Supplementary Information

Androgen-mediated sex bias in the efficiency of leukotriene biosynthesis inhibitors

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Supplemental Materials and Methods

Materials

The following compounds were generous gifts: BWA4C by Dr. L. G. Garland (Wellcome Res. Laboratories, Beckenham, Kent, UK), ZM230487 by Dr. R. M. McMillan (Zeneca Pharmaceuticals, Macclesfield, UK), licofelone by Merckle GmbH (Ulm, Germany), MK886 and MK591 by Dr. A. W. Ford-Hutchinson and Dr. Richard Friesen, respectively (Merck Frosst, Kirkland, Canada). Celecoxib was synthesized by WITEGA Laboratorien Berlin-Adlershof GmbH (Berlin, Germany). Ethyl 2-(3-chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (compound 11a) was synthesized by Dr. R. Troschütz, University Erlangen (Erlangen, Germany). Bay-X 1005 was from Tocris Bioscience (Bristol, UK). The cPLA$_2\alpha$ inhibitor *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide · HCl (indicated in the text as pyrrolidine-1 derivative, RSC-3388) was from Calbiochem (Bad Soden, Germany). PAF (C-16) was from Cayman Chemical (Ann Arbor, MI). Zileuton was from Sequoia Research Products (Oxford, UK). AA-861, Ssi, fMLP, LPS, 5α-DHT, λ-carrageenan type IV isolated from *Gigartina aciculaire* and *Gigartina pistillata* and all other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany), unless stated otherwise. HPLC solvents were from VWR (Darmstadt, Germany).

Determination of LO products by UPLC-MS/MS

Chromatography was carried out on an Acquity UPLC BEH C_{18} column (1.7 µm, 2.1 × 50 mm, Waters, Milford, MA) using an Acquity^{TM} Ultraperformance LC system (Waters, Milford, MA, USA). Eicosanoids (4 µL injection) were separated at a flow rate of 0.8 mL min^{-1} and a column temperature of 45 °C. The solvents for the mobile phase were acetonitrile (A) and water/acetonitrile (90/10; B) both acidified with 0.07% (v v^{-1}) formic acid. Isocratic elution at A/B = 30% was performed for 2 min and followed by a linear gradient to 70% B within 5 min.

The LC system was coupled to a QTRAP 5500 Mass Spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo V™ Source and ESI probe. Eicosanoids were detected by multiple reaction monitoring in the negative ion mode using a dwell time of 10 ms. The ion spray voltage was set to 4500 V, the heater temperature to 500 °C, the declustering potential to 50-120 eV, the entrance potential to 10 eV and the collision cell exit potential to 11-22 eV, the spray gas pressure to 50 psi, the Turbo V gas pressure to 80 psi and the curtain gas pressure to 20 psi. Monitored transitions of eicosanoids (Q1 and Q3) and their collision energies are given in supporting Table S3. The transition first mentioned ('transition 1') was used for quantification. Automatic peak integration was performed with Analyst 1.6 software (AB Sciex, Darmstadt, Germany) using IntelliQuan default settings. Data were normalized on the internal standard PGB_1 and are given as relative intensities. The reported method was optimized for analysis of the comparison of eicosanoid levels between male and female samples and not for absolute quantification.

Extraction and determination of MK886 by UPLC-MS/MS

MK886 was extracted from plasma by solid phase extraction (SPE) following the procedure described for extraction of 5-LO products, with minor alterations. In brief, 400 µL of plasma were added to 1 mL of methanol and kept over night at -20°C to precipitate the plasma protein. After centrifugation (2000×g, 10 min, 4°C), the supernatant was diluted with 6 mL of acidified (pH 3.5) water and subjected to a C18-SPE column. After washing with water, the samples were eluted with 500 µL of methanol and dried (nitrogen stream). The residue was dissolved in 50 µL of 50% aqueous methanol. Chromatography was carried out on an Acquity UPLC BEH C_{18} column (1.7 µM, 2.1 × 50 mm) using an Acquity^{TM} Ultra Performance liquid chromatography system from Waters (Milford, MA). After injection of 10 µL sample, MK886 was separated at 0.8 ml/min and 50°C by isocratic elution with 70% eluent A (acetonitrile/water/0.07% HCOOH) for 2 min followed by a linear gradient to 100% eluent B

(acetonitrile/0.07% HCOOH) within 7 min. The chromatography system was coupled to a QTRAP 5500 Mass Spectrometer (Sciex, Darmstadt, Germany) equipped with an electrospray ionization source. For identification, the precursor-to-product ion transitions in multiple reaction monitoring were m/z 470 → 258 (collision energy: -51 eV), 470 → 395 (collision energy: -33 eV), 470 → 380 (collision energy: -39 eV), 470 → 244 (collision energy: -40 eV). Quantification of MK886 was based on the transition m/z 470 → 258 based on an external calibration curve ($R^2 = 0.999$). The ion spray voltage was set to -4500 V, the heated capillary temperature to 650°C, the curtain gas pressure to 35 psi, the sheath gas pressure to 60 psi, the auxiliary gas pressure to 50 psi, the declustering potential to -109 V and the entrance potential to -10 V. Analyst software 1.6 (Sciex, Darmstadt, Germany) was used for the processing of analytical data.

Carrageenan-induced pleurisy in rats

Zileuton, MK886 or vehicle (1.5 mL of 0.9% saline solution containing 4% DMSO) were given i.p. at the indicated doses 30 min before λ-carrageenan. Rats were anaesthetized with enflurane 4% mixed with O_2, 0.5 L min^{-1}, and N_2O, 0.5 L min^{-1} and subjected to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 mL) or λ-carrageenan type IV 1% (w v^{-1}; 0.2 mL) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. The animals were killed by inhalation of CO_2 at the indicated time point after the injection of λ-carrageenan. The chest was carefully opened, and the pleural cavity was rinsed with 2 mL saline solution containing heparin (5 U mL^{-1}). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the injected volume (2 mL) from the total volume recovered. The amounts of LTB_4 in the supernatants of centrifuged exudates (800 × *g* for 10 min) were assayed by an enzyme immunoassay kit (Cayman Chemical,

Ann Arbor, MI), according to manufacturer's instructions. All vehicle controls were summarized (n = 35). The results are expressed as ng per rat.

Mouse PAF–induced mortality

PAF C-16 was dissolved in chloroform and stored at -20 °C. The PAF working solution was freshly prepared prior use. To this aim, chloroform was evaporated under N_2 and PAF was dissolved in 0.9% saline solution containing 0.25% bovine serum albumin. Mice were challenged with 200 μg kg^{-1} PAF in a volume of 200 μL via a tail vein injection, 30 min after an i.p. injection of either vehicle (0.5 mL 2% DMSO) or compounds at the indicated dose. Death (determined by cessation of breathing) was then recorded over a period of two hours. All animals surviving the 2-hour test session were euthanized by CO_2 inhalation. In the experiments where the effect of testosterone was evaluated, mice were treated daily by subcutaneous injection of sesame oil (vehicle control, 100 μL) or testosterone propionate (3 mg kg^{-1}) for 10 days prior to PAF injection.

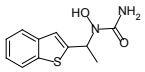
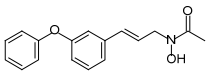
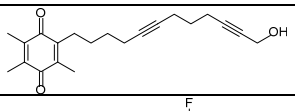
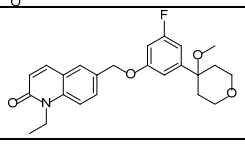
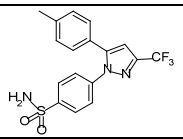
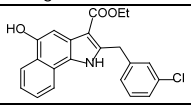
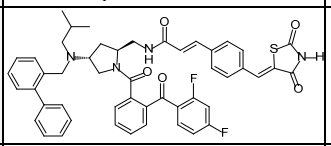
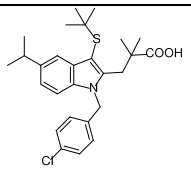
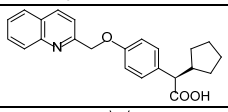
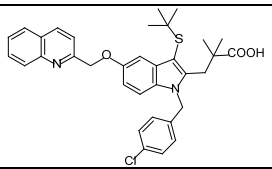
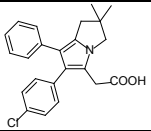
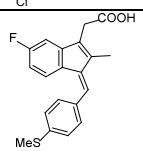
Supplemental Table 1. Sex differences in the biosynthesis of individual 5-LO products in human whole blood and effects of 5α-DHT. Human whole blood from male or female donors was incubated with 30 μM A23187 for 10 min at 37°C and 5-LO products were analyzed by LC-MS/MS. Data are means + SEM, *n*=6.

5-LO products	male[a] (ng mL^{-1})	female[a] (ng mL^{-1})	ratio (male/female)	female + 5α-DHT[a,b] (% of untreated female)
LTB_4	31 ± 11	53 ± 16	0.58	48
LTB_4 isomers	4.4 ± 1.1	9.1 ± 1.5	0.48	48
20-OH-LTB_4	3.9 ± 1.4	9.3 ± 3.4	0.41	48
5-HETE	41 ± 12	69 ± 19	0.59	53

[a] Data are means + SEM, *n*=6.

[b]Blood from female donors was pre-incubated with 10 nM 5α-DHT for 15 min and stimulated with 30 μM A23187 and 5-LO products were analyzed by LC-MS/MS.

Supplemental Table 2. IC_{50} values for 5-LO product formation in LPS/fMLP-stimulated human whole blood. 5-LO products include LTB_4, its trans-isomers, and 5-HETE.

compound	chemical structure	IC_{50} [µM][a] male	IC_{50} [µM][a] female	P value[b]
zileuton		0.87 ± 0.20	0.87 ± 0.17	> 0.9999
BWA4C		0.06 ± 0.02	0.15 ± 0.05	0.1813
AA861		0.83 ± 0.23	0.35 ± 0.09	0.1820
ZM230487		0.026 ± 0.004	0.017 ± 0.003	0.1835
celecoxib		19 ± 5	17 ± 2	0.4830
11a		1.2 ± 0.2	0.8 ± 0.1	0.1994
pyrrolidine-1		4.0 ± 1.7	2.7 ± 0.3	0.4808
MK886		1.9 ± 0.4	0.2 ± 0.1	0.0497
Bay-X 1005		30 ± 4	9.3 ± 2.3	0.0249
MK591		0.47 ± 0.09	0.27 ± 0.06	0.0742
licofelone		> 30	5.8 ± 1.1	n.d.[c]
Ssi		> 100	38 ± 14	n.d.[c]

[a]Given as mean ± SEM; n = 4. [b]Student's t test for paired observations; male vs female.

[c]n.d., not determinable (IC_{50} was not achieved in males).

Supplemental Table 3. Conditions for multiple reaction monitoring

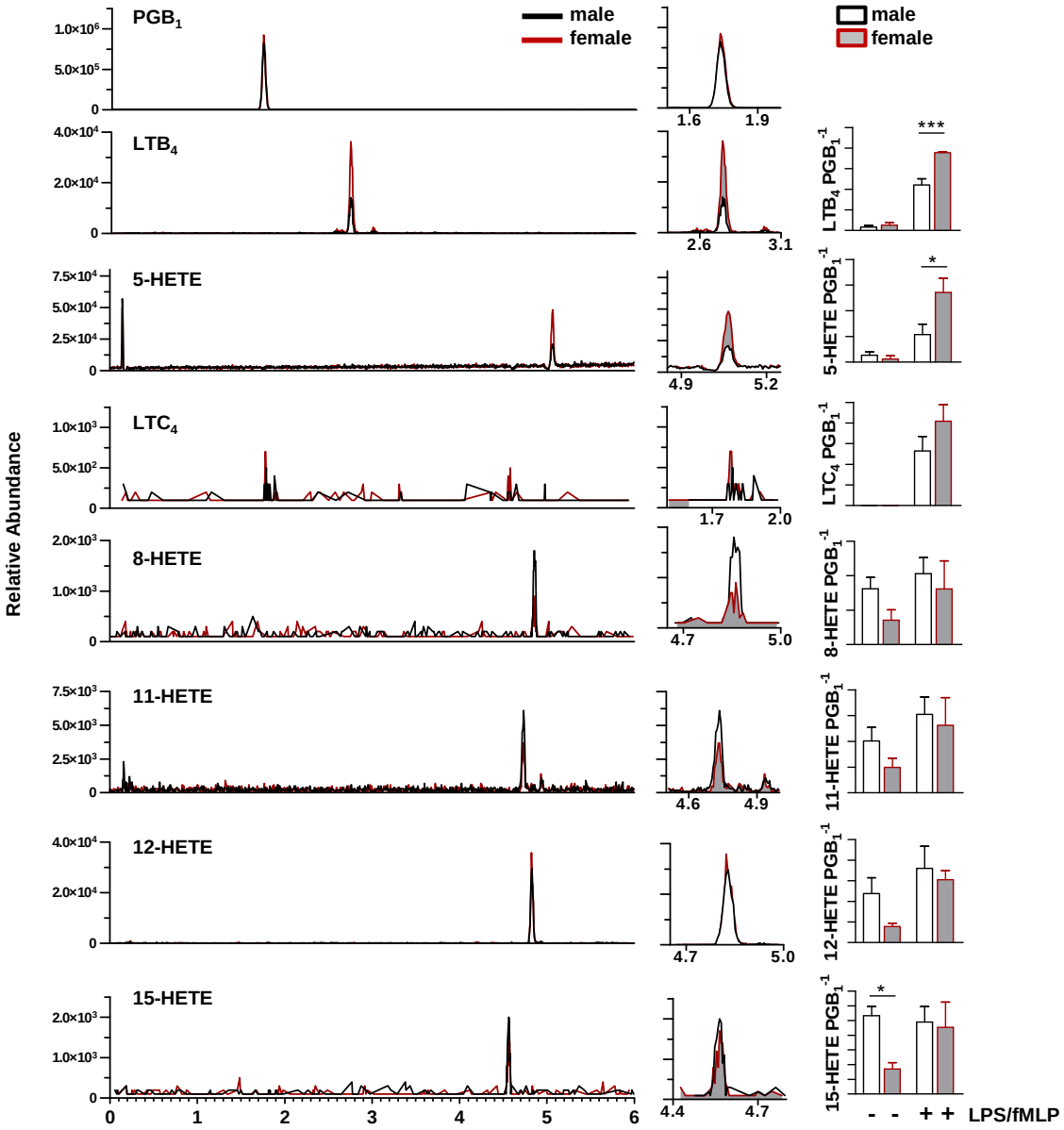
compound	transition 1[a]			transition 2		
	Q1 ($m\ z^{-1}$)[b]	Q3 ($m\ z^{-1}$)[c]	collision energy (eV)	Q1 ($m\ z^{-1}$)[b]	Q3 ($m\ z^{-1}$)[c]	collision energy (eV)
PGB_1	335	113	31	335	221	28
LTB_4	335	129	26	335	195	22
5-HETE	319	115	20	319	203	20
LTC_4	624	272	30	-	-	-
8-HETE	319	155	18	-	-	-
11-HETE	319	167	21	-	-	-
12-HETE	319	179	18	-	-	-
15-HETE	319	219	18	-	-	-
20-OH-LTB_4	351	195	24	-	-	-

[a] transition used for quantification

[b] first quadrupol

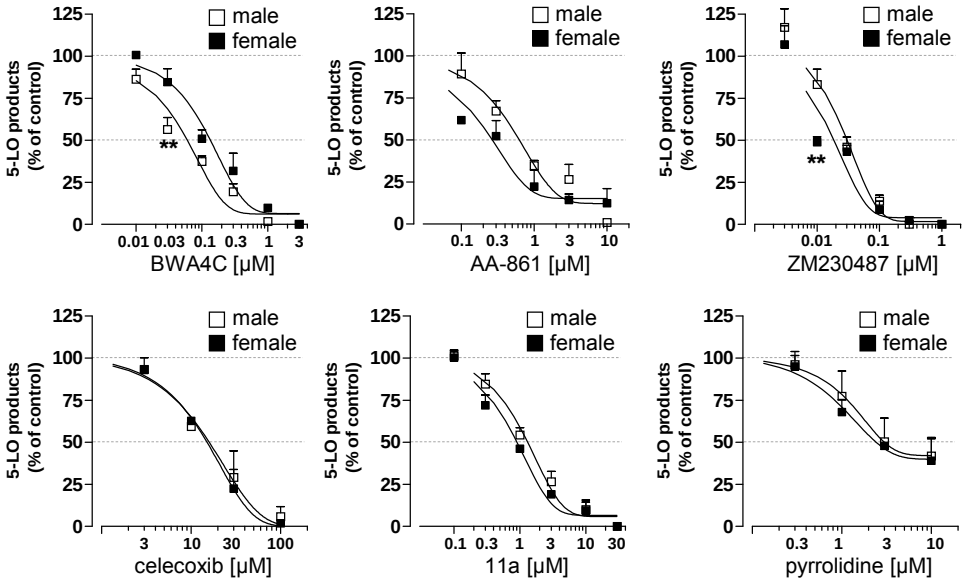
[c] third quadrupol

Supplemental Figure 1



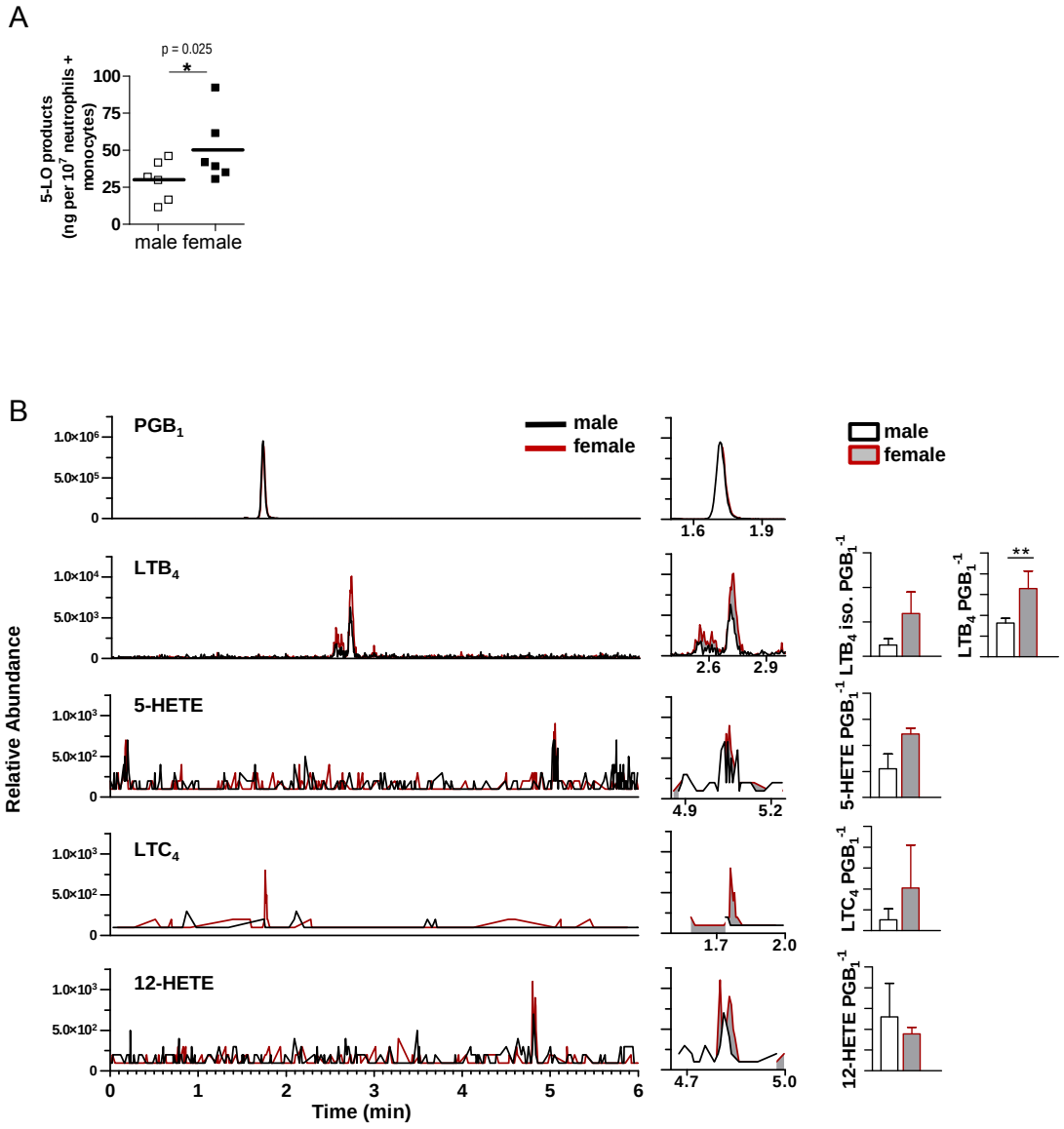
Supplemental Figure 1. LO products in male and female blood. LO product formation in male and female human whole blood stimulated with LPS (1 µg mL^{-1}; 30 min) plus fMLP (1 µM; 15 min), analyzed by LC-MS/MS. Representative chromatograms for male (black line, white fill) and female (red line, gray fill) blood were superimposed. The histograms show the peak area of the indicated metabolite normalized on the peak area of PGB_1 as internal standard; means + SEM; $n = 3$. *$p < 0.05$, ***$p < 0.001$, ANOVA plus Bonferroni.

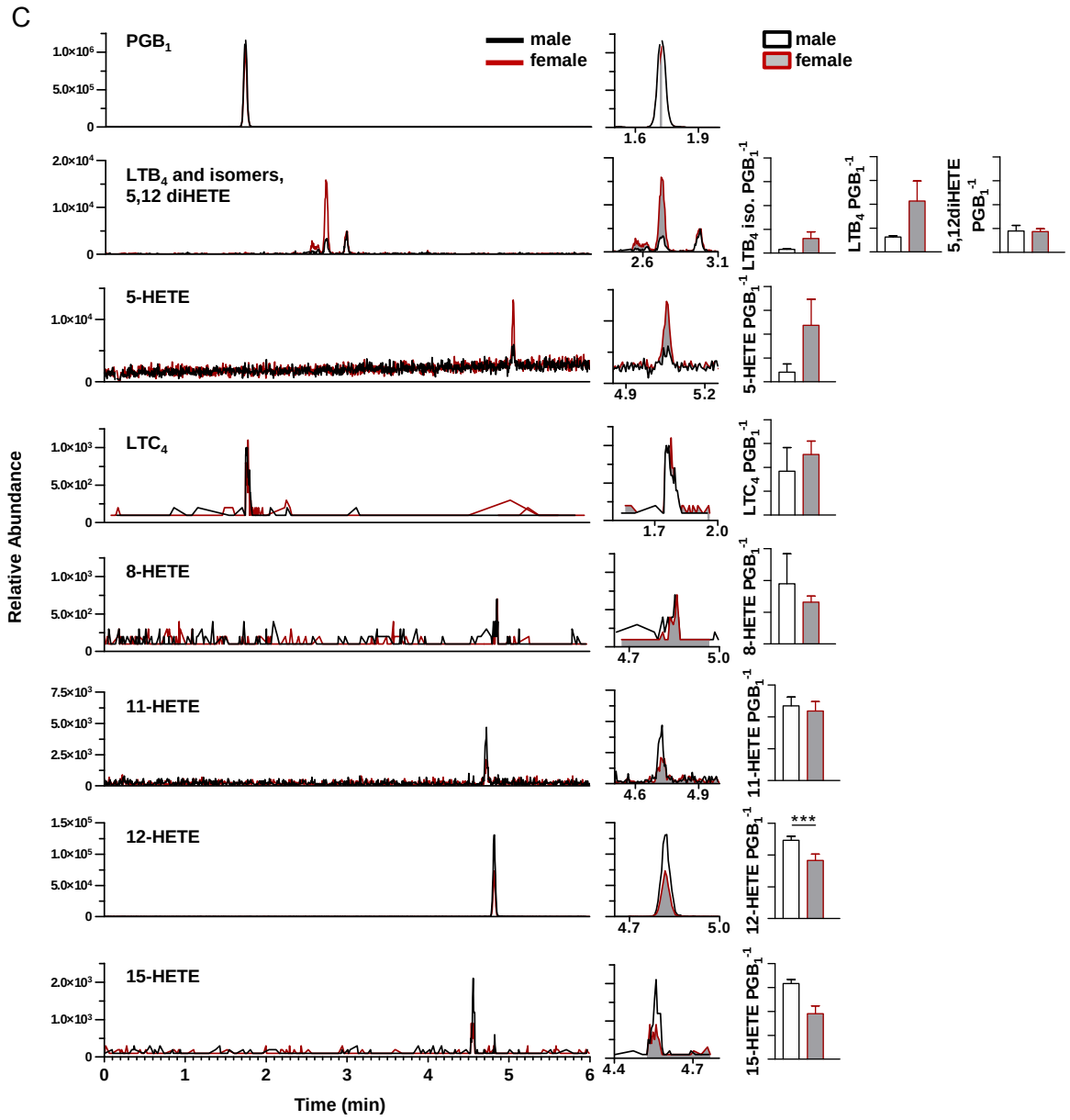
Supplemental Figure 2



Supplemental Figure 2. Effects of direct 5-LO inhibitors and of a cPLA$_2$ inhibitor on 5-LO product formation in male and female blood. Whole blood was stimulated at 37 °C with (1 µg mL^{-1}; 30 min) plus fMLP (1 µM; 15 min). 5-LO products include LTB$_4$, its trans-isomers, and 5-HETE. Data are expressed as percentage of control; means + SEM; $n = 4$. $^{**}p < 0.01$, vs corresponding male, ANOVA plus Bonferroni.

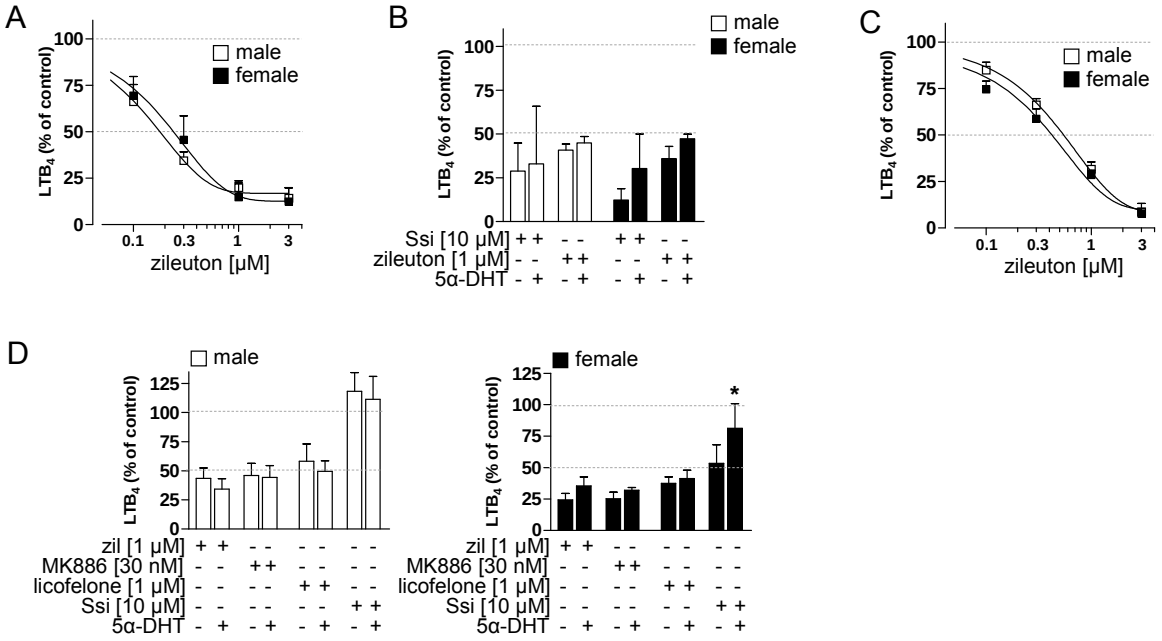
Supplemental Figure 3.





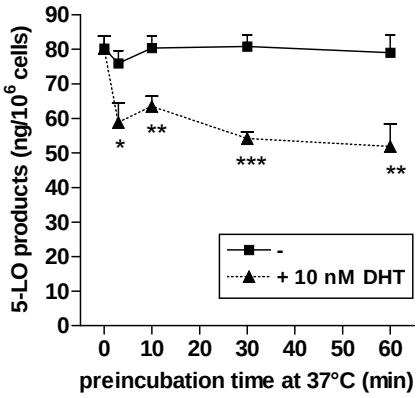
Supplemental Figure 3. Analysis of LO product formation in male and female neutrophils and monocytes. (A) 5-LO product formation in male and female whole blood stimulated at 37 °C with LPS (1 µg mL^{-1}; 30 min) plus fMLP (1 µM; 15 min) after normalization on the sum of neutrophil and monocyte counts in blood. 5-LO products include LTB_4, its trans-isomers, and 5-HETE. Data are given as ng per 10^7 neutrophils plus monocytes; bars are means; $n = 6$; paired t test. (B) LO product formation in male and female isolated human neutrophils stimulated at 37 °C with LPS (1 µg mL^{-1}; 30 min), Ada (0.3 U mL^{-1}; 20 min) plus fMLP (1 µM; 5 min), analyzed by LC-MS/MS. 8-, 11- and 15-HETE were not detected. (C) LO product formation in male and female isolated human monocytes stimulated at 37 °C with LPS (1 µg mL^{-1}; 15 min) plus fMLP (1 µM; 10 min) as analyzed by LC-MS/MS. In (B, C), representative chromatograms for male (black line, white fill) and female (red line, gray fill) cells were superimposed. The histograms show the peak area of the indicated metabolite normalized on the peak area of PGB_1 as internal standard; all-trans isomers of LTB_4 were analyzed together (LTB_4 iso.); means + SEM; $n = 3$. $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA plus Bonferroni.

Supplemental Figure 4.



Supplemental Figure 4. Effect of LT synthesis inhibitors in male and female neutrophils and monocytes. Effects of 5-LO and FLAP inhibitors (as indicated, at the indicated concentrations) on LTB_4 formation in male and female (A, B) neutrophils stimulated at 37 °C with LPS (1 μg mL^{-1}; 30 min), Ada (0.3 U mL^{-1}; 20 min) plus fMLP (1 μM; 5 min) or (C, D) monocytes stimulated at 37 °C with LPS (1 μg mL^{-1}; 15 min) plus fMLP (1 μM; 10 min). In (B) and (D), cells were preincubated for 10 min with vehicle (0.05% EtOH) or 5α-DHT (10 nM). Data are expressed as percentage of control, means + SEM; $n = 3$. In (D), $*p < 0.05$, ANOVA plus Bonferroni. The amount of LTB_4 in 100% controls was (ng per 10^7 cells): (A) male, 1.0 ± 0.3; female, 2.4 ± 1.0; (B) male, 1.4 ± 0.1 (veh) and 1.2 ± 0.2 (5α-DHT); female, 3.3 ± 0.5 (veh) and 2.1 ± 0.2 (5α-DHT); (C) male, 4.8 ± 1.8; female, 11.9 ± 2.7; (D) male, 2.4 ± 0.4 (veh) and 2.9 ± 0.6 (5α-DHT); female, 4.3 ± 1.5 (veh) and 3.1 ± 1.1 (5α-DHT).

Supplemental Figure 5.



Supplemental Figure 5. Time course of 5α-DHT to impact 5-LO product biosynthesis in female monocytes. Human monocytes were pre-incubated with 10 nM 5α-DHT or vehicle (0.05% EtOH) at 37 °C for the indicated periods and then stimulated with 2.5 μM A23187. After 10 min, 5-LO product formation was determined. Data are means + SEM, n=3; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA plus Bonferroni.