# ERK-mediated regulation of leukotriene biosynthesis by androgens: A molecular basis for gender differences in inflammation and asthma

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**5-Lipoxygenase initiates the biosynthesis of leukotrienes, lipid mediators involved in normal host defense and in inflammatory and allergic disorders. Despite an obvious gender bias in leukotriene-related diseases (e.g., asthma), gender aspects have been neglected in studies on leukotrienes and 5-lipoxygenase. Here, we show that leukotriene formation in stimulated whole blood or neutrophils from males is substantially lower compared with females, accompanied by changed 5-lipoxygenase trafficking. This is due to gender-specific differential activation of extracellular signal-regulated kinases (ERKs). The differences are directly related to variant male/female testosterone plus 5$\alpha$-dihydrotestosterone levels, and addition of 5$\alpha$-dihydrotestosterone to female blood or neutrophils reduced the high (female) LT biosynthesis capacity to low (male) levels. In conclusion, regulation of ERKs and leukotriene formation by androgens constitutes a molecular basis for gender differences in the inflammatory response, and in inflammatory diseases such as asthma.**

5-lipoxygenase | arachidonic acid

Leukotrienes (LTs) are proinflammatory lipid mediators formed from arachidonic acid (AA) (1). The most established effects of LTs in the inflammatory response involve phagocytes and vascular endothelium, leading to leukocyte extravasation and migration, and increased vascular permeability. Thus, LTs are established within the innate immune system. However, effects of LTs on dendritic cells and on T cells suggest roles also in the adaptive immune response. The functions of LTs in normal inflammatory reactions imply LTs in inflammatory diseases (2). Associations are established for asthma and allergic rhinitis, and anti-LTs are used to treat these disorders. Ample support implies roles of LTs also in autoimmune diseases and atherosclerosis (2).

Sex hormones regulate sexual differentiation and reproduction, but also influence the immune system (3). Gender differences regarding susceptibility to certain autoimmune diseases are well known, for example rheumatoid arthritis and systemic lupus erythematosus (SLE) more often affect women, which has been associated with sex hormone levels, major histocompatibility complex genetic background, and cytokine production (3, 4). However, female sex can also protect against inflammatory disease. Thus, hepatocellular carcinoma, strongly linked to chronic liver inflammation, is less common among females because of estrogen inhibiting secretion of IL-6 from Kupffer cells (5). In asthma, sex differences are apparent with a male predominance in childhood, whereas after puberty females predominate, because of a drop in males (6), reflecting a protective function of androgens. However, the biochemical mechanisms for this gender disparity are unknown.

The capacity for LT biosynthesis depends on the availability of free AA and the subcellular compartmentalization of 5-LO (7, 8). In neutrophils, 5-LO resides in the cytoplasm of resting cells, and redistributes to the nuclear membrane upon activation by $Ca^{2+}$ where it colocalizes with $cPLA_2$ and the 5-LO-activating protein (FLAP) (7). The C-terminal catalytic domain can be phosphorylated by p38 mitogen-activated protein kinase (MAPK)-regulated MAPKAPK-2, extracellular signal-regulated kinases (ERKs), and protein kinase A, which modulate 5-LO nuclear localization and product synthesis (8–13). Moreover, coactosin-like protein (CLP) binds to 5-LO and promotes LT formation, apparently by functioning as chaperone or scaffold for 5-LO (14).

Despite an obvious gender bias in LT-related diseases [*e.g.*, asthma (15)], gender aspects or effects of sex hormones have been neglected in studies on LT biosynthesis or 5-LO cell biology. Neutrophils are the major source of 5-LO products in blood (16) and these cells are important early effectors of the innate immune response (17). Only few studies have addressed gender differences in neutrophil biology, and effects of sex hormones on typical neutrophil functions are largely unknown (3). Here, we show that variant testosterone levels in males and females cause a differential activation status of ERKs in human neutrophils, which mediates sex differences in LT biosynthesis by regulating the subcellular localization of 5-LO.

## Results

**Gender-Dependent Capacities for 5-LO Product Formation.** Formation of LTs and 5-H(P)ETE in female blood stimulated with lipopolysaccharide (LPS) plus *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Fig. 1*A*) or with $Ca^{2+}$-ionophore A23187 (Fig. 1*B*) was significantly higher than in blood from males. Only traces of 5-LO products were formed in unstimulated blood regardless of gender (data not shown) and generation of the cyclooxygenase (COX)-derived 12(S)-hydroxy-5-*cis*-8,10-transheptadecatrienoic acid (12-HHT) did not vary [see supporting information (SI) Fig. S1].

Next, 5-LO product formation was analyzed in human neutrophils, which are the major 5-LO product-forming cells in blood (16). To preserve properties of blood neutrophils because of sex hormones we isolated neutrophils at 4°C. The low temperature should minimize cellular and biochemical alter-

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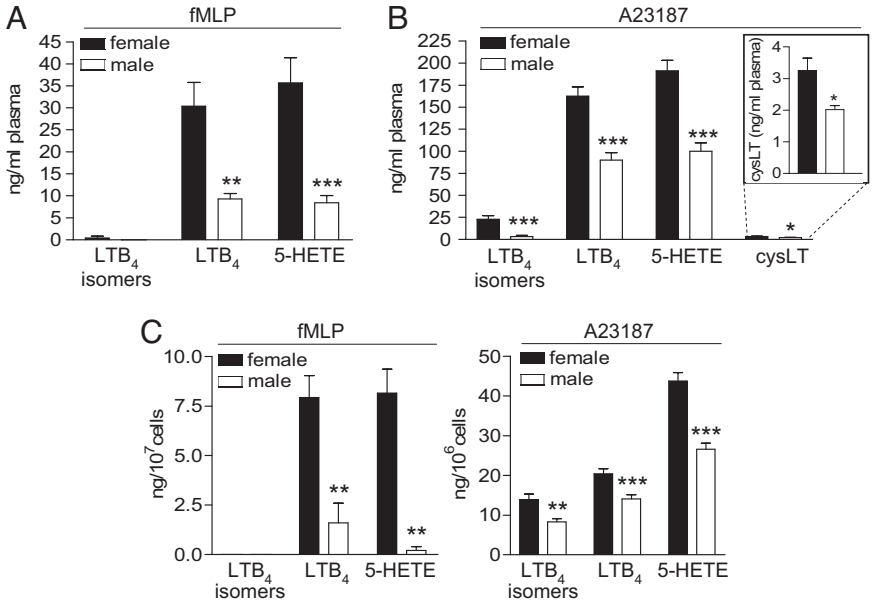


**Fig. 1.** Gender-dependent differences in 5-LO product formation. 5-LO product formation in human whole blood induced by (*A*) fMLP (1 $\mu$M; 15 min, 37°C) after 30 min priming with LPS (1 $\mu$g/ml) or by (*B*) A23187 (30 $\mu$M; 10 min, 37°C). Data are means + SEM; $n = 11$ (fMLP/LPS), $n = 13$ (A23187), duplicates, each. (*C*) 5-LO product formation in human neutrophils induced by fMLP (1 $\mu$M; 5 min, 37°C) after 30 min priming with LPS (1 $\mu$g/ml) plus Ada (0.3 U/ml), or with A23187 (2.5 $\mu$M; 10 min, 37°C). Data are means + SEM; $n = 6$ (LPS/Ada/fMLP), $n = 14$ (A23187), duplicates, each. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; male vs. female, Student *t* test.

ations when the extracellular milieu is changed (blood to buffer). Generation of $LTB_4$ and 5-H(P)ETE in female neutrophils after stimulation with fMLP (after priming with LPS/adenosine deaminase (Ada)) was 4.9- and 10.1-fold higher than in male cells, respectively. Similarly, A23187 caused the synthesis of 1.6-fold more 5-LO products in female versus male neutrophils (Fig. 1*C*).

5-LO protein levels and 5-LO activity in homogenates of blood or isolated neutrophils were not different between genders (Fig. S1). It appeared reasonable that the endogenous substrate supply for 5-LO could vary, and in fact, supplementation of whole blood or neutrophils with excess of exogenous AA abolished unequal 5-LO product synthesis (Fig. S1). However, the amounts of AA liberated from activated neutrophils were equal for male/female cells (Fig. S1), implying that instead the accessibility of endogenously released AA for 5-LO could differ between the genders, because of different subcellular localization of 5-LO.

**Gender-Dependent Subcellular Localization and Trafficking of 5-LO.** To assess 5-LO subcellular distribution, we used subcellular fractionation techniques [i.e., mild-detergent lysis (9) or sonication (18) combined with centrifugation steps] coupled to 5-LO immunodetection as well as indirect immunofluorescence (IF) microscopy (19). In neutrophils from females, 5-LO resided in the cytoplasm of resting cells and redistributed to the nucleus upon stimulation, in accordance with the well established model for 5-LO activation (9, 20). In contrast, in neutrophils from males, 5-LO was detected in both the cytosol and the nuclear compartment of resting cells, and upon stimulation, the compartmentalization of 5-LO was not significantly altered (Fig. 2*A* and Fig. S2). IF analysis of 5-LO confirmed cytoplasmic 5-LO in resting female neutrophils and, after cell activation 5-LO accumulated in the perinuclear region (Fig. 2*B*). However, for male neutrophils, a prevalent punctuate staining in the perinuclear region was observed in both resting and activated cells (Fig. 2*C*). Together, in female neutrophils, 5-LO translocates from the cytosol to the nucleus upon cell challenge, whereas in male cells, a fraction of 5-LO is associated with the nucleus in resting cells, and there is no obvious change in 5-LO distribution upon cell stimulation.

FLAP was exclusively detected in the nuclear membrane

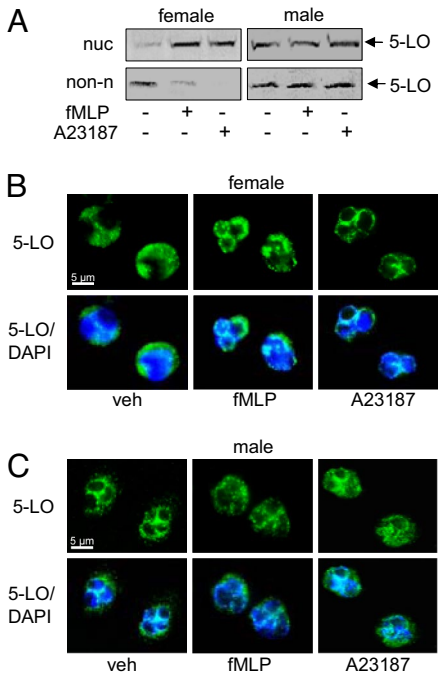


**Fig. 2.** Subcellular localization and trafficking of 5-LO in neutrophils are regulated in a gender-dependent manner. 5-LO subcellular localization after activation of neutrophils with fMLP (1 $\mu$M; 5 min, 37°C) after 30 min priming with LPS (1 $\mu$g/ml) plus Ada (0.3 U/ml), or with A23187 (2.5 $\mu$M; 5 min, 37°C), analyzed by immunodetection of 5-LO in the nuclear (nuc) and non-nuclear (non-n) fractions of mild-detergent (0.1% Nonidet P-40)-lysed cells (*A*), or analyzed by IF microscopy (*B* and *C*). Results are representative of 6–9 independent experiments. In *B* and *C*, pictures with single staining for 5-LO (green) and merged with DNA-stain (diamidino-2-phenylindole, DAPI, blue), are shown.

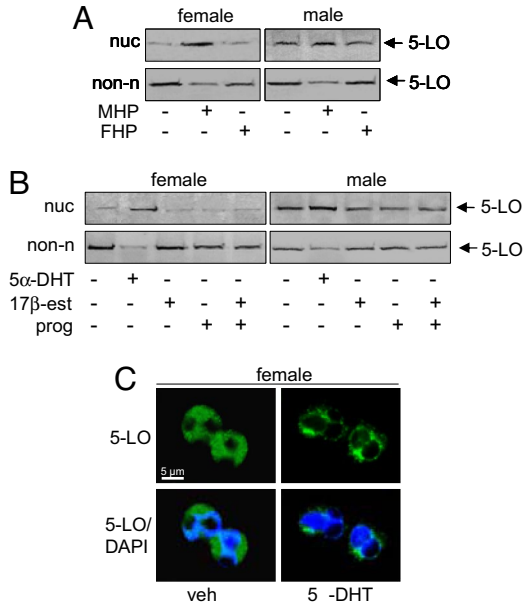


**Fig. 3.** Androgens induce nuclear localization of 5-LO. (*A*) Neutrophils were incubated in male (MHP) or female (FHP) human plasma for 30 min at 37°C. 5-LO was analyzed by immunodetection in the nuclear (nuc) and non-nuclear (non-n) fractions. (*B*) Neutrophils were treated with 5α-DHT (10 nM; 30 min, 37°C), 17β-estradiol (100 nM; 30 min, 37°C) and/or progesterone (10 μM; 30 min, 37°C). 5-LO was analyzed by immunodetection in nuclear (nuc) and non-nuclear (non-n) fractions. (*C*) 5α-DHT (10 nM, 30 min, 37°C) induces association of 5-LO with the nucleus in neutrophils from females, as analyzed by IF microscopy. Pictures with single staining for 5-LO (green) and merged with DNA-stain (diamidino-2-phenylindole, DAPI, blue), are shown. Results are representative of $n = 3$–4 independent experiments.

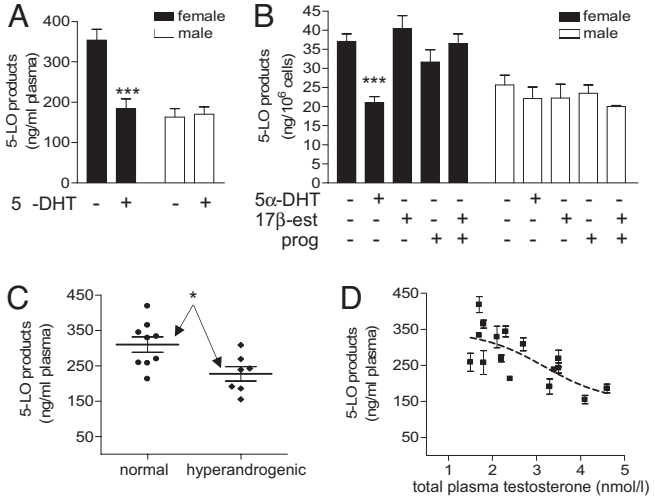


**Fig. 4.** 5α-DHT suppresses 5-LO product formation. (*A*) Whole blood from females was treated with 5α-DHT (100 nM; 30 min, 37°C) and stimulated with A23187 (30 μM, 10 min) for 5-LO product formation. Data are means + SEM; $n = 10$, duplicates. ***, $P < 0.001$ vs. vehicle (0.05% EtOH) control, Student's *t* test. (*B*) Effects of 5α-DHT (10 nM; 30 min, 37°C), 17β-estradiol (100 nM; 30 min, 37°C) and/or progesterone (10 μM; 30 min, 37°C) on 5-LO product formation in A23187 (2.5 μM, 10 min)-stimulated neutrophils. Data are means + SEM; $n = 6$, duplicates. ***, $P < 0.001$ vs. vehicle (0.05% EtOH) control, ANOVA + Tukey HSD post hoc tests. (*C*) Dot plot of 5-LO activity in whole blood from females with normal testosterone levels (total plasma testosterone <2.6 nmol/l) and hyperandrogenic females (total plasma testosterone >2.6 nmol/l) stimulated with 30 μM A23187 (10 min, 37°C). Bars show the group mean ± SEM. *, $P < 0.05$. (*D*) Correlation between plasma testosterone levels in females and 5-LO activity in A23187-challenged whole blood. Data are means ± SEM of duplicates. The broken line represents the fitted sigmoidal curve ($R^2 = 0.52$).

fraction, irrespective of the gender or the cellular activation state (Fig. S2). $cPLA_2$ colocalized with 5-LO in female neutrophils and the same subcellular distribution pattern was obvious in male neutrophils, implying that $cPLA_2$ subcellular compartmentalization does not differ between genders (Fig. S2). Interestingly, the subcellular distribution of CLP was gender-dependent, as it consistently colocalized with 5-LO in female and in male neutrophils (Fig. S2).

**Testosterone and 5α-Dihydrotestosterone Cause Nuclear Localization of 5-LO.** The gender-related difference in 5-LO regulation could be related to variant plasma levels of sex hormones in men and women. Exposure of female neutrophils to male plasma induced 5-LO redistribution to the nucleus, whereas for male neutrophils, addition of female plasma was essentially ineffective and male plasma induced only a moderate increase of 5-LO in the nuclear compartment (Fig. 3*A*). Treatment of female neutrophils with 5α-dihydrotestosterone (5α-DHT) caused translocation of 5-LO to the nuclear compartment in a rapid (within 5 min) and concentration-dependent manner (starting at 10 pM with maximum at 10 nM), whereas 17β-estradiol (100 nM) and progesterone (10 μM) had no effect (Fig. 3*B* and Fig. S3). Interestingly, 5α-DHT -treated neutrophils from females showed a prevalent punctuate staining of 5-LO in the perinuclear region, similar to that observed in resting cells from males (Fig. 3*C*). In male neutrophils, 17β-estradiol and/or progesterone had no influence on 5-LO subcellular localization, but 5α-DHT caused a slight increase of nuclear 5-LO (Fig. 3*B*). Conclusively, 5α-DHT causes a rapid redistribution of 5-LO from the cytosol to the nucleus, resulting in differential 5-LO subcellular localization in male and female neutrophils.

After removal of 5α-DHT, the effect on 5-LO in female neutrophils was reversed (Fig. S3). Moreover, when neutrophils isolated from males were incubated at 37°C for 120 min (instead of keeping on ice), a prevalent cytosolic localization of 5-LO was observed and 5-LO was susceptible to A23187 and redistributed to the nucleus (Fig. S3), as observed for female cells. Hence, the effect of 5α-DHT on neutrophils is reversible, and constant cooling on ice during neutrophil isolation (and thus androgen removal) obviously preserves the influence of plasma androgens.

**5α-DHT Suppresses Cellular 5-LO Product Formation.** Preincubation of whole blood or neutrophils from males with sex hormones caused no significant change in 5-LO product synthesis upon A23187-stimulation (Fig. 4 *A* and *B*). In contrast, treatment of blood or neutrophils from females with 5α-DHT suppressed 5-LO product synthesis to levels found for male-derived samples (Fig. 4 *A* and *B*). Exposure of female neutrophils to 5α-DHT did not affect 5-LO protein levels or agonist-evoked release of AA (data not shown). Interestingly, 5-LO activity in A23187-challenged whole blood from hyperandrogenic females (testosterone >2.6 nmol/l) was significantly lower compared with blood from females with normal testosterone levels (< 2.6 nmol/l) (Fig. 4*C*), and there was a correlation between androgen levels and 5-LO activity (Fig. 4*D*).

**Testosterone and 5α-DHT Cause Activation of ERK.** To elucidate the molecular mechanisms underlying the gender/5α-DHT -dependent regulation of 5-LO, crucial pathways and cofactors regulating 5-LO were examined. The p38 MAPK-regulated MAPKAPK-2 as well as ERKs phosphorylate and regulate 5-LO in leukocytes (10, 21). Pretreatment of male neutrophils with the ERK activation inhibitors U0126 and PD98059 gave cytosolic localization of 5-LO (Fig. 5 *A* and *B*), and upon A23187-

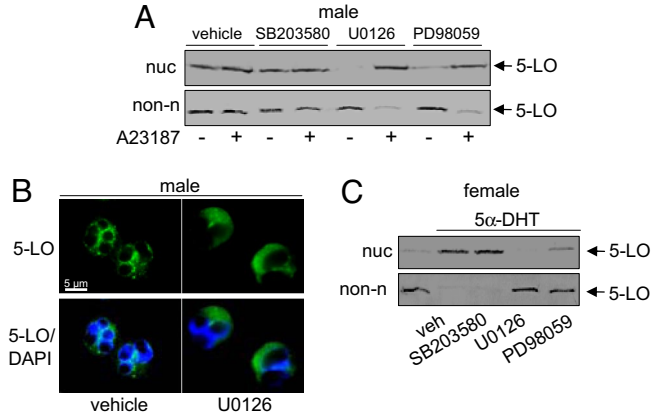


**Fig. 5.** ERKs cause 5α-DHT/gender-dependent regulation of 5-LO subcellular localization. Effects of U0126 (3 μM), PD98059 (30 μM), and SB203580 (10 μM) on 5-LO subcellular localization. Neutrophils from males were pretreated with the kinase inhibitors (or 0.3% DMSO as vehicle) for 15 min at 37°C prior stimulation with A23187 (2.5 μM, 5 min, 37°C). 5-LO was analyzed by (*A*) immunodetection in the nuclear (nuc) and non-nuclear (non-n) fractions and (*B*) by IF microscopy. In *B*, pictures with single staining for 5-LO (green) and merged with DNA-stain (diamidino-2-phenylindole, DAPI, blue), are shown. (*C*) U0126 (3 μM) or PD98059 (30 μM), but not SB203580 (10 μM) block nuclear localization of 5-LO induced by 5α-DHT (10 nM; 30 min, 37°C) in neutrophils from females, as analyzed by immunodetection of 5-LO in the nuclear (nuc) and non-nuclear (non-n) fractions. Results are representative of at least three independent experiments.

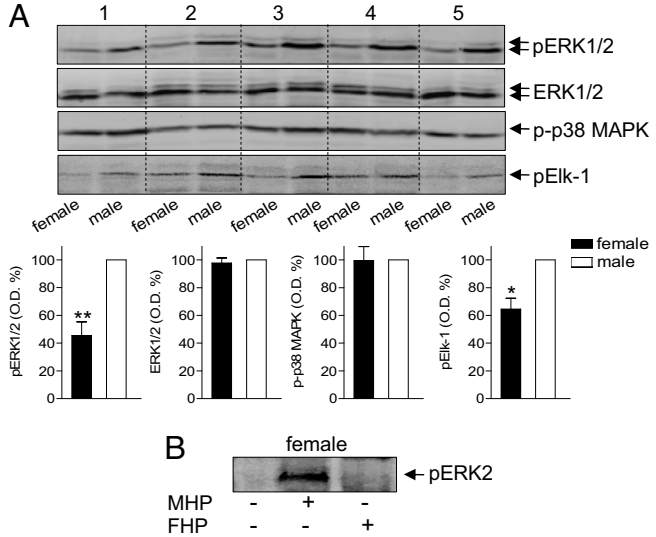


**Fig. 6.** ERKs show a differential activation status in female and male neutrophils and are activated by androgens (*A*) Analysis of phosphorylated ERK1/2 (pERK1/2), total ERK1/2, phosphorylated p38 MAPK (*p*-p38 MAPK) and phosphorylated Elk-1 (pElk-1) in male and female neutrophils. In the pairwise analyses, optical densities measured for male samples were set as 100%, five representatives are shown. Data are means + SEM; $n = 7$; *, $P < 0.05$, **, $P < 0.01$ vs. male, Student *t* test. (*B*) Incubation of neutrophils from females in male human plasma (MHP), but not in female human plasma (FHP), for 1.5 min at 37°C, induce ERK2 activation, as analyzed by immunodetection of phosphorylated ERK2 (pERK2). Results are representative of $n = 3$ independent experiments.

challenge, 5-LO redistributed to the nuclear compartment (Fig. 5*A*). The p38 MAPK inhibitor SB203580 failed in this respect. Pharmacological suppression of ERKs in female neutrophils did not influence 5-LO (Fig. S4*A*). Accordingly, U0126 and PD98059 (but not SB203580) blocked 5α-DHT-induced 5-LO nuclear redistribution in female neutrophils (Fig. 5*C* and Fig. S4).

Our data suggest that male neutrophils should have higher levels of ERK activity versus female cells. The basal phosphorylation (= activation) status of ERK2 and of its substrate Elk-1 was significantly higher in male neutrophils versus female cells (Fig. 6*A*). ERK2 expression and phosphorylation of p38 MAPK did not vary between genders. 5α-DHT caused a rapid (within 0.5 min) and concentration-dependent (starting at 10 pM) phosphorylation of ERK2 in female neutrophils (Fig. S5), whereas 17β-estradiol and/or progesterone were not effective. Finally, incubation of female neutrophils with male (but not female) plasma caused activation of ERKs (Fig. 6*B*).

## Discussion

We show that in human neutrophils 5-LO product synthesis and 5-LO subcellular compartmentalization depend on gender, connected to a differential activation status of ERKs. Thus, stimulated blood or neutrophils from males generate less 5-LO products compared with females, accompanied by changed 5-LO subcellular trafficking. Androgens cause these gender differences, affecting 5-LO via ERKs. Because ERKs are central signaling kinases, regulating multiple neutrophil functions (17), these androgen actions may have profound effects on neutrophil biology.

Differences in 5-LO product formation between male and female blood or neutrophils were more pronounced in response to LPS/fMLP compared with A23187. LPS and fMLP use defined cellular signaling pathways to activate 5-LO (20, 21) and this is of pathophysiological relevance at sites of inflammation (16). In contrast, the extreme elevation of $[Ca^{2+}]_i$ induced by A23187 may circumvent regulatory mechanisms of 5-LO. Hence, the striking differences in the capacities of LPS/fMLP-evoked 5-LO product synthesis between genders may better reflect pathophysiological conditions in the body.

Major factors governing 5-LO product synthesis, for example, levels of enzymatically active 5-LO and of FLAP and substrate availability (7, 9) were not different between the genders. However, 5-LO subcellular distribution clearly varied. Numerous studies have shown that 5-LO resides in the cytoplasm of resting neutrophils and redistributes to the nuclear envelope in response to fMLP (with or without LPS priming) or A23187 (9, 19, 20, 22). For female neutrophils, we confirmed this pattern of 5-LO subcellular redistribution. However, in male neutrophils, a substantial part of 5-LO is located to the perinuclear region already in resting cells, and 5-LO only marginally redistributed upon stimulation, accompanied by reduced 5-LO product synthesis. Previous findings suggest that intranuclear localization of 5-LO confers an increased capacity for subsequent LT biosynthesis (19), whereas 5-LO already associated with the nuclear envelope at the time of cell stimulation is less active (23). Thus, the perinuclear 5-LO localization induced by 5α-DHT may be a regulatory mechanism in males that attenuates LT formation at sites of inflammation.

5-LO subcellular compartmentalization is governed by ERKs (12) that phosphorylate 5-LO (21). We provide evidence that ERKs mediate the male pattern of 5-LO subcellular distribution caused by androgens. First, male (resting) neutrophils exhibit a higher ERK activation status versus females. Second, pharmacological suppression of ERK activity in male cells gave the 5-LO female pattern. Third, exposure of female neutrophils to male plasma or to 5α-DHT enhanced ERK activation, accompanied by the male pattern of 5-LO subcellular distribution, which was reversed by ERK inhibition.

Inconsistent capacities of LT formation and 5-LO subcellular distribution were reported before and have been attributed to individual donor-related variability (16, 24), but gender aspects were never considered. One reason that the gender-difference was not observed before could be the reversibility of the androgen effects after neutrophil isolation from plasma at ambient tem-

perature. We rapidly isolated neutrophils at 4°C, which preserved the plasma androgen effect on 5-LO and ERKs. ERKs have significant impact on numerous neutrophil functions (17) and, as found here for 5-LO, the different activation state of ERKs may lead to striking gender differences. Our results strongly suggest that reversible sex hormone effects should be considered in studies of neutrophil biology. Although activation of ERKs by exogenous androgens in nonhematopoietic cells was shown before [*e.g.*, prostate cancer cells (25)], gender differences in basal ERK activation in humans are thus far unknown. Androgens classically mediate their effects through an intracellular androgen receptor (AR) via transcriptional pathways. Expression and functionality of the AR in neutrophils is not definite and studies investigating androgen effects on neutrophil functions (*e.g.*, oxidative burst) yielded contradictory data (3). Our results suggest the existence of a high affinity receptor for 5$\alpha$-DHT on neutrophils that rapidly causes activation of ERKs. For murine splenic T cells lacking the AR, the existence of a GPCR for testosterone in plasma membranes was demonstrated (26). The identification of the androgen receptor mediating ERK activation and thus neutrophil functions in response to 5$\alpha$-DHT is a major future task.

In conclusion, LT formation in human blood and neutrophils depend on the gender, connected to a suppressive effect by androgens. These findings fit well with the reduction of asthma observed in males during puberty (6). Accordingly, in adolescence, LTs should be more important for asthma symptoms in afflicted females, than in males. This is supported by the better efficiency for the cysLT-1 antagonist montelukast as asthma treatment in girls reaching puberty, compared with boys at same age (27). Also, in a European multicenter study, females dominated among severe asthma patients (28). In patients with active SLE, LTs are strongly increased versus healthy controls, and pharmacological 5-LO inhibition was beneficial in patients with mild SLE (29). Interestingly, in a mouse model resembling human SLE (MRL-*lpr*/*lpr*) female mice had earlier mortality compared with male mice, but the male advantage was abolished after knockout of the 5-LO gene (30). The beneficial androgen effects in asthma and autoimmune diseases are certainly multifaceted, our data indicate that suppression of LTs is one contributing mechanism. Our findings also suggest that inflammatory responses in general, for which LTs are of relevance, may be more vigorous in females. LTs function in antimicrobial defense (2), in such context a high (female) capacity for LT production may be advantageous. For example, females resist bacterial pulmonary infections better than men (31). Finally, the sex-specific regulation of 5-LO implies that gender should be considered in the use of anti-LTs, to optimize pharmacological therapy, in men and women.

## Methods

**Preparation of Whole Blood and Plasma and Isolation of Neutrophils.** Venous blood was collected from fasted (12 h) adult male and female healthy volunteers, with consent (Blood Center, University Hospital, Tuebingen, Germany). The subjects had no apparent inflammatory conditions and had not taken sex hormones or anti-inflammatory drugs for at least 10 days before blood collection. Total serum testosterone was analyzed by an automated chemiluminescence immunoassay system (ADVIA Centaur, Siemens Medical Solution) according to the manufacturer's instructions. For isolation of plasma, blood was centrifuged at 600 $\times$ *g*/10 min/4°C, plasma was centrifuged again (800 $\times$ *g*/10 min/4°C), and the resulting supernatant was analyzed to confirm the absence of cellular contaminations.

For isolation of neutrophils, venous blood (see above) was subjected to centrifugation (4,000 $\times$ *g*/20 min/20°C) for preparation of leukocyte concentrates. Neutrophils were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes as described previously (11). The isolation procedure was strictly performed at 4°C. Neutrophils (purity >96–97%) were finally resuspended in ice-cold PBS plus 1 mg/ml glucose (PG buffer) or in PG buffer supplemented with 1 mM $CaCl_2$ (PGC buffer).

**Determination of 5-LO Product Synthesis and Formation of 12-HHT.** Freshly withdrawn blood (2 ml) was preincubated with test compounds at 37°C, and formation of 5-LO products and 12-HHT was started by addition of the respective stimuli. The reaction was stopped on ice, and the samples were centrifuged (600 $\times$ *g*/10 min/4°C). Aliquots of the resulting plasma (500 $\mu$l) were then mixed with 2 ml of methanol and 200 ng of prostaglandin $B_1$ (internal standard). Samples were placed at −20°C for 2 h and centrifuged again (600 $\times$ *g*/15 min/4°C). The supernatants were collected and diluted with 2.5 ml of PBS and 75 $\mu$l of 1 N HCl.

For determination of cellular 5-LO product formation, neutrophils (5 $\times$ $10^6$/ml PGC buffer) were preincubated with test compounds at 37°C, and the respective stimuli were added. The reaction was stopped with 1 ml of methanol and 30 $\mu$l of 1 N HCl, 200 ng of prostaglandin $B_1$, and 500 $\mu$l of PBS were added. Formed 5-LO metabolites and 12-HHT were extracted and analyzed by HPLC as described (11). 5-LO product formation is expressed as ng of 5-LO products ($LTB_4$ and its all-*trans* isomers, and 5-H(P)ETE) per ml of plasma or per $10^6$ or per $10^7$ cells. Formation of cysteinyl-LTs $C_4$, $D_4$ and $E_4$ in whole blood was analyzed by an enzyme immunoassay kit (Assay Designs Inc.) after extraction of plasma, according to the manufacturer's instructions.

For determination of 5-LO product formation in homogenates, 1 mM EDTA was added to whole blood or freshly isolated neutrophils resuspended in ice-cold PBS. Samples were sonicated (3 $\times$ 10 sec) at 4°C, 1 mM ATP was added, and prewarmed for 30 sec at 37°C. After addition of 2 mM $CaCl_2$ and AA, the reaction was stopped after 10 min at 37°C by addition of 1 ml of ice-cold methanol. Formed metabolites were analyzed by HPLC as described above.

**Determination of 5-LO Protein Expression.** Neutrophils (3 $\times$ $10^7$/ ml ice-cold PBS plus 1 mM EDTA) were sonicated (5 $\times$ 10 sec) at 4°C. Total cell lysates were centrifuged (12,000 $\times$ *g*/15 min/4°C), the supernatant was collected and mixed 1:1 with ice-cold 2 $\times$ SDS/PAGE sample loading buffer (SDS-b), heated for 6 min at 95°C, and analyzed for 5-LO protein by SDS/PAGE and immunoblotting.

**Measurement of the Release of Arachidonic Acid.** Neutrophils (5 $\times$ $10^7$ in 1 ml of PGC buffer) were preincubated (10 min) with the 12-LO inhibitor CDC (10 $\mu$M) and the 5-LO inhibitor BWA4C (1 $\mu$M) to avoid the conversion of released AA to LO metabolites. The reaction was started by addition of the indicated stimuli, and was stopped with 2 ml of methanol and 60 $\mu$l of 1 N HCl, and 2 ml of PBS were added together with 60 $\mu$g of margaric acid, used as internal standard. Released AA was extracted, coupled to dimethoxyaniline hydrochloride in presence of *N*-ethyl-N′-(3-dimethylaminopropyl)carbodiimide and the derivatized AA was analyzed by RP-HPLC at 272 nm.

**Analysis of Subcellular Redistribution of 5-LO, $cPLA_2$, CLP, and FLAP by Cell Fractionation and Immunoblotting.** Neutrophils (3 $\times$ $10^7$/ml PGC buffer or 3 $\times$ $10^7$/500 $\mu$l of human plasma) were incubated with test compounds at 37°C. Samples were chilled on ice and centrifuged (200 $\times$ *g*/5 min/4°C). Subcellular fractionation was performed either by mild detergent (0.1% nonidet P-40) lysis, yielding a nuclear and a non-nuclear fraction (9), or by sonication of the cells and preparation of soluble (S100) and membrane (P100) fractions by 100,000 $\times$ *g* centrifugation (18). For more details, see *SI Text*. Nuclear and non-nuclear fractions and S100 and P100 fractions were analyzed by SDS/PAGE and Western blotting for 5-LO, CLP, FLAP, or $cPLA_2$ as described in *SI Text*.

**Indirect Immunofluorescence Microscopy.** Neutrophils (1.5 $\times$ $10^6$/500 $\mu$l of PGC buffer) were incubated with test compounds at 37°C. Cells were centrifuged at 30 $\times$ *g* for 1 min onto polyL-lysine (MW 150,000–300,000; Sigma–Aldrich)-coated glass coverslips, and activated by addition of the stimuli for 3 min at 37°C. Cells were fixed in methanol (-20°C, 30 min) and permeabilized with 0.1% Tween 20 in PBS (RT, 10 min), followed by 3 washing steps with PBS. Samples were blocked with 10% non-immune goat serum (Invitrogen) for 10 min at RT, washed with PBS, and incubated with anti-5-LO serum (1551, affinity purified) for 30 min at RT. The coverslips were washed 10 times with PBS, incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) for 10 min at RT in the dark, and washed 10 times with PBS. The DNA was stained with 0.1 $\mu$g/ml diamidino-2-phenylindole (DAPI) in PBS for 3 min at RT in the dark. The coverslips were then washed 10 times and mounted on glass slides with Mowiol (Calbiochem) containing 2.5% n-propyl gallate (Sigma). The fluorescence was visualized with a Zeiss Axiovert 200M microscope.

**Determination of MAPK Activation and Phosphorylation of Elk-1.** Neutrophils ($10^7$/100 $\mu$l of PGC buffer) were incubated with the indicated compounds at 37°C and the reaction was stopped by addition of 100 $\mu$l of ice-cold SDS-b and heated for 6 min at 95°C. When the effects of human plasma were analyzed, cells were resuspended in 100 $\mu$l of plasma and incubated at 37°C as indicated.

The reaction was stopped on ice and cells were washed twice with ice-cold PBS. Total cell lysates (20 $\mu$l) were analyzed for ERK1/2, phosphorylated ERK1/2 (Thr-202/Tyr-204), phosphorylated p38 MAPK (Thr-180/Tyr-182), and phosphorylated Elk-1 (Ser-383) by SDS/PAGE and western blotting. Visualization of the immunocomplexes was carried out using an Ettan DIGE imaging system (GE-Healthcare), and densitometry was performed with ImageQuant TL image analysis software (GE Healthcare). For more technical details, see *SI Text*.

**Statistics.** Results are expressed as mean $\pm$ standard error (SEM) of the mean of *n* observations, where *n* represents the number of experiments performed on different days in duplicates. Statistical evaluation of the data were performed by Student *t* test for paired observations. Where appropriate, one-way ANOVA for independent or correlated samples, followed by Tukey HSD post hoc tests, was applied. A *P* value $<0.05$ (*) was considered significant.

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