.

Materials

LP105 [2-{5-[3,5-bis-(2,2,2-trifluoro-ethoxy)-phenylamino]-4-chloro-pyrimidin-2-ylsulfanyl-octanoic acid] was synthesized as previously reported (Popescu *et al.*, 2007). The sEH inhibitor 1-adamantyl-3-cyclohexylurea (ACU) was from Sigma and angiotensin II was from Bachem, Bubendorf, Switzerland. Drug and receptor nomenclature follows Alexander *et al.*, 2009.

Results

LP105 attenuates 5-LOX activity in vitro

In order to establish LP105 as a 5-LOX inhibitor, we determined its acute effect on 5-LOX activity in the murine monocyte/macrophage cell line RAW 264.7. LP105 reduced 5-LOX activity in a dose-dependent manner with an IC_{50} around 2 μM (Figure 1A). Also in the S100 extract of RAW 264.7 cells, LP105 was an effective 5-LOX inhibitor, although with a higher IC_{50} value of 10 μM. In rat aortic tissue, the inhibitory effect of LP105 on LTB_4 production was similar to that observed in RAW 264.7 cells. Moreover, in rat aortic tissue, 5(S)-HETE production, as a measure for 5-LOX activity was inhibited with a similar IC_{50} whereas LP105 had no effect on 12(S)-HETE and 15(S)-HETE formation (data not shown). These observations characterize LP105 as a direct 5-LOX inhibitor and not as a compound that interferes with other regulatory components of cellular 5-LOX product formation.

Oral administration of LP105 to mice results in plasma levels sufficient to suppress 5-LOX product formation

As our goal was to test LP105 in murine vascular disease models *in vivo*, different preparations of the compound were tested in a limited pharmacokinetic screen. Subcutaneous injection of the compound as 200 μL of a 100 μM solution failed to yield detectable concentrations. In contrast, after oral administration, adequate plasma levels were detected in all preparations applied (Figure 1B). Among these preparations, the highest plasma concentrations were obtained in response to gavage of 5 mg LP105 dissolved in corn oil. After oral gavage, however, plasma level declined quickly with an estimated half-life of 4 h. As prolonged feeding of corn oil might have cardiovascular side effects and as mice continuously ingest food during the night, administration via the rodent chow had clear advantages. Plasma levels of LP105 in mice given chow containing 1 mg LP105·g^{-1}, remained above the IC_{50} of 2 μM for intact cells even after 12 h of fasting. This mode of administration was therefore chosen for our experiments, allowing continuous food intake overnight by the mice.

In order to estimate efficiency of this approach, we measured albumin binding and tissue concentration of LP105 in the heart. Although albumin binding was >97% (n = 5), the cardiac concentrations of LP105 measured at the end of the treatment period of the ApoE mice was measured as 69.1 ± 12.1 μmol·kg^{-1} tissue (n = 6). These data suggest that LP105 accumulated in cells and that, despite the high albumin binding, therapeutic levels could be attained intracellularly.

LP105 attenuates vascular remodelling

In order to determine whether LP105 has inhibitory effects on cardiovascular disease development, we treated ApoE$^{-/-}$ mice with AngII for 4 weeks and gave the compound in chow, to a subgroup of animals. AngII induced a marked vascular inflammation in ApoE$^{-/-}$ mice which resulted in vascular hypertrophy and, as a consequence of the macrophage activation, in formation of aortic aneurysms (Figure 2A,B). Indeed, vascular weight, as a marker for hypertrophy and vessel diameter which characterizes aneurysm formation,

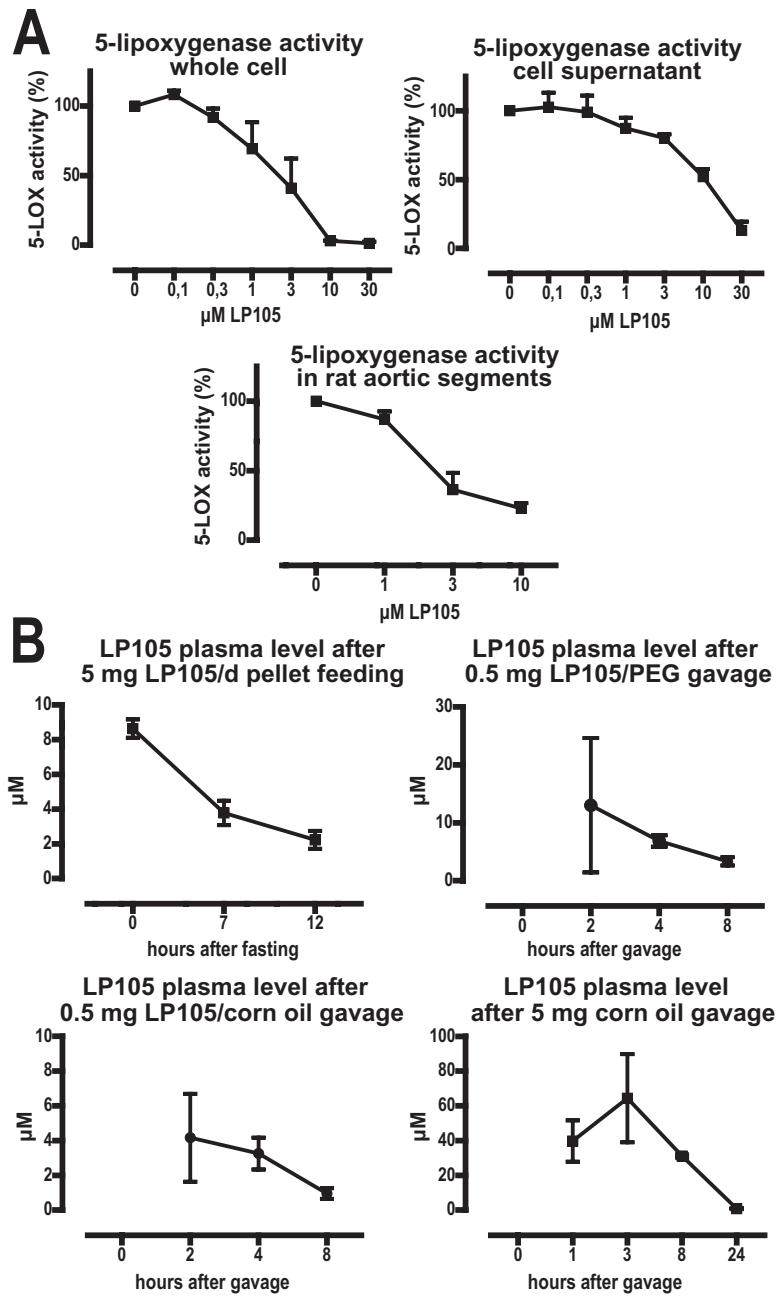

Figure 1

Inhibition of 5-LOX product formation by LP105 *in vitro* (A) and (B) pharmacokinetics of LP105 after oral administration in mice. (A) The assay was performed in intact RAW 264.7 cells (left, total activity 9.1 ± 1.1 ng per 10^6 cells) and in 100 000× *g* supernatants of homogenates of the cells (right, total activity 21.8 ± 1.2 ng per 10^6 cells) or rat aortic segments (bottom). (B) Mice were treated with LP105 as shown and LP105 in plasma determined at the time points indicated. Mean ± SEM, $n = 3$.

were increased in AngII-treated ApoE$^{-/-}$ mice, compared with control animals. Moreover, AngII treatment resulted in four deaths from spontaneous aortic rupture of eight animals treated, whereas in the group co-treated with LP105 only one mouse died. Less severe aneurysms appeared to be more frequent in the LP105 than in the AngII only group when classified according to Daugherty (Daugherty *et al.*, 2001) (Figure 2C). The weight of the abdominal aorta in animals treated with AngII was significantly lower in mice co-treated with LP105 (Figure 2D). Moreover, the aortic diameter was significantly increased in ApoE$^{-/-}$ mice treated with AngII but not in animals treated with the combination of AngII and LP105 (Figure 2E). Collectively, these data suggest that LP105 attenuated vascular remodelling and aneurysm formation.

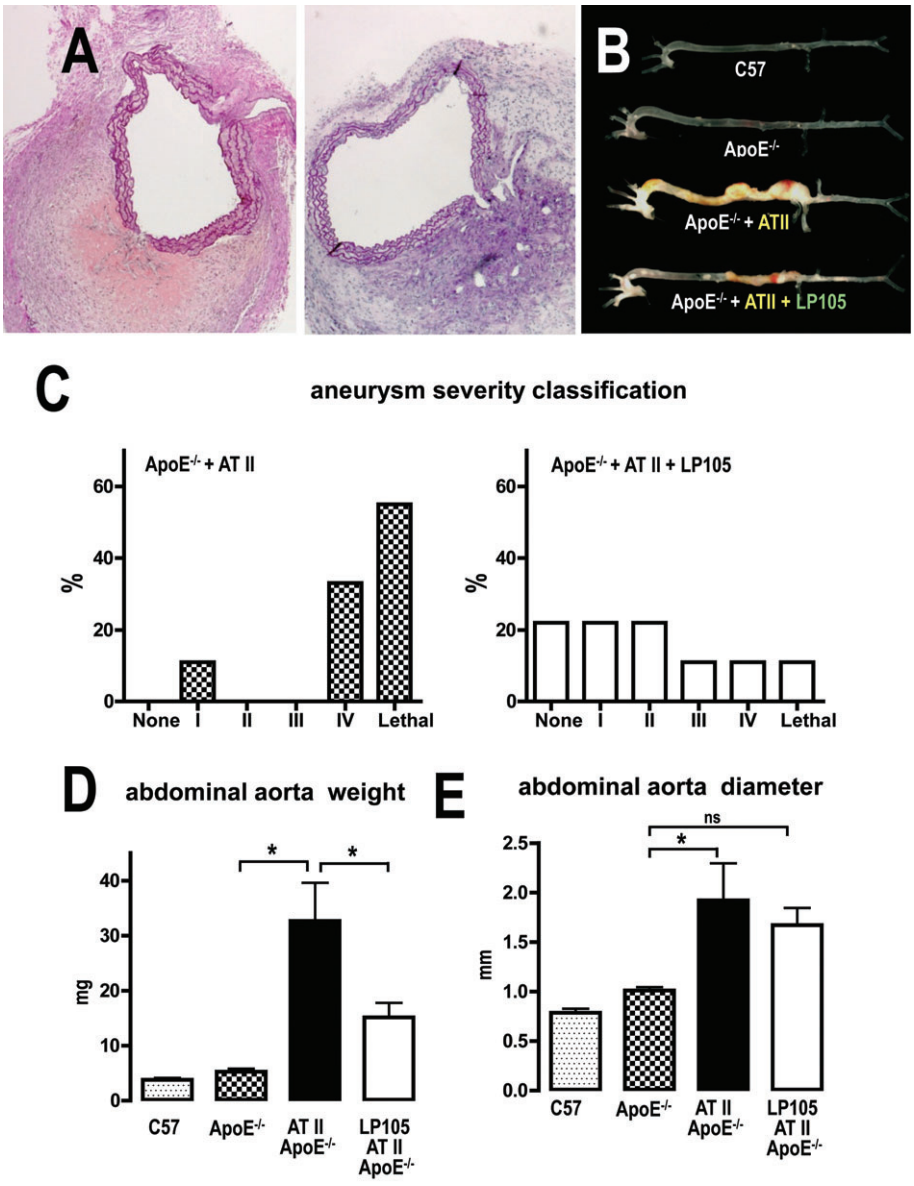

Figure 2

Effect of angiotensin II (ATII) and LP105 on aortic aneurysm formation: Representative histological section of aortic aneurysms occurring in the ATII-treated group (Elastica van Gieson staining) (A). Aneurysm formation was studied in ApoE$^{-/-}$ mice treated with (ApoE$^{-/-}$/ATII) or without (ApoE$^{-/-}$) AngII (1.44 mg·kg^{-1}·bw^{-1}·day^{-1} via osmotic minipump) and with (ApoE$^{-/-}$/ATII/LP105) or without LP105 (1 mg·g^{-1} chow) for 4 weeks. Representative photographs (B), survival and aneurysm severity score (C), abdominal aortic weight (D) and abdominal aortic diameter (E) are also shown. C57: C57/BL6 control mice, Mean ± SEM,*P < 0.05, ns = not significant, n = 4–8 animals per group.

LP105 lowers heart rate but not blood pressure in ApoE$^{-/-}$ mice

The AngII-induced vascular remodelling process is largely dependent on local inflammation, but AngII-induced hypertension also contributes to the process. We therefore determined whether LP105 affected blood pressure. As shown in Figure 3A, this was not the case. Unexpectedly, LP105, however, lowered the heart rate in mice under basal conditions as well as during AngII treatment (Figure 3C). Moreover, AngII induced a significant cardiac hypertrophy as determined by heart to body weight ratio and this effect was absent from the group co-treated with LP105 (Figure 3B). From the experimental design, it is however impossible to extrapolate whether this is either a consequence of an attenuated hypertrophy response to AngII or of the reduced cardiac work as a result of the decreased heart rate.

LP105 lowers aortic inflammation

In order to understand the molecular mechanisms underlying the protective effect of LP105, we determined inflammatory markers by qRT-PCR in aortic sections not directly affected by the aneurysms (upper thoracic aorta) (Figure 4).

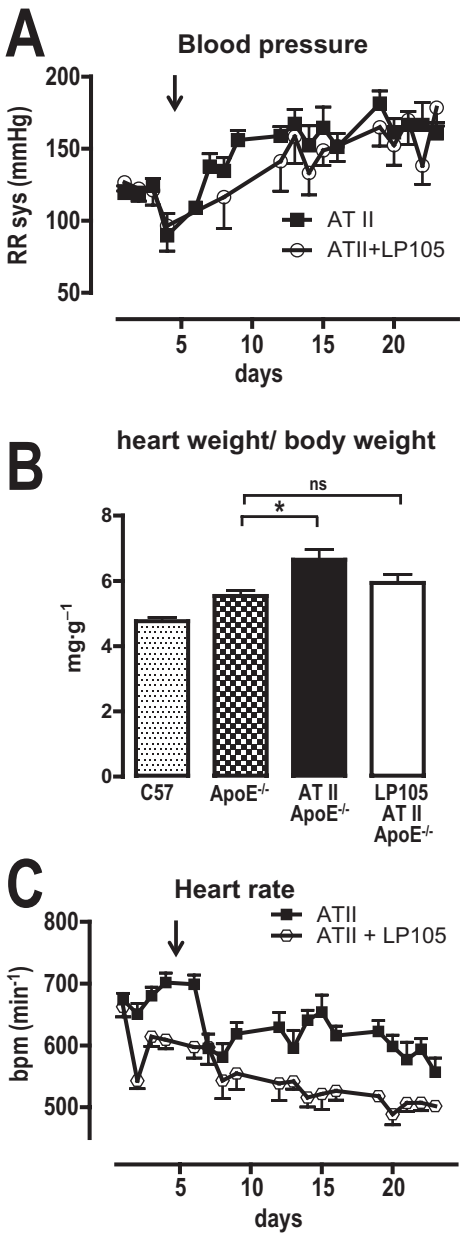

Figure 3

Effect of LP105 on cardiac parameters: (A) Blood pressure (RR sys) as measured by tail cuff technique in ApoE$^{-/-}$ mice. At the time point indicated by the arrow, AngII treatment (ATII, 1.44 mg·kg^{-1}·day^{-1} via osmotic minipump) was started. LP105 feeding (1 mg·g^{-1} chow) was initiated on day 1 of the experiment. (B) Heart to body weight ratio after 4 weeks of treatment. (C) Heart rate obtained during blood pressure measurements. Mean ± SEM,*$P < 0.05$, ns = not significant, $n = 4$–8 in each group per day.

Using this aortic tissue, we could exclude the marked effects on gene expression of deleterious processes such as thrombus formation and dissection within the aneurysms. AngII induced a significant increase of the aortic mRNA expression of TGFβ$_1$, IL-6, TNFα and IL-1β. The latter two are macrophage-derived and indeed, the expression of the macrophage marker EMR1 (F4/80) paralleled that of the two cytokines. Importantly, these effects of AngII were not observed in the group receiving LP105. In contrast, LP105 failed to prevent MMP2 induction by AngII, whereas there was a trend towards MMP9 down-regulation which, however, did not reach significance level.

LP105 lowers LTB$_4$ plasma levels

In order to ascertain that LP105 treatment was effective against 5-LOX *in vivo*, LTB$_4$ plasma levels were measured. Unfortunately, in the ApoE-AngII group, these values were highly variable which resulted in non-significant differences. A direct comparison between all treated against non-treated animals, however, showed that LP105 was effective in lowering the LTB$_4$ levels (from 2.7 ± 0.3 to. 1.6 ± 0.4 ng·mL^{-1}, $P < 0.05$). We also determined plasma levels of 15-HETE, which is produced by 15-LOX and also by non-enzymatic oxidation of arachidonic acid. Surprisingly, 15-HETE plasma levels were increased suggesting that LP105 shifted arachidonic acid metabolism from the 5-LOX to the 15-LOX pathway (Figure 5A).

LP105 increases 14,15-EETs

Within the range of arachidonic acid metabolites, particular EETs have vasoprotective properties. Bearing in mind the possible shift in arachidonic acid metabolism, the plasma levels of 14,15-EET and 5,6-EET were measured. Importantly, we observed a significant increase in 14,15-EET and a trend towards enhanced level of 5,6-EETs in the plasma of LP105-treated animals (Figure 5B). The levels of the degradation products of the EETs studied, the DHETs were not affected by LP105 (Figure 5C). Unlike the 14,15-EETs, 5,6-EETs are also metabolized by COX, suggesting that at least for the plasma level of arachidonic acid derivatives, a substantial LP105-mediated inhibition of COX isoforms was not very likely.

14,15-EETs are hydrolysed by the enzyme sEH, and LP105-mediated inhibition of sEH could be a potential explanation for the increase in 14,15-EETs. Measurements in liver homogenates, however, revealed that the compound at a concentration of 10 µmol·L^{-1} had no effect on sEH activity, whereas the standard sEH inhibitor ACU at 1 µmol·L^{-1} achieved a 90% inhibition of the enzyme. Moreover, treatment with LP105 had no effect on the sEH mRNA expression in the kidney of ApoE$^{-/-}$ mice receiving AngII infusion. These data exclude the sEH pathway as a direct target of LP105 (Figure 6A).

In order to determine whether the increased formation of EETs is a consequence of an altered expression of EET generating enzymes, mRNA expression studies were performed in the kidney, a site of high EET generation. LP105, however, did not increase 5-LOX, 15-LOX, sPLA$_2$, CYP2C38, CYP2C40 or CYP2C44 expression (Figure 6B). This suggests that the increased formation of EETs and 15-LOX products is either a consequence of a shifting of arachidonic acid into these pathways or an increased activation of phospholipase A$_2$.

Finally, we determined whether LP105 had effects on PPAR-regulated genes (Figure 6C). In the presence of AngII, LP105 resulted in a down-regulation of the expression of epithelial sodium channel γ subunits (SCNN1G) and an induction of the solute carrier family 27 member 1 (SLC27A1), whereas LP105 had no effect on the expression of

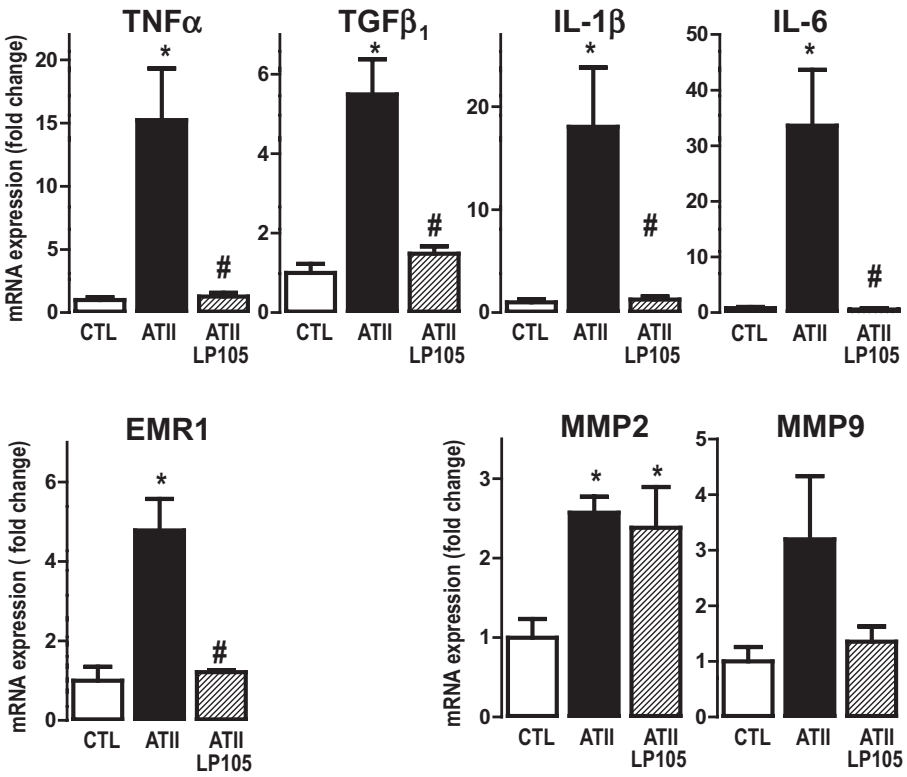

Figure 4

Effect of LP105 on aortic gene expression: mRNA expression of TNFα, transforming growth factor β1 (TGFβ$_1$), IL-1β, IL-6, the macrophage marker EMR1, MMP2 and MMP9, as determined by quantitative RT-PCR from the thoracic aorta. The mRNA expression was measured by the 2^-ΔΔCT technique and is expressed as fold increase of control (CTL). Mean ± SEM, n = 4–6, *P < 0.05 vs. CTL #P < 0.05 vs. angiotensin II (ATII).

the carnitine palmitoyltransferase IA (CPT1A). SCNN1G and CPT1A are thought to be induced by PPARγ and PPARα and SLC27A1 by PGC1α. These divergent effects suggest that LP105 is unlikely to elicit a uniform stimulatory effect on PPAR signalling.

Discussion

In the present study, we have characterized the pirinixic acid derivative LP105 as a 5-LOX inhibitor in mice *in vitro* and *in vivo* and studied its effect on vascular function. We could demonstrate that the compound inhibited 5-LOX in cellular homogenates, intact monocytic cells, vascular segments and *in vivo*, and that pharmacologically relevant LP105 plasma and tissue levels could be attained by giving the compound in the normal diet. Moreover, LP105 prevented vascular disease development and inflammation in ApoE$^{-/-}$ mice treated with AngII, without affecting the AngII-induced hypertensive response. The compound also increased the level of 15-HETE and 14,15-EET, but the latter effect was not a consequence of a direct inhibition of the sEH or a modulation of sEH and 15-LOX mRNA expression.

As we had previously found that some pirinixic acid derivatives interfered with pro-inflammatory signalling pathways, for example 5-LOX, mPGES-1 and COX-1 and activated PPARγ-activity *in vitro*, we investigated whether LP105 could be exploited therapeutically *in vivo*. We here report that LP105 inhibited the murine 5-LOX pathway with a potency similar to that against the human enzyme (Werz *et al.*, 2008). The IC$_{50}$ values in cultured cells, vascular tissue and the cell-free assays were all comparable, indicating that the compound enters the cells in adequate amounts and that it acts as a direct 5-LOX inhibitor rather than interfering with cellular components or processes that regulate 5-LOX activity. Although albumin binding of LP105 was high, tissue levels of the compound were well above the IC$_{50}$ for 5-LOX. With such properties LP105 fills an important gap in strategies to inhibit LT signalling as only one direct 5-LOX inhibitor, zileuton, is currently available on the market (Peters-Golden and Henderson, 2007). Indeed, most strategies to interfere with LTs either focused on LT receptor antagonists, such as montelukast (Riccioni *et al.*, 2008) or on the inhibition of the 5-LOX activating protein (FLAP inhibitors) (Evans *et al.*, 2008). Other frequently used compounds like nordihydroguaiaretic acid have strong anti-oxidant properties (Chen, 2009) or, like MK-886, strongly affect the PPARs (Kehrer *et al.*, 2001). Interestingly, LP105 down-regulated the γ-subunit of the epithelial sodium channel, which is under the control of PPARγ and PPARα (Guan *et al.*, 2005; Mittra *et al.*, 2007), but induced the transporter SLC27A1, whereas it had no effect on CPT1A, suggesting that activation of the PPARγ or PPARα pathways was not the primary mode of action of LP105. This should, however, not imply that the effects of LP105 in the

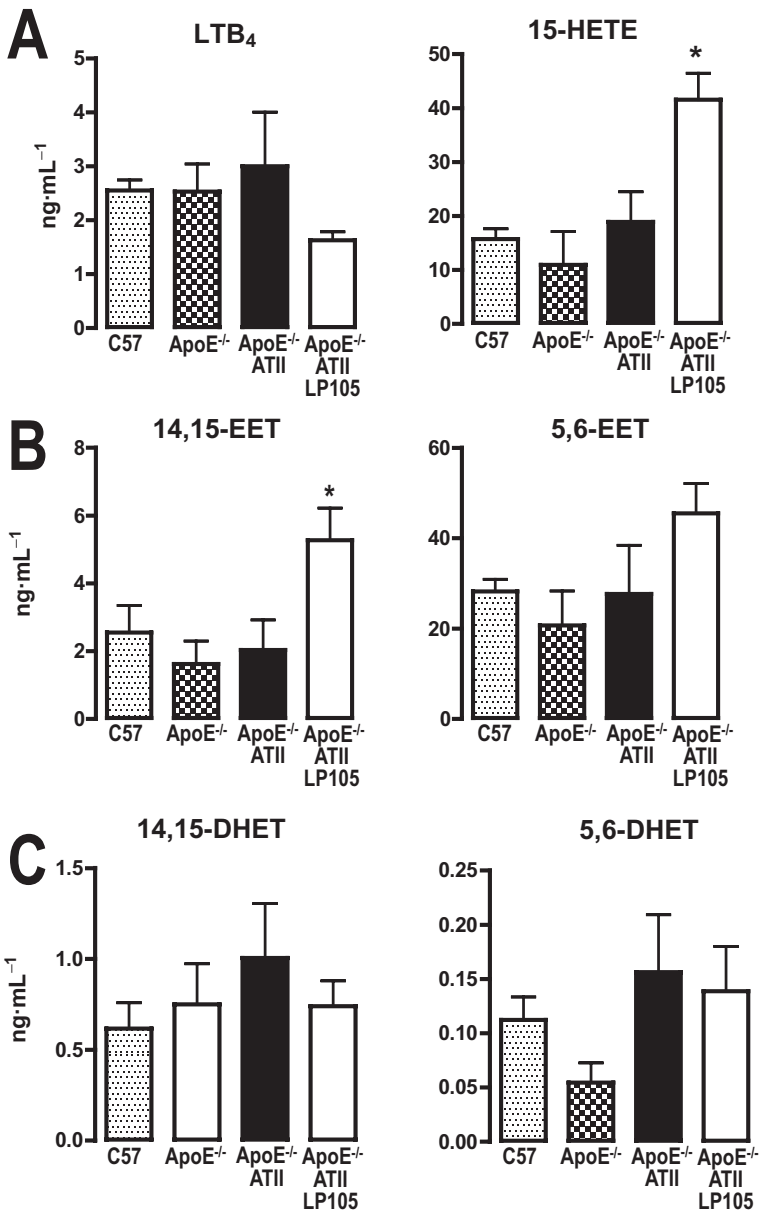

Figure 5

Plasma concentrations of arachidonic acid metabolites. Plasma concentrations of the lipids indicated as measured by LC/MS-MS in C57/BL6 control mice (C57) and ApoE$^{-/-}$ mice treated with or without AngII (ATII by osmotic minipump, 1.44 mg·kg^{-1}·bw^{-1}·day^{-1}) and with or without LP105 (1 mg·kg^{-1} chow) after 4 weeks of treatment. Mean ± SEM, *$P < 0.05$ (15-HETE & 14,15-EET: ApoE$^{-/-}$/ATII vs. ApoE$^{-/-}$/ATII/LP105). $n = 4$–8.

present study were necessarily due to 5-LOX inhibition only, as mPGES-1 and COX-1 are central for inflammatory responses. Genetic knockout of mPGES-1 attenuated aneurysm development in LDL-receptor knockout mice treated with AngII and high fat diet (Wang *et al.*, 2008), and a role of COX for aneurysm development has been suggested, although this effect was predominantly linked to COX-2 (Holmes *et al.*, 1997; Gitlin *et al.*, 2007). Thus, it is a limitation of the present study that the effect of LP105 on aneurysm development was not compared to that of a FLAP inhibitor. Another limitation is that we did not treat control mice with LP105. This decision was based on the fact that remodelling does not occur in the absence of AngII but complicates the interpretation of the changes in plasma lipids.

Indeed, despite the theoretical background for 5-LOX being a causal factor for aneurysm development, the experimental situation is less clear. Genetic knockout of 5-LOX in ApoE$^{-/-}$ mice, given a high fat diet, attenuated aneurysm formation but the total disease activity in the model was somewhat low (Zhao *et al.*, 2004). Similarly, AngII-induced aneurysm formation in ApoE$^{-/-}$ mice was attenuated after genetic deletion (Ahluwalia *et al.*, 2007) or pharmacological inhibition (Kristo *et al.*, 2010) of the BLT1-receptor, which is the predominant receptor for LTB_4 in some leukocytes. In

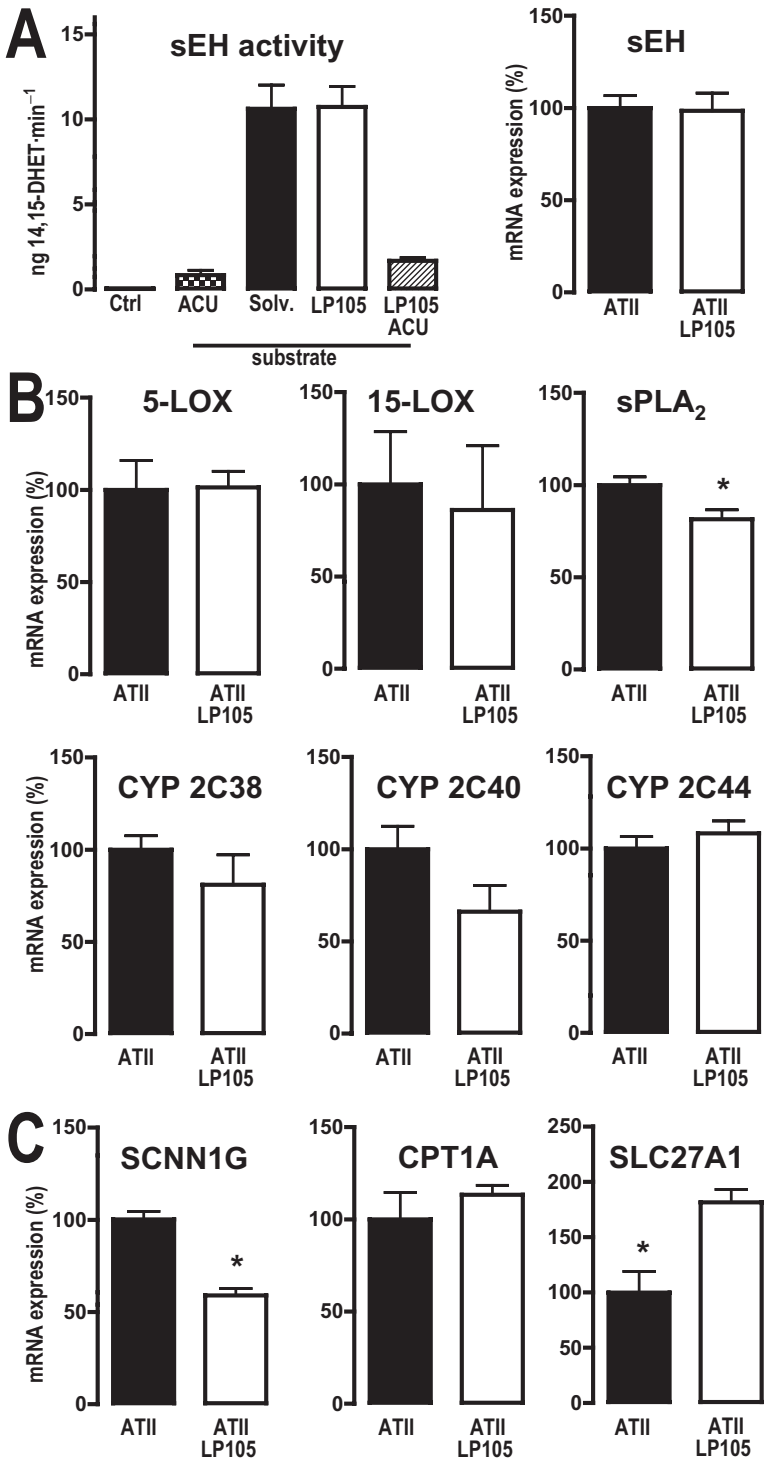

Figure 6

Effect of LP105 on arachidonic acid metabolizing enzymes. (A) Effect of LP105 (10 μmol·L^{-1}) and the soluble epoxide hydrolase (sEH) inhibitor 1-adamantyl-3-cyclohexylurea (ACU; 10 μmol·L^{-1}) on the sEH activity as measured by the conversion of 14,15-EET to 14,15-DHET in liver microsomes and renal sEH mRNA expression of ApoE$^{-/-}$ mice treated with AngII (1.44 mg·kg^{-1}·bw^{-1}·day^{-1}, 4 weeks via osmotic minipumps) with and without LP105 (1 mg·g^{-1} chow). (B) Renal mRNA expression of 5-lipoxygenase (5-LOX), 15-LOX, soluble phospholipase A_2 (sPLA2), cytochrome P450 isoforms CYP2C38, CYP2C40 and CYP2C44 as determined by quantitative RT-PCR and (C) of the PPAR-responsive genes, epithelial sodium channel γ subunits (SCNN1G), carnitine palmitoyltransferase IA (CPT1A) and solute carrier family 27 member 1 (SLC27A1). The mRNA expression was measured by the 2^-ΔΔCT technique. Mean ± SEM, n = 4–6, *P < 0.05.

contrast, knockout of 5-LOX and FLAP inhibition failed to have an effect on aneurysm formation in ApoE$^{-/-}$ mice treated with AngII (Cao *et al*., 2007). The conflicting results between BLT1 blockade and 5-LOX knockout are potentially mediated by a shifting of the LT signalling after selective blockade of only one of the four LT receptors (Peters-Golden and Henderson, 2007). Alternatively, it should be mentioned that there were some discrepancies between the AngII model in the ApoE-5-LOX double knockout study (Cao *et al*., 2007) and our current work. For instance, the original model of aneurysm used mice at 6 months (Daugherty *et al*., 2000), whereas the study by Cao *et al*. used mice as young as 8 weeks. Moreover, the incidence of aneurysms was relatively low (approx. 30%) and a lower amount of AngII (0.7 mg·kg^{-1}·bw^{-1}·day^{-1}) was infused compared to the 1.44 mg·kg^{-1}·bw^{-1}·day^{-1} administered in the present study. Consequently, the vascular changes in the present study were not only the consequence of vascular distention or enlargement but also of aortic rupture. Irrespective of these differences, we cannot exclude the possibility that the predominant part of the effect of LP105 was mediated by potential additional effects.

It was an unexpected observation of the present study that LP105 significantly lowered the heart rate in mice. Although we can only speculate about the underlying mechanism, it is interesting to note that this had no effect on blood pressure suggesting that LP105 may increase the peripheral resistance, which is in line with our difficulties to measure blood pressure by tail cuff technique (M. Revermann, unpubl. obs.). As the tail cuff technique is however influenced by many factors, we currently cannot address this aspect usefully. Despite this effect, the AngII-induced cardiac remodelling, as determined by heart weight/body weight ratio, was largely prevented by LP105. This effect is potentially a consequence of the negative chronotropic action of the compound, as an augmented cardiac output through the Frank-Starling mechanism optimizes cardiac performance. To our knowledge, the direct effect of the LOX isoforms and leukotrienes on cardiac hypertrophy has not been reported previously.

An interesting observation of this study was that LP105 treatment increased the plasma level of 15-HETE and 14,15-EETs. Given that the renal mRNA expression of the enzymes involved in this pathway were not altered by LP105, these data suggest that the effects observed are a consequence of a shifting of arachidonic acid metabolism away from COX and 5-LOX towards the 15-LOX pathway. This suggestion, however, is based on the assumption that renal mRNA expression of these enzymes reflects the activity in other organs and that plasma levels of 15-HETE and 14,15-EET would correlate with tissue activities. Unfortunately, due to the very limited amount of tissue available from the mouse aorta, we cannot confirm whether changes in plasma lipids or the renal gene expression reflect the situation in the vasculature. In previous studies, we tried to measure vascular concentrations of EETs, which, however, was unsuccessful due to the low abundance of the compounds and the limited amount of murine tissue available (Revermann *et al*., 2010). In the present work, we could exclude the direct inhibition of sEH by LP105 or that the compound, at least in the kidney, down-regulates sEH protein. In accordance with this observation, AngII, at least in endothelial cells, is known to induce sEH (Ai *et al*., 2007).

Several of the observations in this study are compatible with a beneficial action of EETs. These arachidonic acid derivatives are known to prevent vascular remodelling (Revermann *et al*., 2010) and to reduce plaque formation in ApoE$^{-/-}$ mice treated with AngII (Ulu *et al*., 2008). Moreover, increasing EET levels by inhibition of sEH prevented AngII-induced cardiac hypertrophy (Ai *et al*., 2009). The role of increased plasma level of 15-HETE are less clear. 15-HETE elicits a broad spectrum of effects, some potentially beneficial, such as the activation of PPARs (Naruhn *et al*., 2010) or induction of angiogenesis, others more pro-inflammatory (Ma *et al*., 2010). Importantly, we observed a strong anti-inflammatory effect of LP105, which is compatible with increased EET signalling or inhibition of the 5-LOX system.

In conclusion, in the present study we report that the newly developed 5-LOX inhibitor LP105 interfered with development of aneurysms in the AngII-infusion model in ApoE$^{-/-}$ mice. LP105 elicited multiple potential beneficial effects, such as reduction of inflammation and increasing EET plasma levels.

Nevertheless, we currently cannot prove that these effects induced by LP105 are a direct consequence of the reduction of 5-LOX products and/or of a shift in the arachidonic acid metabolic cascade, induced by inhibition of 5-LOX. Additional work is required to dissect the mechanism of the protective effect of the compound. Despite this, LP105 is a promising novel therapeutic agent for vascular inflammatory diseases.

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Conflicts of interest

None.

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