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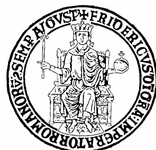
FACOLTÀ DI FARMACIA



DIPARTIMENTO DI FARMACOLOGIA SPERIMENTALE

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GENDER-SPECIFIC REGULATION OF 5-LIPOXYGENASE

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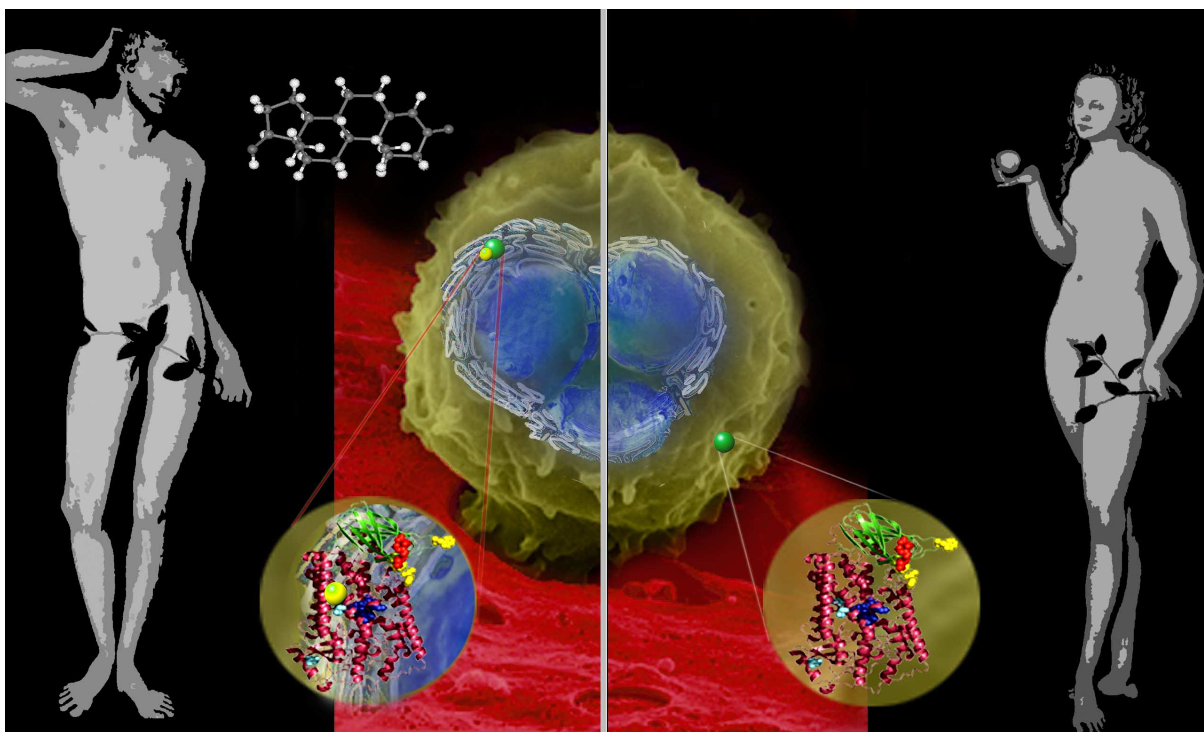

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1. INTRODUCTION

1.1. Inflammation

Inflammation is the biological response of the immune defence to harmful stimuli, both internal (injury or lesion) and external (infection, toxins, irritants). It is a protective attempt carried out by the organism to remove the injurious stimuli as well as to initiate the healing process for the tissues. Inflammation is a complex process occurring through a variety of mechanisms, leading to changes in local blood flow (resulting in blood vessel dilatation and enhanced capillary permeability) and in the recruitment and infiltration of leukocytes. As a consequence, the clinical signs of inflammation are *rubor* (redness), *dolor* (pain), *calor* (heat), and *tumor* (swelling).

A cascade of biochemical events propagates and maintains the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Normal inflammation is a self-limiting process, because the production of anti-inflammatory agents follows the pro-inflammatory mediators closely. However, prolonged inflammation leads to a progressive shift in the type of cells and the healing of the tissue could be accompanied by a simultaneous destruction due to the inflammatory process and, therefore, to a loss of function (*functio laesa*). Several agents or factors can induce an inflammatory response and the concomitant production of inflammatory mediators, such as cytokines, complement factors and lipid mediators. These mediators can cause vasodilatation and increased vascular permeability (vasoactive mediators), or recruit circulating leukocytes to the inflammatory site (chemotactic mediators).

The metabolites arising from the arachidonic acid (AA) cascade have been shown to play a substantial role in the different phases of inflammatory responses. Thus, AA released from phospholipids by the action of phospholipases (PL) serves as precursor for the generation of mediators of inflammation, the so called eicosanoids, by cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-LO, 12-LO and 15-LO), and cytochrome p450 enzymes (CYP) **(Fig. 1)**. The respective products bind and act via selective G protein-coupled receptors (GPCRs) that eventually mediate functional responses.

1.2. 5-Lipoxygenase

1.2.1. Discovery of 5-lipoxygenase

Lipoxygenases are dioxygenases, which contain a *non-heme* iron and stereospecifically catalyze the insertion of molecular oxygen into a l,4-pentadiene structure of a polyunsaturated fatty acid. In 1976, Borgeat, Hamberg and Samuelsson (Borgeat et al., 1976) described the transformation of AA to 5-hydroxyeicosatetraenoic acid (5-HETE) by rabbit polymorphonuclear leukocytes (PMNL), thus providing the first evidence for the existence of a 5-LO enzyme. This novel pathway of AA metabolism attracted even more attention as the formation of 5-HETE and leukotriene (LT) B_4 was observed also in human leukocytes (Borgeat and Samuelsson, 1979a). Soon it became evident that the unknown substances with contractile properties released after immunological challenge of sensitized lungs (the so-called “slow-reacting substance of anaphylaxis” (Kellaway, 1940)) consisted of a mixture of LTC_4, D_4 and E_4, which are products of the 5-LO pathway (Samuelsson, 1983).

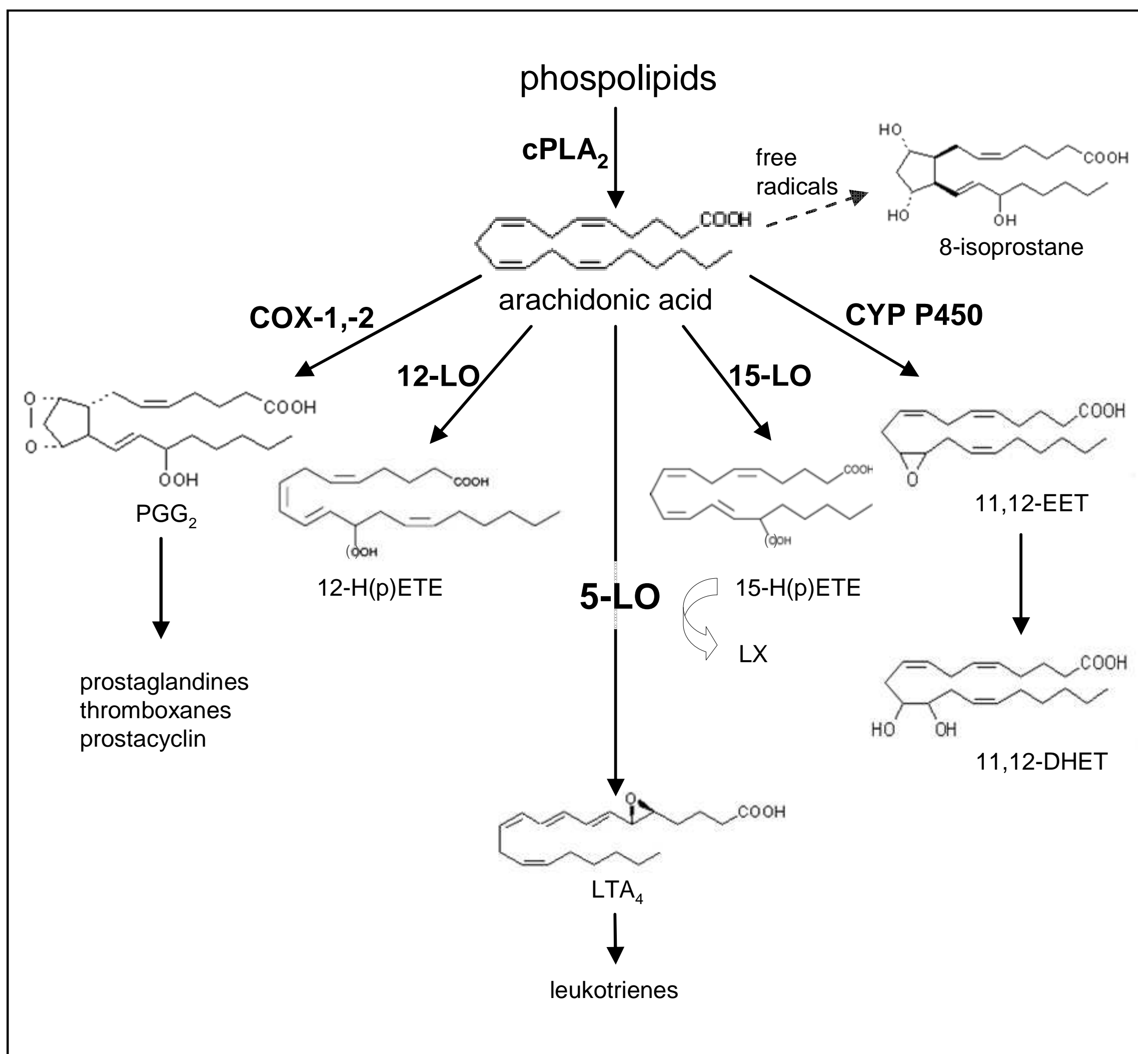


Fig. 1: AA cascade. AA can be released from phospholipids by the action of a phospholipase, foremost $cPLA_2$. AA itself constitutes a second messenger with impact on signalling pathways in the cell, but serves also as a precursor for the generation of eicosanoids by cyclooxygenases (COX-1 and COX-2) that produce the prostanoids PG, Tx and prostacyclin, by lipoxygenases (5-LO, 12-LO, and 15-LO) that synthesize LT, LX and H(p)ETE, or by CYP P450 enzymes that produce EET and DHET. Moreover AA can react with free radicals to give isoprostane. *Abbreviations:* AA, arachidonic acid; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; CYP P450, cytochrome P450; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; H(p)ETE, hydro(pero)xy-eicosatetranoic acid; LO, lipoxygenase; LT, leukotrienes; LX, lipoxins.

1.2.2. 5-Lipoxygenase catalysis and leukotriene biosynthesis

5-LO (EC 1.13.11.34) possesses two distinct enzymatic activities: it catalyses the incorporation of molecular oxygen into AA (oxygenase activity) and the subsequent formation of the unstable epoxide LTA_4 (LTA_4 synthase activity) (Shimizu et al., 1984; Rouzer et al., 1986) **(Fig. 2)**. 5-LO first catalyses the abstraction of the pro-S hydrogen at C-7 of the fatty acid, followed by the insertion of molecular oxygen at position C-5, leading to the hydroperoxide 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid (5-HpETE). Subsequent conversion of 5-HpETE to LTA_4 involves the abstraction of the pro-R hydrogen from C-10 and allylic shifts of the radical to C-6, resulting in the formation of the 5,6 epoxide (Shimizu et al., 1986). Alternatively, 5-HpETE can be reduced to the corresponding alcohol 5-HETE. The ratio of LTA_4 to 5-HpETE formed depends on the assay conditions, e.g. the relative concentrations of free AA and 5-HpETE, membrane association, amount of 5-LO, and the presence of the 5-LO activating protein (FLAP) (Wiseman et al., 1987; Hill et al., 1992; Abramovitz et al., 1993; Noguchi et al., 1994).

Depending on the cell type and the enzymes present, the unstable intermediate LTA_4 can be converted to LTB_4 by LTA_4-hydrolase, or conjugated with glutathione to LTC_4, by LTC_4-synthase, whose crystal structure was recently identified (Ago et al., 2007; Molina et al., 2007), or by other members of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). Release of LTC_4 into the extracellular environment and successive amino acid (γ-glutamyl residue and glycine) cleavage yields LTD_4 and then LTE_4. LTC_4, D_4 and E_4 are collectively denominated cysteinyl-LT (cys-LT) due to the presence of a cysteinyl residue in their structure. Moreover, the combined action of 5-LO and 12-LO or 15-LO can lead to lipoxins, which are bioactive trihydroxytetraene-containing lipid mediators that appear

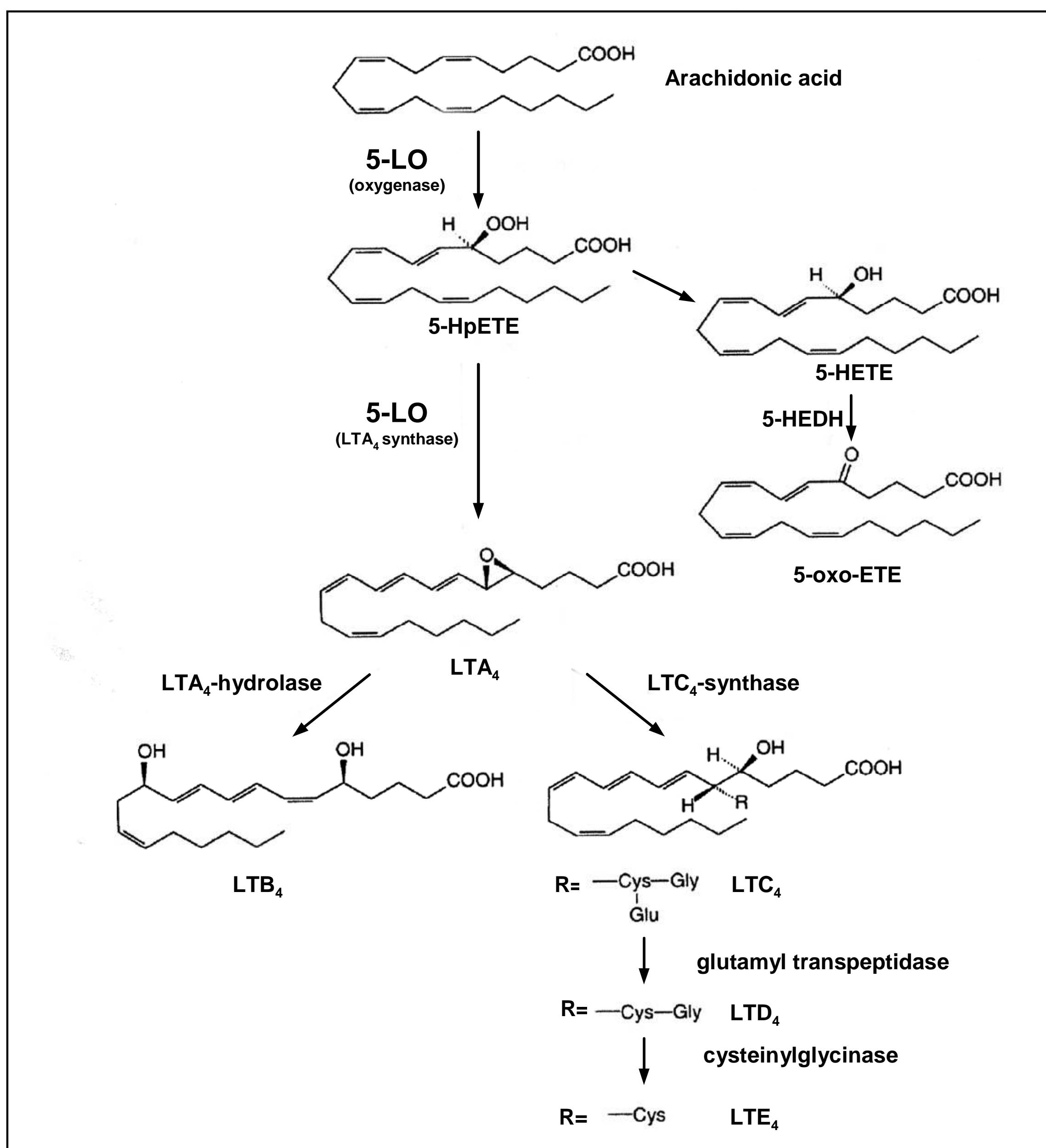


Fig. 2: 5-LO pathway. 5-LO is a dioxygenase that possesses two distinct enzymatic activities: it catalyses the incorporation of molecular oxygen into AA (oxygenase activity) and the subsequent formation of the unstable epoxide LTA_4 (LTA_4 synthase activity). 5-LO first catalyses the abstraction of the pro-S hydrogen at C-7 of the fatty acid, followed by the insertion of molecular oxygen at position C-5, leading to 5-HpETE. Subsequent conversion of 5-HpETE to LTA_4 involves the abstraction of the pro-R hydrogen from C-10 and allylic shifts of the radical to C-6, resulting in the formation of the 5,6 epoxide. Alternatively, 5-HpETE can be reduced to the corresponding alcohol 5-HETE and oxidation of 5-HETE by 5-HEDH leads to 5-oxo-ETE. The unstable intermediate LTA_4 can be converted to LTB_4 by LTA_4-hydrolase, or conjugated with glutathione to LTC_4, by LTC_4-synthase. Release of LTC_4 into the extracellular environment and successive amino acid cleavage yields LTD_4 and then LTE_4. *Abbreviations:* 5-HpETE, hydroperoxide 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; 5-LO: 5-lipoxygenase; LT, leukotriene.

to function as stop signals for inflammatory responses and to promote repair and wound healing. Finally, oxidation of 5-HETE by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) leads to 5-oxo-ETE.

5-LO contains a *non-heme* iron in the active site, coordinated by His367, His372, His550, Asn-554 and the C-terminal Ile, that acts as an electron acceptor or donator during catalysis (Radmark, 1995). Using electron paramagnetic resonance (EPR) spectroscopy, it was shown that the iron of isolated, inactive enzyme is in the ferrous state (Fe^{2+}) and that treatment with fatty acid hydroperoxides gave the active ferric (Fe^{3+}) form (Chasteen et al., 1993; Hammarberg et al., 2001). The reaction sequence of 5-LO consists of an initiation phase, where 5-LO is converted to the ferric state, a linear propagation phase with the highest conversion rate, and an irreversible inactivation phase **(Fig. 3)**. The turnover-dependent inactivation is an inherent property of the enzyme and is therefore also termed suicide inactivation. Although lipid hydroperoxides (LOOHs) are needed to generate active ferric enzyme, such oxidants formed during catalysis may be responsible for rapid inactivation of 5-LO (Aharony et al., 1987).

For the reduction of 5-HpETE, a pseudoperoxidase activity of 5-LO has been accounted (Riendeau et al., 1991). It was shown that certain 5-LO inhibitors (N-hydroxyureas, hydroxamic acids) serve as reducing agents for the pseudoperoxidase activity, indicating that to some extent the inhibitory potency of such inhibitors might be due to the reduction of Fe^{3+} to Fe^{2+} (Falgueyret et al., 1992).

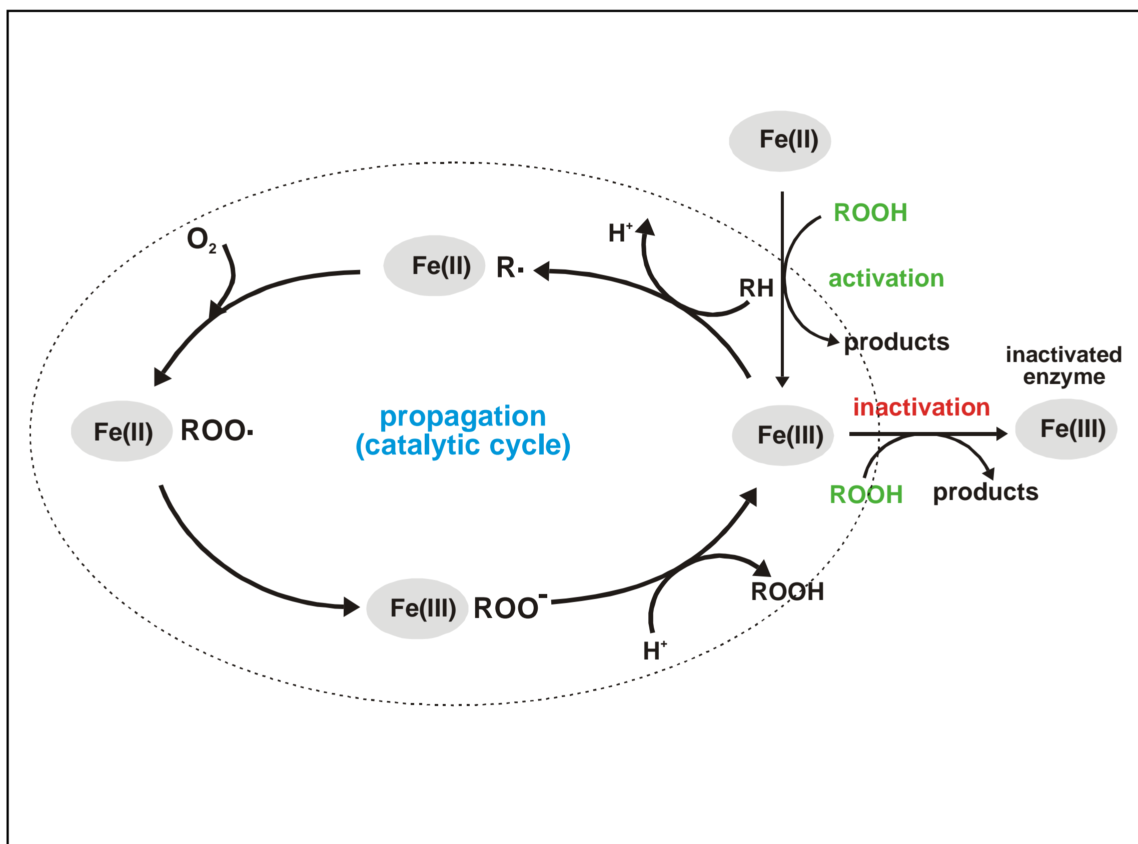


Fig. 3: 5-LO catalytic cycle and redox regulation. Activation of resting 5-LO requires the oxidation of the active site iron from the ferrous to the ferric state. Among various lipid hydroperoxides, 5- and 12-hydroperoxyeicosatetraenoic acid, and 13-hydroperoxyoctadecadienoic acid activate crude 5-LO in homogenates, whereas hydrogen peroxide and several other organic hydroperoxides fail in this respect. Lipid hydroperoxides can shorten the lag phase of 5-LO, which is observed after addition of substrate to crude 5-LO in homogenates or for purified enzyme, and conditions that promote lipid peroxidation stimulate 5-LO activity in leukocytes. Selenium-dependent GPx reduce the cellular peroxide content and are potent suppressors of 5-LO activity. *Abbreviations:* 5-LO, 5-lipoxygenase; ROOH, hydroperoxide.

1.2.3. 5-Lipoxygenase gene

The human 5-LO gene is located on chromosome 10, contains 14 exons, and the promotor lacks typical TATA and CCAAT sequences **(Fig. 4)**. The promotor is highly G + C rich and contains several consensus–binding sites for transcription factors, such as c-myb, AP-2, nuclear factor-κB (NF-κB), Sp1, Sp3, Egr-1, Egr-2, and the nuclear receptors retinoid Z receptor alpha (RZRα) as well as retinoic acid receptor-related orphan-receptor alpha (RORα). Particular attention has been focused on a series of 5 GC-boxes in tandem, which can specifically bind the transcription factors Sp1 and Egr-1, thereby activating 5-LO promotor-reporter constructs (In et al., 1997; Silverman et al., 1998). Examination of genomic DNA from asthmatic as well as non-asthmathic subjects revealed the occurrence of natural mutations within these functional promotor regions (Silverman and Drazen, 2000). The nuclear receptors RZRα and RORα may repress 5-LO gene expression in B-lymphocytes in response to melatonin (Steinhilber et al., 1995).

1.2.4. 5-Lipoxygenase expression

5-LO is expressed mainly in leukocytes, in line with the function of LTs as mediators of immune reactions. Granulocytes, monocytes/macrophages, mast cells, dendritic cells and B lymphocytes express 5-LO, whereas platelets, endothelial cells and erythrocytes do not. In T lymphocytes, evidence for 5-LO expression on both transcriptional and translational levels has been recently provided, although these cells are incapable of synthesizing LTs in absence of exogenous AA (Cook-Moreau et al., 2007). In the skin, Langerhans cells strongly express 5-LO (Steinhilber, 1999).

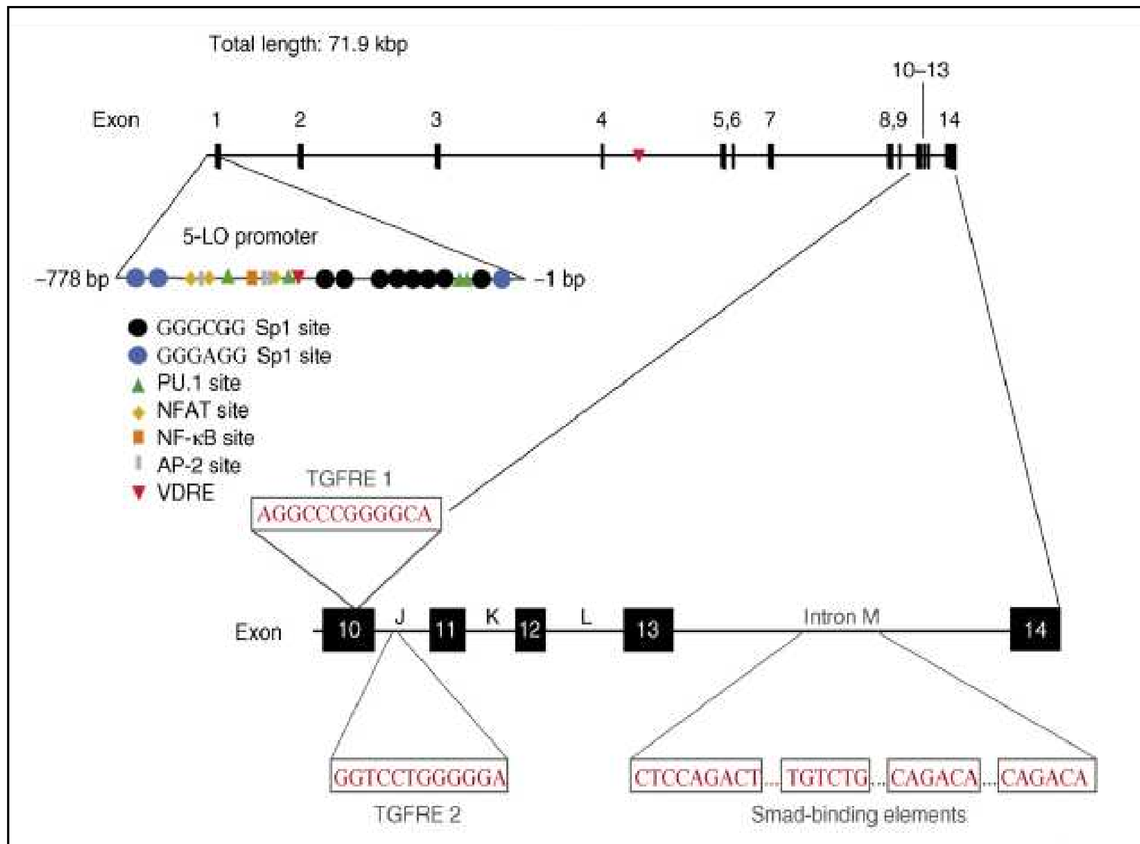


Fig. 4: The human 5-LO gene. The large human 5-LO gene (71.9 kbp) is located on chromosome 10 and is divided into 14 exons. Several transcription initiation sites have been described in the 5-LO gene: the principal one (position 0) is located 65 nucleotides upstream of ATG. Regulatory elements in the promoter, and in the distal part of the gene, are indicated. The promoter is GC-rich, indicated by the multiple Sp1-binding sites. *Abbreviations:* TGFRE, TGF-β-responsive element. (from *Radmark, O. et al. Trends Biochem Sci 32, 332-41, 2007*)

The observation that expression of 5-LO is much higher in differentiated myeloid cells and cell lines as compared to undifferentiated cells initiated studies on the mechanism of 5-LO upregulation. Differentiation inducers, such as dimethyl sulfoxide (DMSO), retinoic acid, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and transforming growth factor β (TGFβ), lead to an increase in 5-LO mRNA and protein (Radmark et al., 2007). This increase is thought to reflect the upregulation of 5-LO expression during leukocyte differentiation in the bone marrow. Upregulation of 5-LO has also been reported in mature blood leukocytes. Thus, granulocyte/macrophage colony-stimulating factor (GM-CSF) can increase 5-LO expression (two- to three-fold) in human granulocytes through both upregulated transcription and post-transcriptional effects. Increased 5-LO expression under pro-inflammatory conditions was also indicated by the increase in 5-LO mRNA in peripheral blood leukocytes from asthmatics (Koshino et al., 1999).

Expression of 5-LO also increases during another differentiation process – namely, when blood monocytes enter tissues and differentiate into macrophages (Pueringer et al., 1992), whereas human monocytes lose 5-LO and FLAP when kept in cell culture for 7 days (Ring et al., 1996). Thus, it seems that additional growth factors, that are not present in typical cell cultures, are required to maintain and to increase 5-LO expression.

Moreover, DNA methylation and histone acetylation can regulate 5-LO expression. In fact, in the 5-LO negative cell lines U937 and HL60TB, the core promoter was heavily methylated whereas upon treatment with an hypomethylating agent, 5-LO expression occurred (Uhl et al., 2003).

1.2.5. 5-Lipoxygenase structure

5-LO cDNAs cloned from human, mouse, rat and hamster, encode mature proteins of 672 or 673 amino acids (Funk, 1996). 5-LO protein has been purified from various leukocytes as a monomeric soluble protein with an estimated molecular mass of 72 to 80 kD (Radmark, 2000). Thus far, the crystal structure of 5-LO has not been solved, hence structural informations are still limited.

Based on the crystal structure of rabbit reticulocyte 15-LO (a 12/15-LO) (Gillmor et al., 1997), the 5-LO structure can be modelled as a monomeric enzyme with two domains **(Fig. 5)**. The catalytic C-terminal domain (residues 121-673) is mainly helical in structure, and contains iron. In particular, five iron ligands (His367, His372, His550, Asn554 and Ile673) were identified, whereas the sixth ligand is assumed to be H_2O, as shown for soybean LO-1. Based on mutagenesis, His372, His550 and the C-terminal Ile673 appear to constitute a 2-His-1-carboxylate facial triad anchoring the iron, a common feature for active sites of mononuclear *non-heme* iron (II) enzymes (Hegg and Que, 1997). In addition, His367 and Asn554 may function as replaceable ligands for iron (Hammarberg et al., 2001). The structural specificity of LOs may depend on the space of the pocket binding the fatty acid substrate, since mutagenesis reducing the pocket space converted 5-LO to a 15-LO (Schwarz et al., 2001).

In recombinant human 5-LO and porcine leukocyte 5-LO, iron as well as enzyme activity was lost when 5-LO was exposed to oxygen (Percival, 1991). EPR spectroscopy showed that the iron of the isolated enzyme is ferrous, while treatment with LOOHs gave ferric iron (see also **1.2.2.**). The multicomponent g6 signal in the EPR-spectrum indicated that several forms of the metal center were present (Chasteen et al., 1993). Not only 5-HpETE, but also 15-HpETE gave EPR-detectable ferric 5-LO. Similar to soybean LO, EPR indicated a flexible iron ligand arrangement in 5-LO.

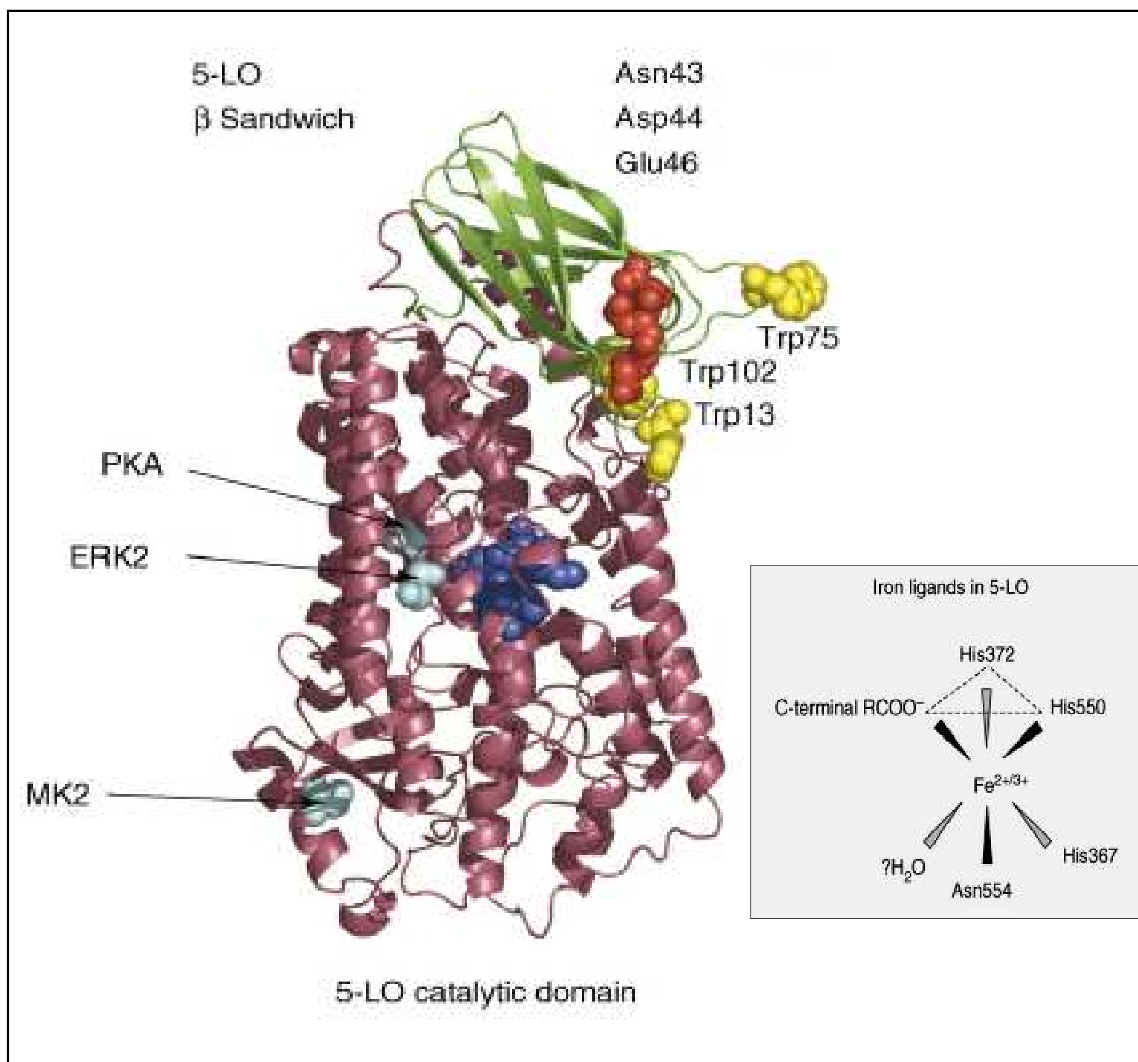


Fig. 5: Model of 5-LO structure. Model of 5-LO based on the crystal structure of the Fe^{2+} form of rabbit reticulocyte 15-LO. Rabbit 15-LO and, consequently, the model of 5-LO comprise two domains: 1) The catalytic C-terminal domain (residues 121–673; magenta) is mainly helical in structure and contains the iron that is central to enzyme activity. In the catalytic domain, the five iron ligands (His367, His372, His550, Asn554 and Ile673) are shown in blue (see also inset). The sixth ligand is assumed to be H_2O; 2) The catalytic domain also contains three kinase motifs for MK2 (*Leu-Glm-Arg-Gln-Leu-**Ser***), PKA (*Arg-Lys-Ser-**Ser***) and ERK2 (*Tyr-Leu-**Ser**-Pro*); the phosphorylated serine residues are shown in light blue. The smaller N-terminal domain (residues 1–114; green) is a C2-like β sandwich with typical ligand-binding loops. Residues in these loops of 5-LO have been shown to bind Ca^{2+} and cellular membranes. Trp13, Trp75 and Trp102 (yellow) mediate the effects of PC, glycerides and CLP. *Abbreviations:* CLP, coactosin-like protein; ERK, extracellular signal-regulated kinase; 5-LO, 5-lipoxygenase; LOOH, lipid hydroperoxide; MK-2/3, mitogen-activated protein kinases-activated protein kinases-2/3; PC, phosphatidylcholine; PKA, protein kinase A. (from *Radmark, O. et al. Trends Biochem Sci 32, 332-41, 2007*)

The catalytic domain also contains three kinase motifs for mitogen-activated protein kinase (MAPK)-activating protein kinase (MK)-2 (*Leu-Glm-Arg-Gln-Leu-**Ser***), protein kinase A (PKA) (*Arg-Lys-Ser-**Ser***) and extracellular signal-regulated kinase (ERK)-2 (*Tyr-Leu-**Ser**-Pro*) (Radmark et al., 2007).

The smaller N-terminal domain (residues 1-114) is a C2-like ß-sandwich with typical ligand binding loops (Hammarberg et al., 2000). Residues in these loops have been shown to bind Ca^{2+} and cellular membranes, and Ca^{2+} can activate 5-LO by inducing membrane association. In the ß-sandwich, mutagenesis of residues 43-46 reduced Ca^{2+} binding and Trp13, 74, and 102 mediate effects of phosphatidylcholine (PC), glycerides (e.g. 1-oleoyl-2-acetyl-sn-glycerol, OAG) and coactosin-like protein (CLP). The N-terminal domain was also modelled on the structure of the C2-like domain of *C. perfringens* α-toxin (1QMD, a phospholipase C) (Kulkarni et al., 2002). Sequence and topology similarities between Polycystin-1, Lipoxygenase and α-Toxin defined the PLAT domain as a subset within the C2 family (Allard and Brock, 2005).

1.2.6. Regulation of 5-lipoxygenase

The regulation of 5-LO activity is complex and depends on many factors that each can influence 5-LO catalysis (see ***1.2.6.1***). Importantly, 5-LO activity in the cell is even more complicated and several additional mechanisms are involved (see ***1.2.6.2***).

1.2.6.1. Factors regulating 5-lipoxygenase activity

The factors regulating 5-LO activity essentially seem to govern the accessibility of 5-LO towards its substrate and facilitate the oxidation of ferrous 5-LO to ferric form. Also,

stabilization of an active form of 5-LO may take place. Among these factors, Ca^{+2}, PC vesicles, glycerides, and CLP act via the C2-like domain, whereas the binding site for ATP has not yet been fully determined. Indeed, LOOHs provide the redox tone required for iron oxidation. These factors are involved in 5-LO regulation in *cell free systems* (isolated 5-LO, whole cell homogenates, subcellular fractions) as well as in intact cells. Studies in *cell free systems* have been helpful in the comprehension of the regulation of 5-LO activity. However, in the cells, other factors, like subcellular localization of the enzyme, interaction with cellular proteins and phosphorylation events, have to be considered **(Fig. 6)**.

Calcium

Initially, LT synthesis was observed after cell stimulation of neutrophils by Ca^{2+} ionophores (Borgeat and Samuelsson, 1979b), which suggested that the increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) is an important determinant for cellular 5-LO activity. However, the role of Ca^{2+} in 5-LO activation is complex.

Although no obvious Ca^{2+}-binding motif in the primary sequence of 5-LO is apparent, reversible binding of Ca^{2+} to 5-LO was found by different techniques, including Ca^{2+} overlay, gelfiltration in the presence of Ca^{2+}, equilibrium dialysis, and Ca^{2+}-induced mobility shift in gel electrophoresis (Hammarberg et al., 2002). 5-LO binds Ca^{2+} in a reversible manner (Hammarberg and Radmark, 1999): for the intact enzyme a K_d close to 6 µM was determined by equilibrium dialysis and the stoichiometry of maximum binding averaged around two Ca^{2+} per 5-LO. Similar results (two Ca^{2+} per 5-LO, K_{Ca} 7-9 µM) were obtained for the His-tagged C2-like domain (residues 1-115) (Kulkarni et al., 2002). Mutagenesis indicated that residues in

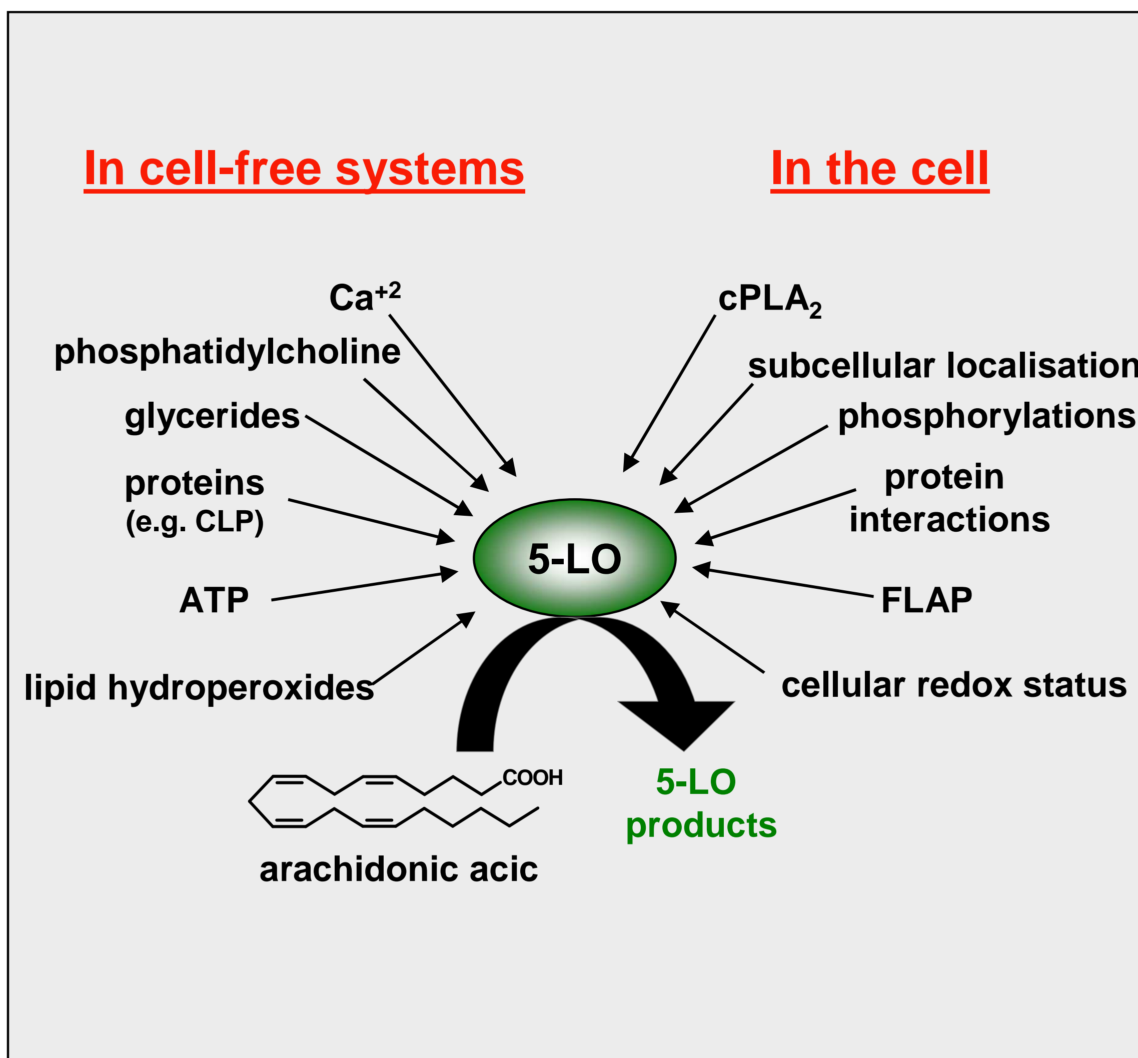


Fig. 6: Stimulating factors that regulate 5-LO catalysis *in vitro* and 5-LO activation in the cell. The factors regulating 5-LO activity result in access of 5-LO towards AA, in oxidation of ferrous 5-LO to the ferric form, or in stabilization of an active conformation of 5-LO. Among these factors, Ca^{+2}, PC vesicles, glycerides, and CLP act via the C2-like domain, whereas the binding site for ATP has not yet been fully determined. Indeed, LOOHs induce the redox tone required for iron oxidation. These factors are involved in 5-LO regulation in *cell free systems* (isolated 5-LO, whole cell homogenates, subcellular fractions) as well as in intact cells. In the cells, other factors, like subcellular localization of the enzyme, interaction with cellular proteins and phosphorylation events, have to be considered. *Abbreviations:* AA, arachidonic acid; CLP, coactosin-like protein; $cPLA_2$, cytosolic phospholipase A_2; 5-LO, 5-lipoxygenase; LOOH, lipid hydroperoxide; PC, phosphatidylcholine. (from *Werz, O. et al. Expert Opin Ther Patents 15, 2005*)

ligand binding loop 2 (Asn43, Asp44, Glu46, *see* **Fig. 3**) of the 5-LO C2-like domain are important for both Ca^{2+} binding, and for Ca^{2+} activation of enzyme activity (Hammarberg et al., 2000).

The C2-like domain seems to be involved in the Ca^{2+}-dependent interaction of the enzyme with membrane structures. Thus, Ca^{2+} stimulates 5-LO translocation to the nuclear envelope as well as association of 5-LO with membranes (Rouzer and Samuelsson, 1987), and preferentially with the nuclear membrane, which is rich in PC (Kulkarni et al., 2002). This scenario leads to a model suggesting that Ca^{2+} promotes membrane association which then facilitates the transfer of AA to 5-LO via FLAP (Dixon et al., 1990). Ca^{2+} may also induce a "productive binding" to some scaffold factors, like CLP. However, *in vitro*, requirement of Ca^{2+} for enzyme activity is not absolute and strongly depends on cellular stimulus, cell type, and assay conditions.

The EC_{50} for Ca^{2+} activation of purified 5-LO is quite low (1-2 µM) and full activation is reached at 4-10 µM (Percival et al., 1992; Noguchi et al., 1994; Hammarberg et al., 2000) whereas lower concentration of Ca^{+2} (200 nM) seems sufficient for 5-LO activation in intact cells. On the other hand, in homogenates of human PMNL and rat basophilic leukemia cells (RBL), considerable 5-LO product synthesis is detected in the absence of Ca^{2+}, whereas Ca^{2+} is required for 5-LO activity in homogenates of monocytic Mono Mac 6 cells under the same assay conditions (Straif et al., 2000; Burkert et al., 2003). In Mono Mac 6 cells, glutathione peroxidase (GPx)-1 was identified as endogenous inhibitor of cellular 5-LO (Straif et al., 2000), that renders 5-LO activity dependent on Ca^{2+} (Burkert et al., 2002) and it was found that the C2-like domain mediates the Ca^{2+}-dependent resistance of 5-LO activity against inhibition by GPx-1 (Burkert et al., 2003).

Ca^{2+} also lowers the K_m of 5-LO for AA and reaction kinetics are changed, leading to substrate inhibition. Apparently, Ca^{2+} can also modify the affinity of 5-LO for LOOHs. Possibly, ferric 5-LO could be formed at a lower concentration of LOOHs, when Ca^{2+} is present. LOs may have two fatty acid binding sites, one catalytic and one regulatory. Recently binding of an AA analogue not only to the active site in the catalytic domain, but also to the ß-sandwich of 15-LO was demonstrated (Romanov et al., 2006). It appears possible that fatty acids or LOOHs can bind to the ß-sandwich also of 5-LO, maybe in a Ca^{2+} dependent manner. Also Mg^{2+}, at millimolar concentrations present in cells, can activate 5-LO *in vitro* (Reddy et al., 2000). However 5-LO has some basal activity also in absence of Ca^{2+}/Mg^{2+}, and the divalent cation seems not be part of catalysis.

Phosphatidyl choline (PC)

Many C2 domains mediate Ca^{2+}-induced membrane association (Rizo and Sudhof, 1998). Early studies have identified cellular membrane fractions that upregulate 5-LO activity (Rouzer and Samuelsson, 1985), which could be replaced by synthetic lipid vesicles consisting of PC (Puustinen et al., 1988) but not by phosphatidylserine (PS), phosphatidylethanolamine, or phosphatidylinositol (Puustinen et al., 1988). PC was required for both basal and Ca^{2+}-stimulated 5-LO activity *in vitro* (Skorey and Gresser, 1998; Reddy et al., 2000). Binding of 5-LO to synthetic PC liposomes was induced by Ca^{2+} (Noguchi et al., 1994), and Ca^{2+} (also Mg^{2+}) increased the hydrophobicity of 5-LO in a phase partition assay. The isolated 5-LO C2-like ß-sandwich had a higher affinity for zwitterionic PC vesicles than for anionic PS and phophatidylglycerol vesicles, and three Trp residues in the ligand binding loops (Trp13, Trp75, Trp102) were important for PC binding. It has been suggested that the PC selectivity directs 5-LO to the nuclear envelope (Kulkarni et al., 2002).

Ca^{2+}-induced binding to PC stabilized the structures of both 5-LO protein and membrane (Pande et al., 2004). This binding occurred also in absence of Ca^{2+}, but Ca^{2+} was required for 5-LO activity. Based on these comparisons, it was suggested that 5-LO can bind to membranes in “productive/non-productive” modes, i.e. membrane binding *per se* may not confer 5-LO activity. Increased membrane fluidity favoured 5-LO association, and it was argued that this should be the factor directing 5-LO to the AA enriched nuclear envelope (Pande et al., 2005). Interestingly, addition of cholesterol to a membrane preparation reduced 5-LO activity in the *cell free* assay (Pande et al., 2005) and cholesterol sulfate could inhibit 5-LO also in *intact cells* (Aleksandrov et al., 2006).

Glycerides

Various glycerides, other types of lipid, were found to activate 5-LO. In particular, OAG was the most potent among the compounds tested (Hornig et al., 2005). It is interesting to note that Ca^{2+}, as well as phospholipids or cellular membranes, prevented the OAG stimulatory effect. Interestingly, the same three Trp residues which mediate binding to PC are involved in the binding to OAG, since the mutant 5-LO-Trp13/75/102Ala was not stimulated by OAG. Apparently, the uncharged glycerides can bind to the C2-like domain of 5-LO in absence of the charge neutralization, or in absence of the changes in side chain orientations, that are important, on the contrary, for Ca^{2+}-induced binding of 5-LO to PC (Kulkarni et al., 2002). As Ca^{2+}, also OAG protected 5-LO against the inhibitory effect of GPx-1 (Hornig et al., 2005).

Coactosin-like protein (CLP)

Human CLP is a protein of 142 amino acids similar to *D. Discoideum* coactosin, a member of the ADF/Cofilin group of actin binding proteins. A direct interaction between CLP and 5-LO

has been demonstrated *in vitro* and *in vivo* (Provost et al., 1999; Provost et al., 2001). CLP binds 5-LO in Ca^{+2}-independent manner with a 1:1 molar stoichiometry. Human CLP, like coactosin, binds also F-actin, with a 1:2 stoichiometry. Although mutagenesis showed the involvement of two different Lys in the CLP structure for the binding to F-actin and 5-LO (Lys75 and Lys131, respectively) (Liepinsh et al., 2004), these Lys are close each other and this implies overlapping binding sites. Indeed, no ternary complex has been observed and 5-LO competed with F-actin for the binding of CLP.

CLP can modulate the 5-LO pathway *in vitro* (Rakonjac et al., 2006). Apparently, CLP can function as a scaffold for 5-LO, similar to membranes, and increase 5-LO activity. These effects required protein interaction via Trp residues in ligand binding loops of the 5-LO ß-sandwich: in fact binding and stimulatory effects of CLP were abolished for the mutant 5-LO-Trp13/75/102Ala. Although, as mentioned above, CLP can bind to 5-LO in absence of Ca^{2+} (Provost et al., 2001), Ca^{2+} was required for 5-LO activity. Thus, the model of binding of 5-LO to CLP may depend on the presence of Ca^{2+}, and could be similar to the "productive/non-productive" modes hypothesized for the membrane binding (see *Phosphatidyl choline*) (Pande et al., 2004). Moreover, CLP was shown to follow 5-LO inside the cell (Rakonjac et al., 2006). Possibly, cellular 5-LO is always in complex with CLP, and when activated by Ca^{2+} (or Mg^{2+}) this complex is capable of producing 5-HpETE. Since formation of LTA_4 is determined by the well established translocation of 5-LO to the nuclear membrane, CLP may comigrate with 5-LO in this translocation. When AA is converted to 5-HpETE and further to LTA_4, 5-LO performs hydrogen abstractions at carbons 7 and 10. It has been speculated that association of 5-LO with both CLP and membrane confers an active 5-LO conformation which promotes the second hydrogen abstraction (at C-10), leading to LTA_4 formation.

ATP

In contrast to other LOs, the 5-LO catalytic activity is stimulated by ATP and, to a lesser extent, by other nucleotides including ADP, AMP, cAMP, CTP, and UTP (Ochi et al., 1983). The extent of 5-LO stimulation at 0.1 - 2 mM ATP is in the range of 2- to 6-fold, is partially Ca^{2+}-dependent, and the K_a value for ATP binding was 31 µM (Aharony and Stein, 1986).
In the cell, most of the ATP binds Mg^{2+}, and it appeared that a $MgATP^{2-}$ complex is the 5-LO activating factor (Reddy et al., 2000). Reactive ATP-analogues bound to 5-LO with stoichiometry close to 1:1, and 2-azido-ATP covalently modified Trp75 and Trp201, indicating that these residues were close to the reactive moiety of the analogue, sitting on the 5-LO nucleotide-binding site (Zhang et al., 2000). Located on the tip of one of the solvent exposed loops of the β-sandwich, Trp75 is one of the three Trp residues involved in membrane association of the human 5-LO (Kulkarni et al., 2002). This residue could be mutated without clear changes in activity and ATP-affinity. On the other hand, the mutant 5-LO-Trp201Arg was barely expressed, and exchange of Trp201 to Ala or Ser resulted in severely reduced activity and decreased ATP-column yield. In addition to being close to (or part of) an ATP-binding site, Trp201 appears to be important for the overall structure of 5-LO (Okamoto et al., 2005). ATP may activate 5-LO by stabilizing the protein structure. Hydrolysis of ATP is not required for the activating effect, and it appears that purified 5-LO is more stable in the presence of ATP.

Lipid hydroperoxides (LOOHs)

For LO catalysis, the ferrous iron of the resting form of the enzyme has to be oxidized to ferric iron by LOOHs. Among various hydroperoxides, 5-HpETE and 12-HpETE stimulated crude 5-LO in homogenates (Hammarberg et al., 2001). LOOHs can reduce the lag phase of 5-LO

after addition of AA to homogenates or purified enzyme, and conditions that promote lipid peroxidation upregulate 5-LO activity (Riendeau et al., 1989).

1.2.6.2. Regulation of 5-lipoxygenase activity in the cell

Biosynthesis of LTs in intact cells requires an orchestrated interplay of the crucial enzymes cytosolic phospholipase A_2 ($cPLA_2$), 5-LO, and the membrane-bound FLAP for formation of the intermediate LTA_4, as well as additional enzymes for the downstream conversion to effector LTs.

The amount of free AA available as substrate for 5-LO (Zarini et al., 2006) as well as its accessibility for 5-LO (Luo et al., 2003) are determinants for LT biosynthesis. Hence, stimuli capable to induce LT formation, like *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF), opsonized zymosan, LTB_4, C5a, IL-8, and ionophores, cause activation of both 5-LO and $cPLA_2$ (Werz, 2002). In fact, both enzymes share structural (C2 domain) and regulatory properties (activated by Ca^{2+} and by MAPK phosphorylations) (Gijon et al., 1999; Radmark and Samuelsson, 2005). The cellular activity of 5-LO is therefore tightly regulated by several additional mechanisms that include the release of AA by $cPLA_2$, localization of the enzyme inside the cells, phosphorylation, interaction with FLAP and/or other cellular proteins and the cellular redox tone.

Cytosolic phospholipase A_2 ($cPLA_2$)

$cPLA_2\alpha$ is a group IVA PLA_2 that catalyzes the hydrolysis of the sn-2 position of glycerophospholipids to release free AA, which is in turn metabolized to prostaglandins by the COX pathway and to LTs by the 5-LO pathway (Shimizu and Wolfe, 1990). The liberation of

free AA acid is tightly regulated and often the initial, rate-limiting step in the biosynthesis of eicosanoids. $cPLA_2$ has been isolated from various sources. The purified enzyme demonstrated maximal activity at alkaline pH (8.0-10.0) and considerable activity at micromolar or submicromolar concentrations of Ca^{+2} (Hirabayashi et al., 2004). The human cDNA sequence for $cPLA_2$ encodes a 85 kDa protein, although the enzyme migrates as an approximately 90-110 kDa protein on SDS-polyacrylamide gels. The enzyme contains two homologous catalytic domains (A and B) interspaced with gene-unique sequences. The lipase consensus sequence (Gly-Leu-Ser-Gly-Ser) is located at the N-terminal region of domain A. Studies by direct mutagenesis and by active site directed inhibitor indicated that the catalityc center contains the catalytically active Ser228 **(Fig. 7A)** (Sharp et al., 1994). Thiol modifying reagents completely inactivate $cPLA_2$ and Cys331 has been involved in the loss of enzyme activity, suggesting a role for this amino acid for the catalytic activity of the enzyme (Li et al., 1994). The amino-terminal portion of the protein (approximately 50 amino acids) has considerable sequence homology with the constant region 2 (C2) of protein kinase C (PKC), and this domain was demonstrated to mediate the Ca^{2+}-dependent translocation to membranes (Clark et al., 1991). In fact in the cell, specific subcellular targeting of $cPLA_2$ is an important step in AA release and eicosanoid production. An increase in $[Ca^{2+}]_i$ concentration promotes binding of Ca^{2+} ions to the C2 domain and then allows $cPLA_2$ to translocate from the cytosol to the perinuclear region, including the Golgi apparatus, endoplasmic reticulum, and nuclear envelope (Glover et al., 1995; Schievella et al., 1995; Hirabayashi et al., 1999). Upon membrane binding, conformational changes in the enzyme might take place to allow the fatty acyl chain of a substrate molecule to enter the active site **(Fig. 7B)**.

The amplitude and duration of intracellular Ca^{2+} increases are important for $cPLA_2$ activation (Hirabayashi et al., 1999). In most cases, Ca^{2+}-dependent translocation of $cPLA_2$ from the

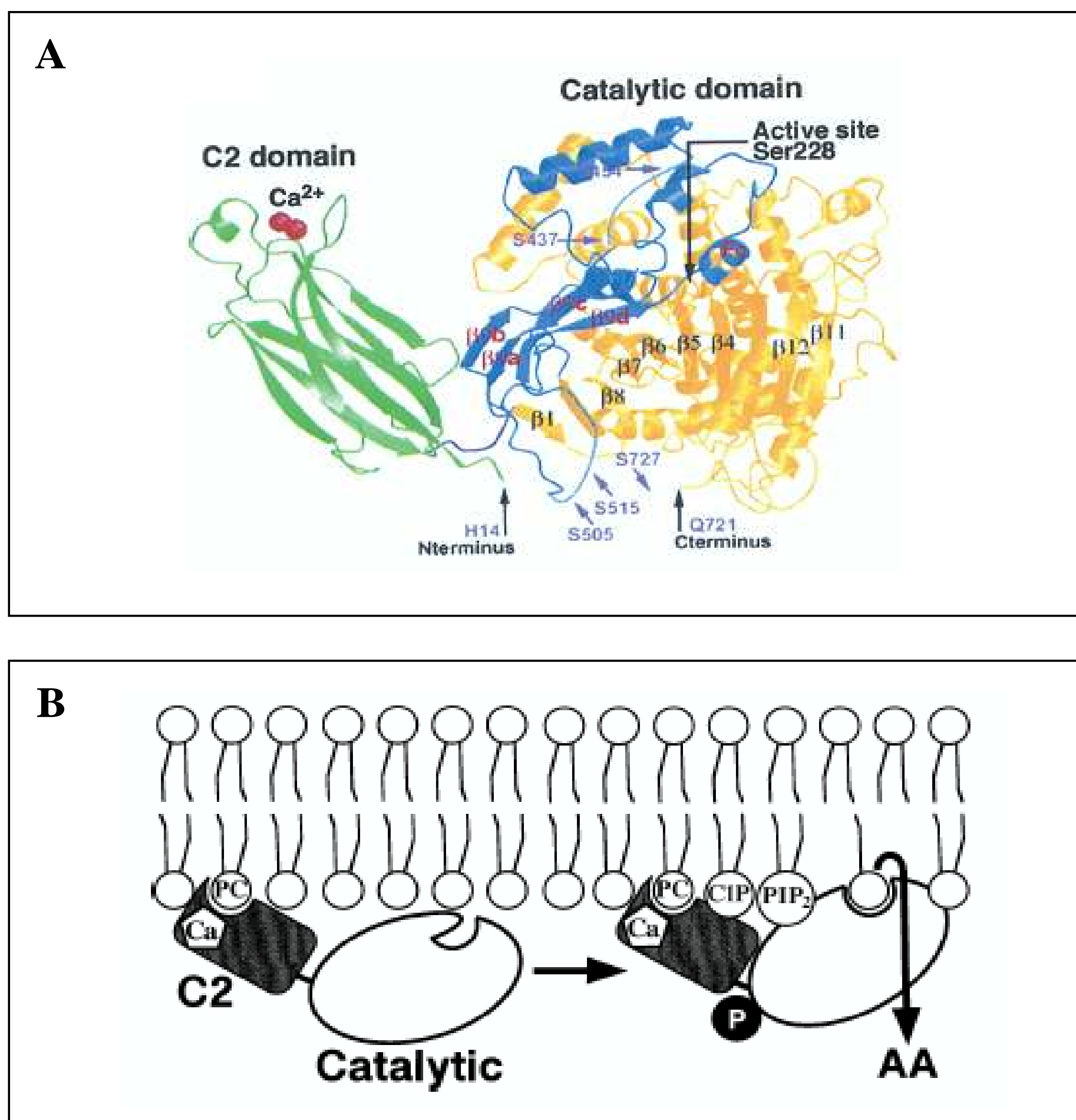


Fig. 7: (A) Three-dimensional structure of $cPLA_2$. The three-dimensional structure of $cPLA_2$ and its isolated C2 domain have been determined by X-ray crystallography. A flexible linker connects C2 and catalytic domains. The C2 domain consists of an eight-stranded antiparallel β-sandwich and binds to two Ca^{2+}-ions through a cluster of aspartic acid residues. The catalytic domain consists of 13α-helices and 14β–strands. The catalytic dyad composed of Ser228 and Asp549 is located at the bottom of a deep, narrow cleft. **(B) Proposed model for $cPLA_2$ activation.** Ca^{2+}-binding to the C2 domain promotes $cPLA_2$ translocation from the cytosol to the membrane containing PC. Phosphorylation on Ser residues and/or binding to anionic phospholipids stabilize the association of $cPLA_2$ with the membrane and increase catalytic activity. The interfacial activation may be caused by conformational changes in the protein leading to removal of the lid from the active site and favorable orientation of the catalytic domain at the membrane surface. *Abbreviations:* AA, arachidonic acid; C1P, ceramide-1-phosphate; $cPLA_2$, cytosolic phospholipase A_2; P, phosphate; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate. (from *Hirabayashi, T. et al. Biol Pharm Bull 27, 1168-73, 2004*)

cytosol to membranes is necessary for its access to phospholipid substrates (Channon and Leslie, 1990). A sustained increase in the cytosolic Ca^{2+} level leads to continuous membrane localization and once the duration of Ca^{2+} increase extends beyond a certain time (2-5 minutes), $cPLA_2$ is kept in the perinuclear region even after the $[Ca^{2+}]_i$ returns to the basal level (Evans et al., 2001).

A Ca^{2+}-independent localization of $cPLA_2$ on the perinuclear membranes has also been described. This may be regulated by phosphorylation, anionic lipids, and hydrophobic binding of the catalytic domain (Hirabayashi et al., 2004). So far, three distinct phosphorylation on serine residues of this enzyme have been reported. Depending on the cell type and stimulus, $cPLA_2$ can be phosphorylated by MAPKs on Ser505 (Lin et al., 1993; Nemenoff et al., 1993; Kramer et al., 1996), by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) on Ser515 (Muthalif et al., 2001) and by MAPK-interacting kinase Mnk1 on Ser727 (Hefner et al., 2000). Phosphorylation of $cPLA_2$ on either Ser505, Ser515, or Ser727 fails to induce AA release in absence of a concomitant increase in intracellular Ca^{+2}, but increases $cPLA_2$ intrinsic enzymatic activity 2- to 3-fold at submicromolar intracellular Ca^{2+} concentrations (Hefner et al., 2000). However, the contribution of $cPLA_2$ phosphorylation to AA release is much less at high concentrations of intracellular Ca^{2+} (Hefner et al., 2000).

Subcellular localization of 5-lipoxygenase: a mobile enzyme

In a generally accepted model **(Fig. 8)**, 5-LO occurs as a soluble enzyme in resting cells, either in the cytosol or in the nucleus, depending on the cell type (Werz and Steinhilber, 2006). In particular, cytosolic 5-LO has been described in neutrophils, eosinophils and peritoneal macrophages, whereas 5-LO is in a nuclear soluble compartment associated with the chromatin in alveolar macrophages, Langherans cells or rat basophilic leukaemia cells (Werz, 2002).

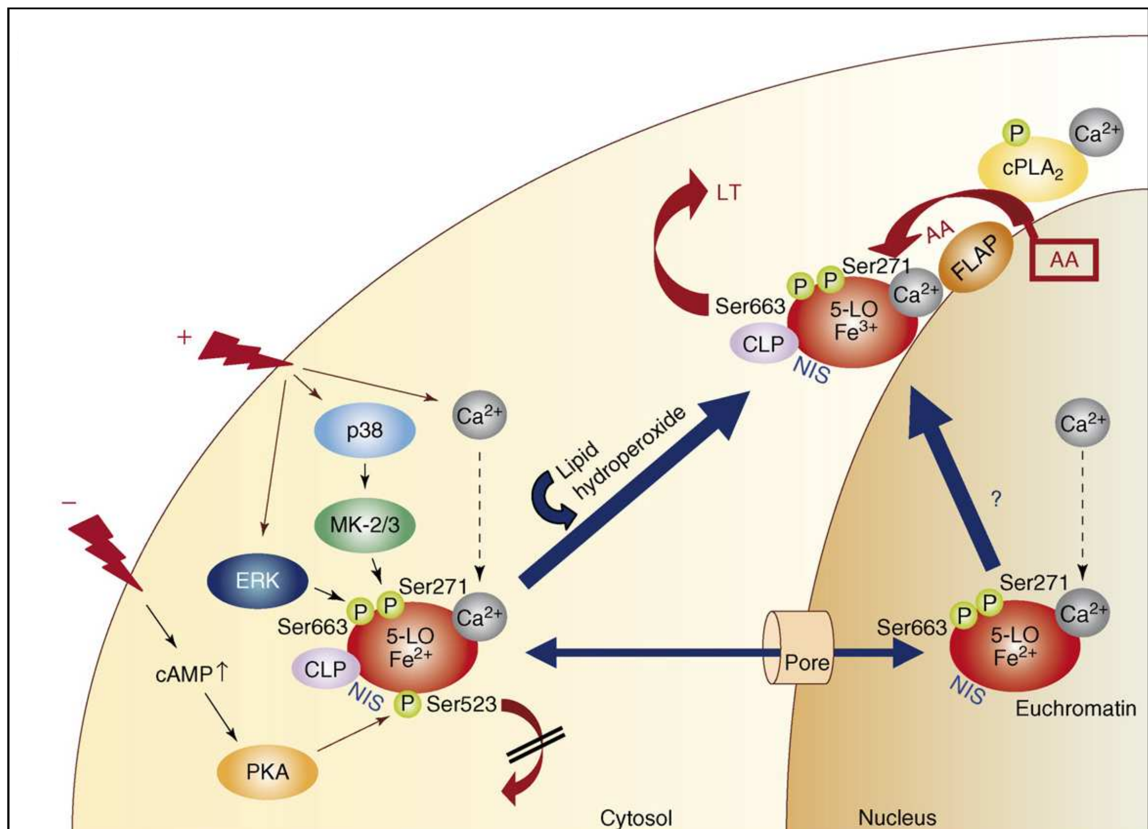


Fig. 8: 5-LO activation in the cell. In the resting cell, 5-LO is localized in either the cytosol or a soluble compartment inside the nucleus. On activation, 5-LO migrates to the nuclear envelope, where $cPLA_2$ and FLAP aid 5-LO in LT biosynthesis. $cPLA_2$ liberates AA from phospholipids. Membrane-bound FLAP is thought to facilitate the transfer of AA to 5-LO. CLP can bind 5-LO. Nuclear import sequences (NIS) in the 5-LO sequence determine import into the nucleus. Stimuli of LT biosynthesis lead to an increase in Ca^{2+} and/or activation of MAPK. When 5-LO is activated, the iron is oxidized by LOOH from Fe^{2+} to Fe^{3+}. In 5-LO, Ser271 is phosphorylated by MK-2/3 downstream of p38 MAPK. ERK phosphorylates 5-LO on Ser663. An increase in cAMP levels activates PKA, which represses 5-LO activity through phosphorylation on Ser523. *Abbreviations:* AA, arachidonic acid; CLP, coactosin-like protein; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular-signal-regulated kinase; FLAP, 5-LO-activating protein; 5-LO, 5-lipoxygenase; LOOH, lipid hydroperoxide; LT, leukotriene; MAPK, mitogen-activated protein kinases; MK-2/3, MAPKAPK-2/3; NIS, nuclear import sequences; PKA, protein kinase A. (from *Radmark, O. et al. Trends Biochem Sci 32, 332-41, 2007*)

Upon extracellular stimulation by relevant agonists (e.g. ionophores, thapsigargin, PAF, LTB_4, fMLP, complement 5a, IL-8, zymosan, or urate and phosphate crystals), signaling molecules activate $cPLA_2$ and 5-LO, which both translocate from the soluble compartment to the nuclear envelope. Here, 5-LO associates with FLAP, and this process is accompanied with substantial LT generation.

Within this model, two distinct nuclear translocation patterns can be distinguished: import/export of cytosolic/nuclear 5-LO, for cells showing an intranuclear localization of 5-LO, and association of 5-LO with the nuclear membrane, after activation.

Import of 5-LO into a nuclear soluble compartment depends on nuclear localization sequences (NLS) and on the phosphorylation site Ser271 (Luo et al., 2003), and does not have to be associated with LT synthesis. Therefore, the import of 5-LO in nuclear soluble compartments seems to be an entirely distinct phenomenon from its ability to move to membranes upon cell stimulation. NLS appear to be present in both the N-terminal part of 5-LO, and close to the C-terminus (Lepley and Fitzpatrick, 1998; Chen et al., 1998; Healy et al., 1999; Jones et al., 2002; Jones et al., 2003). Apparently, nuclear import of 5-LO may reflect the combined functional effects of the three discrete import sequences surrounding the amino acids Arg518, Arg112 and Lys158 (Healy et al., 1999; Jones et al., 2003). Leptomycin B, a specific inhibitor of nuclear export signal (NES)-dependent transport, diminished the cytosolic localization of 5-LO, suggesting that also an NES-system might function in positioning 5-LO (Hanaka et al., 2002). The import process can also be triggered by adherence (Brock et al., 1997; Brock et al., 1999) or by cytokines (Cowburn et al., 1999; Hsieh et al., 2001) and seems to occur during leukocyte recruitment into sites of inflammation (Brock et al., 1997; Brock et al., 2001).

Association of 5-LO to the nuclear membrane after activation depends on several factors, like Ca^{+2} (see above), phosphorylations and interaction with other proteins. Interestingly, the

location of 5-LO in resting cells seems to determine the locale where 5-LO redistributes upon stimulation. Thus, for example, it has been reported that in neutrophils and peritoneal macrophages, cytoplasmic 5-LO associates with the endoplasmic reticulum and the outer nuclear membrane, whereas the intranuclear 5-LO (e.g. in dendritic cells or in alveolar macrophages) translocates to the inner membrane of the nuclear envelope (Luo et al., 2003). The capacity for LT generation is higher when 5-LO is located in the nucleus. In the absence of exogenously added AA, translocation to the nuclear membrane is required for 5-LO to get access to AA released by $cPLA_2$. On the contrary, co-addition of exogenous AA resulted in prominent LT formation, but only marginally increased membrane-binding is observed. Thus, under these conditions, LT formation apparently occurs in the cytosol where 5-LO has ample supply of substrate.

It is interesting to note that free AA could be supplied to the cells also by transcellular mechanisms from neighbouring cells (Folco and Murphy, 2006) and 5-LO may be in different cellular loci when exogenous/endogenous AA is metabolized.

Phosphorylation

In intact cells, phosphorylation events by cellular kinases function together with Ca^{+2} in the regulation of 5-LO activation (Radmark et al., 2007) **(Fig. 9)**.

The 5-LO sequence contains several protein kinase motifs, and 5-LO is phosphorylated *in vitro* by p38 MAPK-regulated MAPKAPK-2/3 (MK2/3) (Werz et al., 2000), ERK1/2 (Werz et al., 2002a), CaMKII (Werz et al., 2002c) and PKA (Werz et al., 2002c; Luo et al., 2004).

There are several isoforms of p38 MAPK, which are activated by cell stress or treatment of cells with inflammatory cytokines (Lee et al., 2000). Activated p38 MAPK in turn phosphorylates and activates MK2/3. By in-gel kinase assays, 5-LO was found to be a

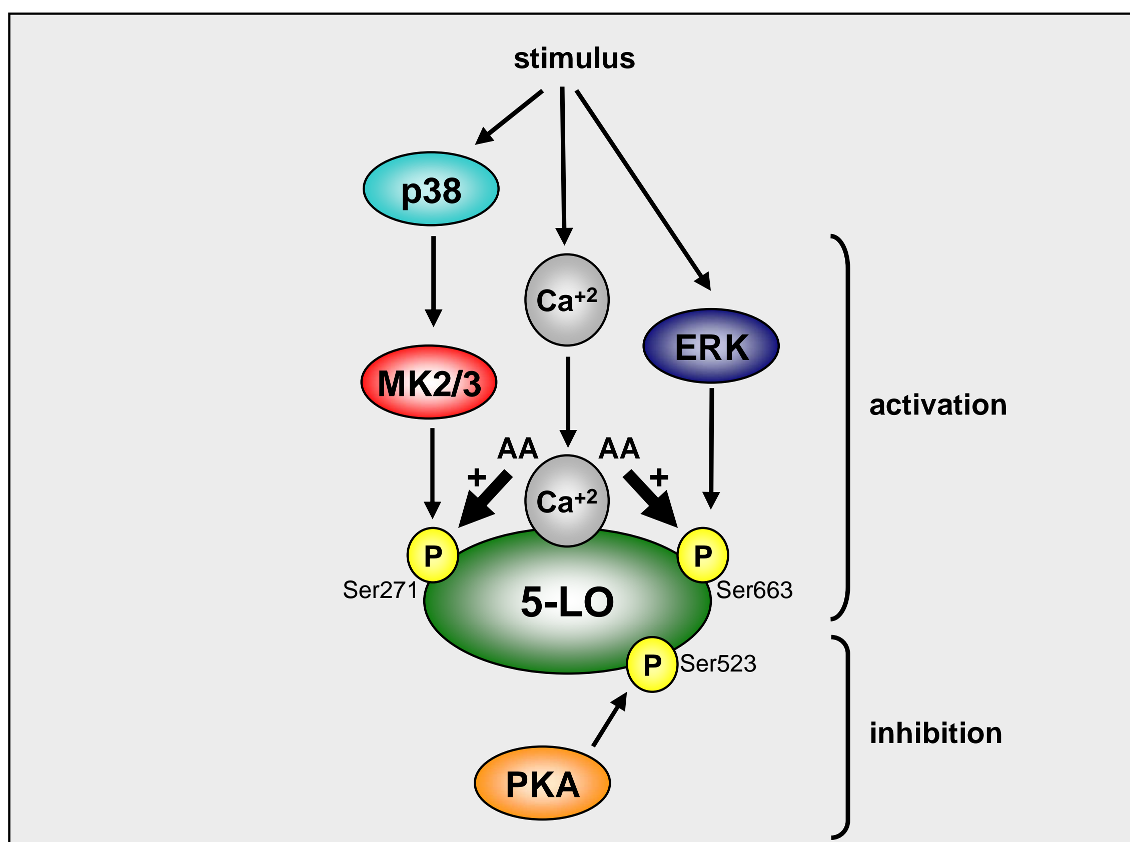


Fig. 9: Activation of 5-LO by Ca^{2+} and by phosphorylation. Depending on the stimulus and the cell-type, cellular 5-LO can be activated by Ca^{2+} and/or phosphorylation to metabolize AA. 5-LO phosphorylation by p38 MAPK-regulated MK at Ser271 and by ERK at Ser663 is promoted by AA. *Abbreviations:* AA, arachidonic acid; ERK, extracellular signal-regulated kinases; 5-LO, 5-lipoxygenase; MK2/3, mitogen-activated protein kinases-activated protein kinases 2/3; PKA, protein kinase A. (from *Werz, O. et al. Pharmacol Ther 112, 701-18, 2006*)

Table 1: Different features between cell stress and Ca^{2+}-induced activation of 5-LO in PMNL

Feature	Cell stress	Ionophore
Ca^{+2} dependency	*None*	*High*
Decreased K_m for AA	*No*	*Yes*
Sensitivity to MAPK inhibitor	*High*	*Low*
Time-course of activity	*Prolonged*	*Quick*
Sensitivity to certain non-redox 5-LO inhibitors	*Low*	*High*

substrate for MK2/3 (Werz et al., 2001a). Mutation of Ser271 to Ala in 5-LO abolished MK2 catalyzed phosphorylation in vitro. Also, phosphorylation by kinases prepared from stimulated PMNL and MM6 cells was clearly reduced, indicating that this is a major site for cellular phosphorylation of 5-LO (Werz et al., 2002c). Compared to the established MK2 substrate heat shock protein 27, 5-LO was only weakly phosphorylated in vitro by MK2.

Another MAP kinase, namely ERK2, was found to phosphorylate 5-LO in vitro, on Ser-663. For PMNL, it appeared that both ERK2 and p38 MAP kinase activities are important for 5-LO product formation in absence of ionophore stimulation (Werz et al., 2002a). Results supporting that phosphorylation is an alternative pathway for stimulation of 5-LO, which is different in character from Ca^{2+} activation in ionophore-treated cells (Radmark and Samuelsson, 2005), are given in **Table 1**.

Conditions that activate MAPKAPKs and ERKs (cell stress, phorbol esters) induce nuclear translocation of 5-LO and enhance product formation in intact cells, and this is susceptible to kinase inhibitors (SB203580, U-0126, calphostin) (Werz et al., 2000; Werz et al., 2001a; Werz et al., 2001b; Werz et al., 2002a; Werz et al., 2002c; Luo et al., 2003). Intriguingly, nuclear translocation of 5-LO in PMNL after activation of p38 MAPK by sodium arsenite occurred also in absence of intra- and extracellular Ca^{+2} (Werz et al., 2002b).

Moreover, mutation of Ser271 and/or Ser663 to Ala impaired cellular 5-LO product formation when transfected cells were selectively activated by AA (which activates MAPK) (Werz et al., 2002a; Werz et al., 2002c). It was suggested that first, already membrane bound 5-LO catalyzes an initial burst of LTB_4 biosynthesis in the absence of Ca^{2+} mobilization. Then, via an autocrine loop, LTB_4 might cause the release of Ca^{2+} from intracellular stores and further LT biosynthesis. One could visualize that AA-induced phosphorylation of 5-LO at Ser residues 271 and 663 could contribute to the initial activation of 5-LO.

In vitro, phosphorylations of Ser271 and Ser663 were both supported by unsaturated fatty acids, including AA and oleic acid (Werz et al., 2002a; Werz et al., 2002c). Possibly, binding of unsatured fatty acids to a putative regulatory fatty acid binding site on 5-LO stimulates phosphorylation by MK2 (Sailer et al., 1998). However, phosphorylation by MK2 *in vitro* does not seem to increase 5-LO activity in subsequent enzyme assays (Werz et al., 2002c), and the same was found for ERK2. Possible mechanisms could be that phosphorylations at Ser271 and Ser663 govern nuclear redistribution more than 5-LO activity itself.

Therefore it seems that phosphorylation rather modulates or regulates the interaction of 5-LO with other cellular components than directly affecting its catalytic properties. Recent data on the effects of 5-LO enzyme phosphorylation by the p38 MAPK pathway on cellular 5-LO localization underscore this point of view. In fact, it was found that 5-LO phosphorylation at Ser271 is responsible for the stress-induced nuclear export of 5-LO in CHO-K1 and HEK293 cells (Hanaka et al., 2005), whereas, in NIH 3T3 cells, phosphorylation at Ser271 stimulates nuclear localization and subsequently cellular 5-LO activity (Luo et al., 2003).

Phosphorylation at Ser523 by PKA directly suppresses 5-LO catalysis *in vitro* as well as in the cell (Luo et al., 2004; Luo et al., 2005), and it has been hypothesized that this phosphorylation could give a direct reduction of 5-LO catalytic activity via allosteric changes close to the active site. Moreover, phosphorylation at Ser523 prevents 5-LO nuclear localisation by inhibiting the nuclear import function of a NLS close to the kinase motif (Luo et al., 2005). This appears to be the molecular basis for the 5-LO suppressive effects of increased cAMP, which activates PKA (Flamand et al., 2002). It was suggested that PKA activation in response to agents, such as adenosine, prostaglandin E_2, and β-adrenergic agonists, is a mechanism for the control of LT biosynthesis which may be important to limit inflammation (Flamand et al., 2002; Luo et al., 2004).

Interestingly, polyunsaturated fatty acids (such as AA), which promote phosphorylation at Ser271 and 663, prevented cAMP-mediated inhibition of 5-LO translocation and product synthesis in activated neutrophils, apparently by interaction with a region close to the catalytic site (Flamand et al., 2006).

Protein interaction of 5-lipoxygenase

A proline-rich region spanning residues 566-577 in 5-LO was identified as a Src homology 3 (SH3)-binding motif, and 5-LO can bind to the SH3-domain of growth factor receptor-bound protein 2, an adaptor protein for tyrosine kinase-mediated cell signaling (Lepley and Fitzpatrick, 1994). Since a peptide corresponding to the SH3-binding motif inhibited the redistribution of 5-LO from the cytosol to the membrane in stimulated neutrophils, the SH3 motif could be involved in nuclear translocation. Also, a role for 5-LO in tyrosine kinase signaling, distinct from its role in lipid mediator formation, is conceivable.

5-LO protein was detected in NF-κB immunoprecipitates prepared from HL-60 cell lysates (Lepley and Fitzpatrick, 1998). The amount of 5-LO in such precipitates was high in ionophore-challenged cells, where 5-LO translocated to the nuclear envelope, but was low when 5-LO translocation was blocked, and it was concluded that 5-LO may have a role in cellular NF-κB responsiveness.

Interaction of 5-LO with three cellular proteins was found by yeast two-hybrid screening of a human lung cDNA library with 5-LO as bait, namely CLP (see above), TGFβ receptor-I-associated protein I (TRAP-1) and the ribonuclease Dicer (Provost et al., 2002; Provost et al., 1999). TGFβ strongly upregulates expression and activity of 5-LO during maturation of myeloid cells and a functional link between TRAP-1 and 5-LO appears reasonable. Dicer

cleaves double-stranded RNA into small interfering RNAs, and interaction with Dicer implies a possible role for 5-LO in RNA interference, which is quite distinct from its function in the synthesis of LTs.

Moreover, numerous experimental data indicate a possible interaction of 5-LO with FLAP, although a direct association between these proteins has not been shown yet.

5-Lipoxygenase activating protein (FLAP)

FLAP is a 18 kDa membrane-bound protein that was discovered as a target of MK886 (Miller et al., 1990). MK886 potently inhibited LT biosynthesis in intact leukocytes, but failed to suppress catalytic activity of 5-LO in broken cell preparations, confirming the role of FLAP in the regulation of 5-LO in intact cells. The cDNAs of FLAP have been cloned from rat and human, encoding a 161-amino acid protein with three transmembrane-spanning regions and two hydrophilic loops (Dixon et al., 1990). Interestingly, there are similarities in the overall amino acid sequence and the ligand-binding domains of the 18-kDa LTC_4-synthase and the other MAPEGs with FLAP (Jakobsson et al., 2000). The crystal structure of human FLAP has been recently determined (Ferguson et al., 2007) **(Fig. 10)**: FLAP contains four transmembrane helices that are connected by two elongated cytosolic loops and one short lumenal loop, and crystallizes as a homotrimer. Expression of 5-LO is consistent with the occurrence of FLAP in myeloid cells (Steinhilber, 1994; Vickers, 1995) and the upregulation of FLAP often correlates with that of 5-LO. On the other hand, also 5-LO negative cells express FLAP (Claesson et al., 1993; Kargman et al., 1993), and increased FLAP expression has also been observed differentially from that of 5-LO (Coffey et al., 1994; Cowburn et al., 1999).

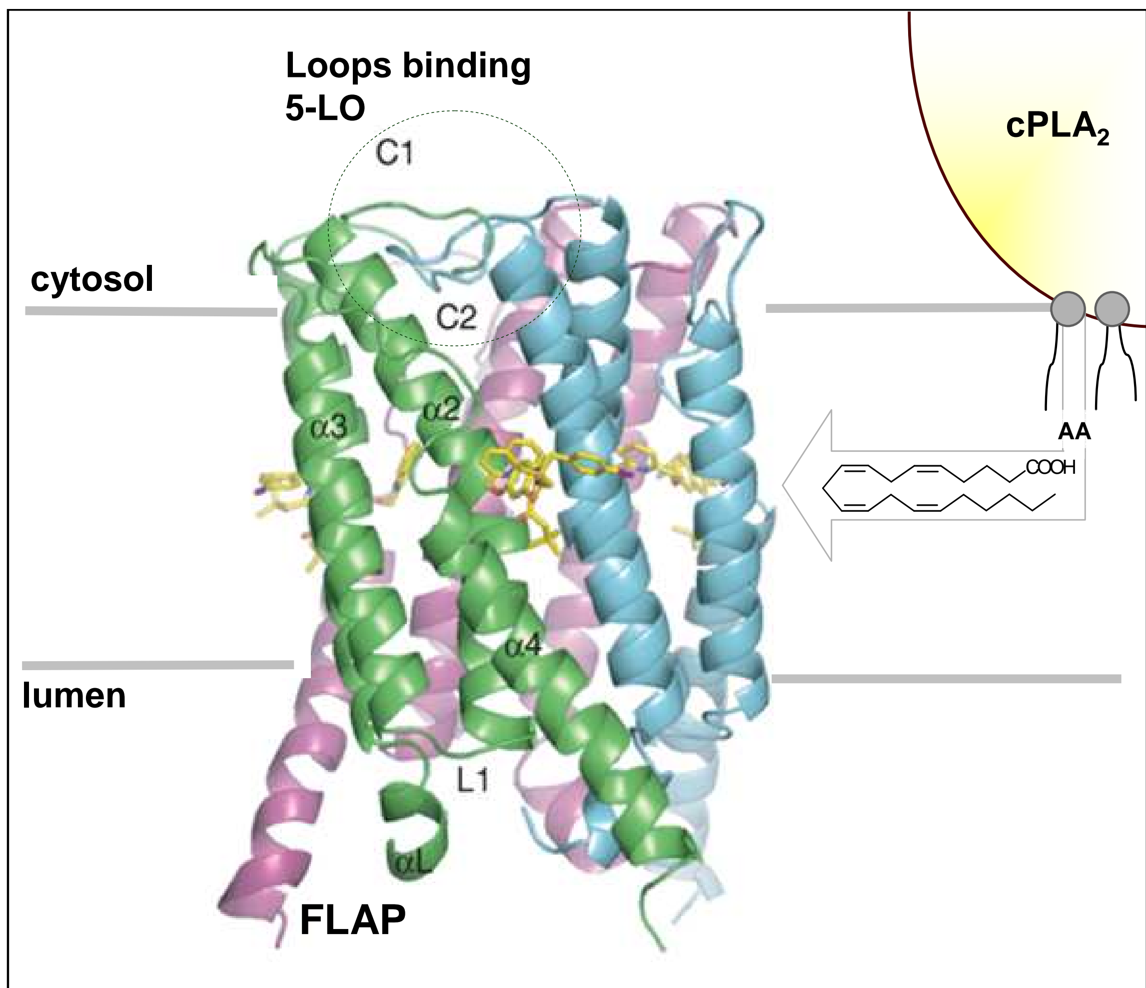


Fig. 10: The FLAP trimer structure. Monomers are colored green, cyan and magenta and the view is given parallel to the nuclear membrane. Each monomer contains four transmembrane helices (α1-α4) that are connected by two elongated cytosolic loops (C1 and C2) and one short lumenal loop (L1). Bound inhibitor molecule is shown in yellow and is located within the nuclear membrane, which provides an appropriate environment for the lateral diffusion of AA molecules released by $cPLA_2$ to FLAP. It is reasonable that the C-terminal catalytic domain of 5-LO binds to the cytosolic loops of FLAP. One 5-LO molecule could bind to each FLAP trimer, so that AA molecules are sequentially transferred to 5-LO. *Abbreviations:* AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-LO-activating protein; 5-LO, 5-lipoxygenase. (from *Ferguson, A. D. et al. Science, 317, 510-12, 2007*)

The requirement of FLAP for cellular LT synthesis could be visualized by transfection experiments using a human osteosarcoma cell line (Dixon et al., 1990). Cotransfection of both 5-LO and FLAP was necessary for cellular LT synthesis, whereas cells transfected with 5-LO alone produced no LTs, although 5-LO was active in corresponding cell homogenates. Also, no LT synthesis was detectable in FLAP-deficient macrophages from knock out mice (Byrum et al., 1997). Therefore, FLAP is considered absolutely required for cellular LT synthesis from endogenous substrate, but it is dispensable for catalytic 5-LO activity in cell homogenates (Rouzer et al., 1990). However, there is no absolute requirement for FLAP when cells are stimulated in presence of exogenous AA (Abramovitz et al., 1993), although it could stimulate the utilization of exogenous AA by 5-LO. Of interest, it has been observed that the potency of the FLAP-inhibitor MK-886 in inhibiting 5-LO product synthesis in human PMNL was impaired in presence of exogenous AA (Fischer et al., 2007). In the light of these observations and since it was shown that FLAP can bind AA and other cis-unsaturated fatty acids (Mancini et al., 1993), it seems reasonable that FLAP serves as an AA transfer protein facilitating the presentation of substrate to 5-LO for optimal synthesis of LTA_4 (Abramovitz et al., 1993). By combining the recently characterized crystal structure with previous data, a model has been proposed to explain the selective transfer of AA to 5-LO by FLAP. After translocation to the nuclear membrane, the Ca^{+2}-binding domain of 5-LO could anchor the nuclear membrane adjacent to FLAP. It is reasonable to assume that the C-terminal catalytic domain of 5-LO binds to the cytosolic loops of FLAP. In the nuclear membrane, AA could then laterally diffuse to FLAP. This event may be coupled to structural changes in FLAP, and AA molecules might be sequentially transferred to 5-LO. Moreover, it has been hypothesized that FLAP could be involved also in the lateral transfer of LTA_4 to LTC_4-synthase (Ferguson et al., 2007).

Cellular redox status

Since 5-LO catalysis requires oxidation of the iron to the active ferric state (see also *lipid hydroperoxides*), the redox tone is an important parameter for cellular 5-LO activity. Conditions that promote lipid peroxidation such as phorbol 12-myristate 13-acetate (PMA)-induced formation of reactive oxygen species, addition of peroxides, inhibition of GPx enzymes, or depletion of glutathione upregulate 5-LO product synthesis. On the contrary, reduction of peroxides by GPx-1 and -4 suppress 5-LO product formation (Werz, 2002). Interestingly, oxidative stress stimulates also p38 MAP kinase and may thus stimulate 5-LO in two ways: by inducing phosphorylation and by promoting formation of the ferric form of the enzyme (Werz et al., 2001a). The efficiency of nonredox type 5-LO inhibitors depended on the presence of intact GPx activity leading to low hydroperoxide concentration (Werz et al., 1998), and this appeared related to oxidative stress and activation of p38 MAP kinase. Ca^{2+}, as well as glycerides (e.g. OAG), may decrease the concentration of LOOHs needed for 5-LO activation, probably via increasing the affinity of 5-LO to LOOHs and therefore allowing cellular 5-LO product formation also at a low redox tone (Burkert et al., 2003; Hornig et al., 2005).

1.2.7. Stimuli inducing cellular 5-lipoxygenase product formation and priming agents

Stimuli that induce cellular 5-LO product synthesis include Ca^{2+}-mobilizing agents (ionophores or thapsigargin), soluble agonists (chemotactic PAF, LTB_4, fMLP and C5a), cytokines (IL-8), and phagocytic paricles (zymosan, urate or phosphate crystals) (Werz, 2002). Ca^{2+} ionophores cause strong 5-LO product formation, whereas the naturally-occurring

ligands, which moderately rise Ca^{2+} by acting through specific cell surface receptors, are much less efficient.

Since the availability of free AA is a critical parameter for the capacity of 5-LO product synthesis, the poor LT synthesis observed after exposure of leukocytes towards naturally-occurring ligands (like fMLP, C5a, PAF, or LTB_4) might be related to a low activity of $cPLA_2$ and substrate supply. Pre-treatment of leukocytes with so-called "priming" agents, including lipopolysaccharide (LPS), growth factors, cytokines, phorbol esters, or Epstein Barr Virus (EBV), which by themselves do not induce LT synthesis, strongly increased LT generation after subsequent stimulation with natural agonists (Werz, 2002). In view of the complex regulation of 5-LO by several cofactors and the involvement of several enzymes within the 5-LO pathway, these priming effects may operate at multiple sites, such as by enhancing AA availability (Liles et al., 1987; McIntyre et al., 1987), increasing expression of FLAP (Pouliot et al., 1994), elevating levels of intracellular Ca^{2+} (McColl et al., 1991), increasing accumulation of 5-LO at the nuclear membrane and enhancing phosphorylation of 5-LO (Werz et al., 2001b). In particular, Ca^{2+} and phosphorylations at serine residues of $cPLA_2$ and 5-LO appear to be important signaling events determining the cellular activation and subcellular distribution of these enzymes.

1.2.8. 5-Lipoxygenase products: receptors and pathophysiological roles

The biological actions of 5-LO products are mediated by specific receptors. LTB_4 is a potent chemotactic and chemokinetic mediator stimulating the migration and activation of granulocytes, leading to adherence of granulocytes to vessel walls, degranulation, release of superoxide (Claesson and Dahlen, 1999), and it was shown to augment phagocytosis of

neutrophils and macrophages (Mancuso et al., 1998; Mancuso et al., 2001). In lymphocytes, LTB_4 stimulates the secretion of IgE, IgG and IgM. Furthermore, LTB_4 has been connected to increased interleukin production (Brach et al., 1992) and neutrophil-dependent hyperalgesia (Levine et al., 1984). These properties imply a significant role for LTB_4 in the pathogenesis of inflammatory diseases (arthritis, psoriasis, inflammatory bowel disease, and asthma). LTB_4 actions are mediated by the BLT_1 and BLT_2 receptors, which are G-protein coupled receptors (GPCRs) with high homology (36-45 %), but distinct tissue distribution and affinity for the ligand (Brink et al., 2003). The BLT_1 receptor mediates the chemotaxis and data from different animal models suggest that LTB_4 signalling through this receptor is related to atherogenesis, bronchial asthma, glomerulonephritis, arthritis and chronic inflammatory bowel diseases (Okuno et al., 2005). The physiological and pathophysiological roles of the BLT_2 receptor are not clear, although it has been hypothesized that BLT_2 receptor is responsible for LTB_4 signalling when LTB_4 concentrations are high and BLT_1 might be desensitized. In addition, LTB_4 binds and activates the peroxisome proliferator-activated receptor-α, a transcription factor that mediates antiinflammatory actions, suggesting a pathway for inflammation control (Devchand et al., 1996).

Cys-LTs induce smooth muscle contraction, mucus secretion, plasma extravasation, vasoconstriction and recruitment of eosinophils (Claesson and Dahlen, 1999). The cys-LTs are recognized by two receptors ($CysLT_1$ and $CysLT_2$), which have been recently cloned, but there are indications for the existence of subclasses of $CysLT_1$ and $CysLT_2$. Moreover, numerous investigators have reported data suggesting the presence of additional CysLT receptor types in human tissues, but conclusive molecular/biological information are still unavailable (Brink et al., 2003; Capra et al., 2007). Also, a number of experimental evidence indicates the possibility that CysLT receptors might exist as homo- and/or hetero-dimers, but

how this can influence their pharmacology and function, or if this is of any importance for the cellular physiology, are issues that still need to be addressed (Capra et al., 2007). Both $CysLT_1$ and $CysLT_2$ receptors are GPCRs coupled with a G_q protein. The $CysLT_1$ is expressed in peripheral blood leukocytes, spleen, lung tissue, smooth muscle cells and macrophages (Lynch et al., 1999), whereas the $CysLT_2$ is expressed more ubiquitously (eosinophils, peripheral blood monocytes, lung macrophages, endothelial cells, etc). The agonist potency at the $CysLT_1$ receptor is $LTD_4 >> LTC_4 > LTE_4$, whereas LTC_4 and LTD_4 exhibit similar potency at the $CysLT_2$ receptor and LTE_4 is a weak agonist. Vascular leakage, bronchoconstriction, dendritic cell maturation and migration seem to be mediated by the $CysLT_1$ receptor, whereas both receptors contribute to macrophage activation, smooth muscle proliferation and fibrosis. Interestingly, endothelial cell activation by CysLTs seem to be predominantly related to $CysLT_2$ (Lotzer et al., 2003; Sjöström et al., 2003)

The 5-oxo-ETE actions, that include chemotaxis, actin polymerisation, Ca^{+2} mobilization, integrin expression and degranulation (Ghosh and Myers, 1998; Powell and Rokach, 2005), are mediated by the OXE receptor, which is G_i protein-coupled highly expressed in eosinophils, neutrophils and monocytes (Brink et al., 2004).

Because of their biological actions, LTs have been identified as mediators of a variety of inflammatory and allergic reactions, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and allergic rhinitis, but their major pathophysiological implication was linked to bronchial asthma (Samuelsson et al., 1987; Lewis et al., 1990). 5-LO pathway has also been associated with shock (Rossi et al., 2007), atherosclerosis (Mehrabian et al., 2002; Spanbroek et al., 2003), osteoporosis and certain types of cancer, like prostate cancer (Werz and Steinhilber, 2006).

1.2.9. Gender differences in leukotriene-related diseases

Intriguing and potentially very informative gender differences have been described in the incidence of LT-related diseases. In particular, for asthma, allergic rhinitis, rheumatoid arthritis and osteoporosis, females have a significant higher risk as compared to males (Wingard, 1984; Osman, 2003). Of interest, epidemiological studies, of both incidence and prevalence of asthma and atopic conditions, have reported a female predominance only after puberty, whereas before puberty a male predominance is observed **(Fig. 11)** (Ninan and Russell, 1992; Zannolli and Morgese, 1997). The reversal of the male/female prevalence conditions at puberty strongly suggests a role for sex hormones. In particular, since this reversion is due to a decrease in the incidence in males and not to an increase in females, a protective role by androgens has been assumed. The immunomodulatory properties of sex steroids have been known for many years in the autoimmunity field. However this knowledge has seldom been applied to asthma and allergy, although there is some evidence for a role of sex hormones as relevant principle.

Generally, androgens exert suppressive effects on both humoral and cellular immune responses and seem to represent natural anti-inflammatory hormones; in contrast, estrogens exert immunoenhancing activities, at least on the humoral immune response (Schmidt et al., 2006). Low levels of gonadal androgens (testosterone/5α-dihydrotestosterone) and adrenal androgens (dehydroepiandrosterone and its sulfate), as well as low androgen/estrogen ratios, have been detected in body fluids (that is, blood, synovial fluid, smears, salivary) of rheumatoid arthritis patients, supporting the possibility of a pathogenic role for decreased levels of the immune-suppressive androgens (Cutolo et al., 2002).

A beneficial effect by androgens was observed also in other leukotriene-related diseases, like atherosclerosis and cardiovascular diseases (CVD). In fact, although the incidence of these

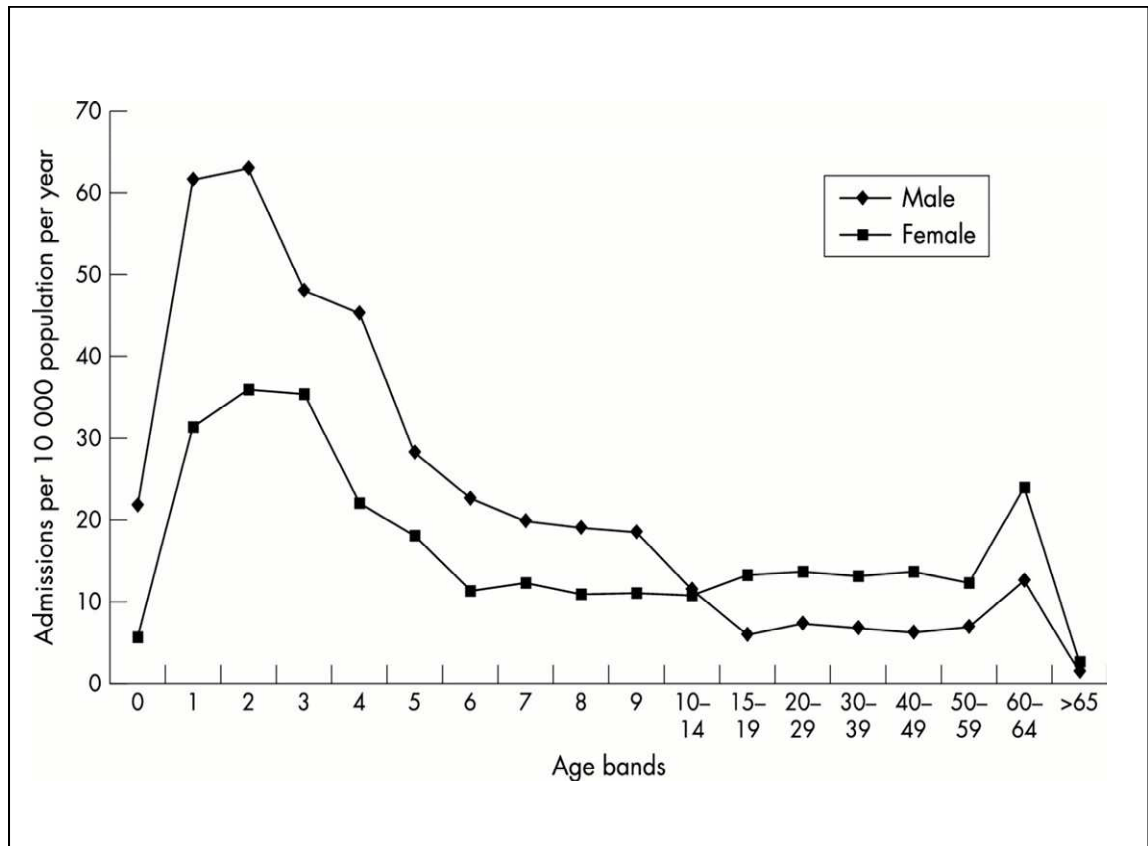


Fig. 11: Occurrence of asthma in male and female subjects. Asthma admissions (ICD J45) for all hospitals in Scotland from April 2000 to April 2001, by selected age groups and sex (Source: Scottish Morbidity Data Information and Statistics Division, Common Services Agency, NHS Scotland), show more boys admitted before puberty and an evident gender reversal post-puberty. (from *Osman, M. Arch Dis Child 88, 587-90, 2003*)

diseases is higher in males than in females, testosterone is a protective factor against atherosclerosis (Malkin et al., 2003), and men with coronary artery disease have lower levels of androgens than men with normal coronary angiograms (English et al., 2000).

A mutual relation between the sex-related difference in autoimmune diseases and 5-LO has been shown by animal studies after targeting disruption of the 5-LO gene. Of interest, it was shown that the higher survival rate of male compared to female MRL/MpJ-Fas*lpr* mice (where mice spontaneously develop a generalized autoimmune disease) was abolished after knock-out of the 5-LO gene, implying differences in 5-LO product synthesis or distinct actions of 5-LO products in males and females (Goulet et al., 1999).

1.3. Sex hormones

Sex hormones include estrogens, progestins, and androgens **(Fig. 12)**. They have been traditionally defined by their role in normal reproductive function: thus, 17β-estradiol and progesterone were considered the major sex hormones produced by the ovary, whereas testosterone and its active metabolite 5α-dihydrotestosterone (5α-DHT) are the major sex hormones produced by the testis. Steroid hormones, however, are also produced by peripheral conversion in target tissues (such as fat and the liver). The typical plasma concentrations of sex steroids are reported in **Table 2**. These hormones may act in a paracrine manner or circulate to act at target tissues in an endocrine fashion. Recently, newly alternative forms of sex steroid receptors have been recognized and different modes of genomic and nongenomic actions have been described. These data have, in turn, given new insights into pathological states. An understanding of this new information can shed light into sex-based differences in diseases and responses to therapeutic interventions.

1.3.1. Sex hormone targets and receptors

Until recently, it has been assumed that the targets for sex hormones are primarily the reproductive organs: the breast, female reproductive tract (uterus and ovary), and male reproductive tract (testes and epididymis) (Santoro et al., 1986; Whitcomb and Crowley, 1993). An expanded list of sex hormone targets became apparent when investigators examined the phenotypes of naturally occurring mutations in humans and genetically altered mouse models. Deficiency of the aromatase complex (that converts testosterone to 17β-estradiol) or knockout of the sex-steroid receptors showed tissue-specific deficits (Eddy et al., 1996; Couse and Korach, 1999; Curtis and Korach, 2000; Conneely, 2001; Matsumoto et al., 2005).

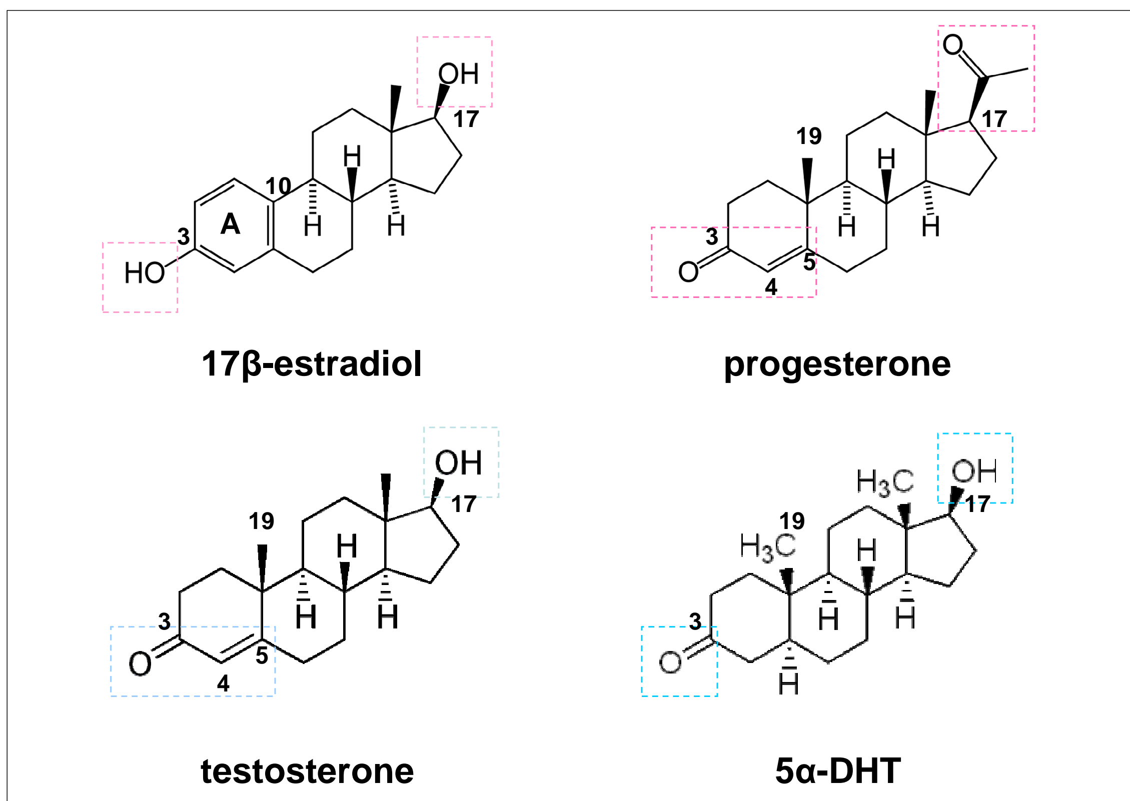


Fig. 12: Structures of sex hormones. The structure of 17β-estradiol contains an aromatic ring in A with hydroxyl group in position 3 and the notable features of a 17-β-OH (hydroxyl). Progesterone contains a keto group in C_3 conjugated with a double bond in $C_{4,5}$, a methyl group in 19β and a acetyl group in17β. In the structure of testosterone, a 17-β-OH (hydroxyl) is present. The double bond in $C_{4,5}$ can be reduced to give 5α-DHT by the enzyme 5-α reductase. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone.

Table 2: Typical plasma concentration of sex steroids

	male	female	Postmenop. female
estradiol	< 40 ng/L	40 – 400 ng/L	< 50 ng/L
progesterone	< 0.2 ng/mL	0.3 - 16 ng/mL	< 0.2 ng/mL
testosterone 5α-DHT	2 -10 ng/mL 0.3 - 0.7 ng/mL	< 0.8 ng/mL < 0.02 ng/mL	< 0.5 ng/mL < 0.02 ng/mL

Together, these data suggested that sex steroid hormones function in an expanded list of target tissues, including the vascular system, central nervous system, gastrointestinal tract, immune system, skin, kidney, and lung. An understanding of the tissue-specific roles of gonadal hormones is important when predicting the benefits or risks of replacing natural ligands or use of steroid hormone antagonists in humans.

Sex hormones have been traditionally considered to act on cells and tissues via the regulation of transcriptional processes, involving the so-called *"classic sex-steroid receptors"*. This involves the nuclear translocation of the receptor followed by the binding to specific response elements, and, ultimately, the regulation of gene expression. However, novel non-transcriptional mechanisms of signal transduction through still uncharacterized steroid hormone receptors have been identified. These are very rapid effects (within minutes), mainly affecting intracellular signalling, and they are clearly incompatible with the genomic model. In fact, these rapid, non-genomic actions do not depend on gene transcription or protein synthesis, but involve steroid-induced modulation of cytoplasmic or cell membrane-bound regulatory proteins and are likely to be transmitted via specific membrane receptors (Falkenstein et al., 2000). A variety of rapid actions, not mediated by the classic nuclear receptors, have been described also for other steroid hormones (glucocorticoids, mineralcorticoids, neuroactive steroids), suggesting that these hormones could share the characteristic to work by both genomic and non-genomic mechanisms (Losel et al., 2003).

1.3.1.1. Genomic effects are mediated by the classic sex-steroid receptors

Classical sex-steroid receptors are hormone-activated transcription factors, whose activities are also modulated by post-translational modifications including phosphorylation (Faus and Haendler, 2006). Two estrogen receptors (ERα and ERβ), two progesterone receptors (PRA

and PRB) and one androgen receptor (AR) have been cloned and characterized (Greene et al., 1986; Loosfelt et al., 1986; Lubahn et al., 1988; Chang et al., 1988; Fuller, 1991). They are modular proteins with distinct functional domains **(Fig. 13A)**. The amino-terminal region contains the activation function (AF)-1, a transcriptional activation surface. The midregion of the molecule contains the DNA binding region (DBD), followed by a hinge region and then the ligand binding domain (LBD). Dimerization interfaces are located in the mid- and COOH-terminal regions. The COOH terminus of the molecule within the LBD contains the AF-2 domain, which is another ligand-dependent transcriptional activation region.

In absence of hormone, receptor monomers associate in the cytosol with heat shock protein complexes **(Fig. 13B)** and, as a rule, are minimally phosphorylated. Binding of steroid hormones to the specific LBD induces a conformational modification of the receptor, followed by the separation of the receptor from cytoplasmic chaperone proteins and by the exposure of nuclear localization sequences. This allows nuclear translocation and homo/heterodimerization of the ligand-bound receptors, and their binding to palindromic hormone response elements (HRE) on the promoter regions of the target genes, thus regulating gene expression by interacting with the transcription machinery and mediating the genomic actions of sex-hormones (Wierman, 2007). In addition, it has become apparent that ligand-bound steroid receptors may function in an alternative mechanism that involves protein-protein interactions to either augment or block the effects of other transcription factors bound to gene promoters (Wierman, 2007).

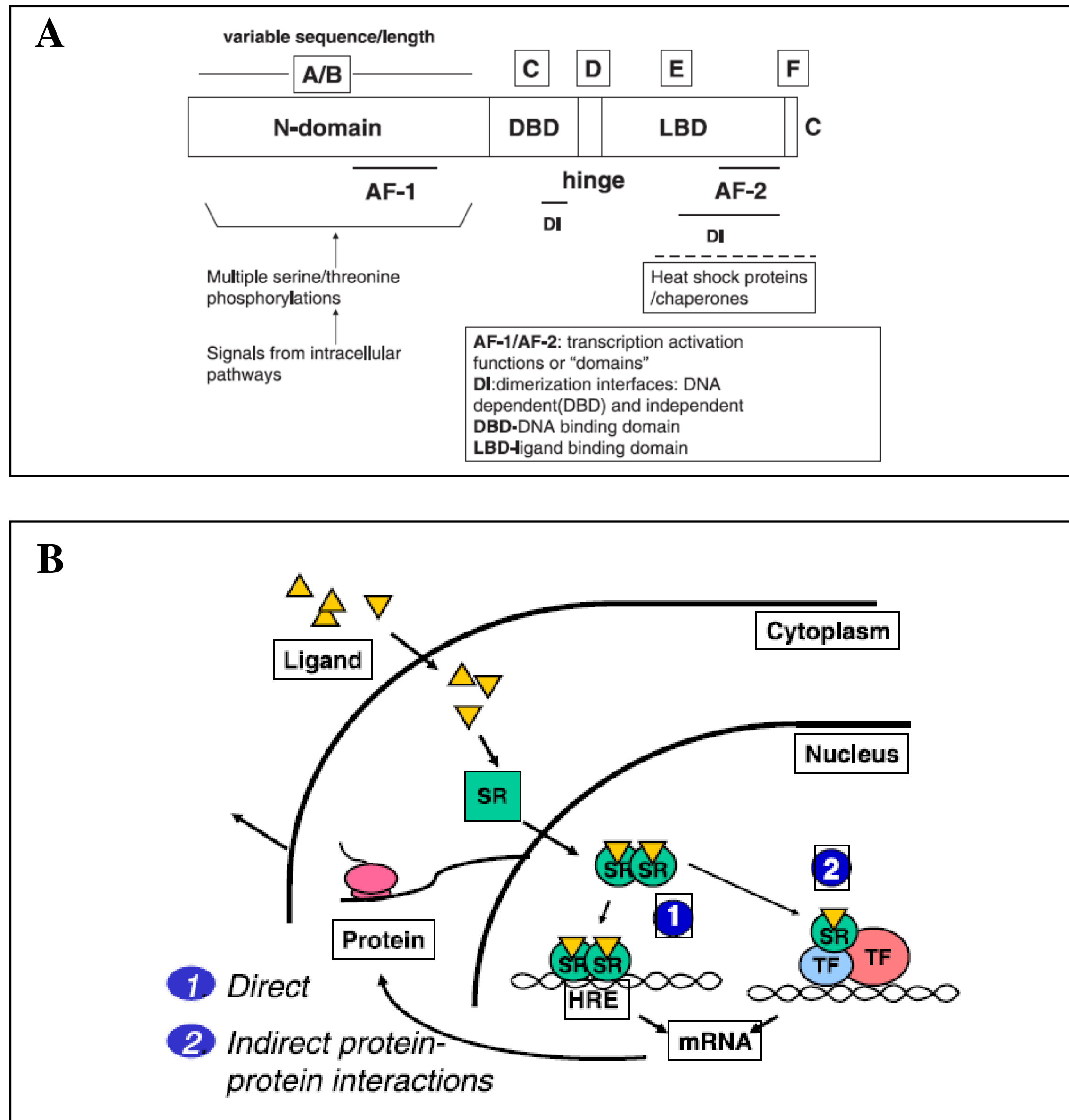


Fig. 13: (A) Modular structure of steroid hormone receptors. The NH_2-terminal region contains AF-1, a transcriptional activation surface. The midregion of the molecule contains the DBD, followed by a hinge region and then the LBD. Dimerization interfaces are located in the mid- and COOH-terminal regions. The COOH terminus of the molecule within the LBD contains the AF-2 domain, which is another transcriptional activation region that is dependent on the ligand. *Abbreviations:* AF, activation function; DBD, DNA binding domain; LBD, ligand binding domain. **(B) Genomic action by sex steroids.** The ligand circulates in the bloodstream, diffuses into cells, and interacts with its cognate receptor in the cytoplasm or nucleus to alter the conformational state. The ligand-bound steroid receptor recognizes the HREs on promoters of target DNA to directly bind this DNA as a transcription factor and ultimately increase gene expression. In addition, it has become apparent that ligand-bound steroid receptors may function in an alternative mechanism that involves protein-protein interactions to either augment or block the effects of other transcription factors bound to promoter DNA. *Abbreviations:* HRE, hormone responsive elements; SR, steroid receptor; TF, transcriptional factor. (from *Wierman, M. E. Adv Physiol Educ 31, 26-33, 2007*)

1.3.1.2. Non-genomic effects and the hypothesis of sex-steroid membrane-bound receptors

In contrast to the genomic action, non-genomic effects of sex-steroid are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and, representing the most obvious experimental evidence, by their rapid onset of action (within seconds to minutes). These rapid effects are likely to be mediated through receptor subfractions of the classic receptor, that can also localize on the cell membrane (Pietras and Szego, 1977; Pietras and Szego, 1980), or through receptors with pharmacological properties distinct from those of the intracellular steroid receptors. In this respect, accumulating evidences indicate the existence of distinct cell membrane receptors, although they have not been yet cloned and characterized (Simoncini and Genazzani, 2003). Membrane receptor-mediated events are typically not blocked by antagonists of the classical receptors and can be stimulated by hormones coupled to high molecular weight compounds (such as bovine serum albumine, BSA), that retard their diffusion across the cell membrane (Heinlein and Chang, 2002). Moreover, for androgens, a binding to the sex hormone binding globulin (SHBG) and a consequent activation of SHBG receptor on the cell surface has been suggested (Heinlein and Chang, 2002).

The hypothesis that the non-genomic actions of sex hormones involve membrane receptors is also supported by the observation that they are able to recruit signaling pathways that are often associated with cell membrane receptors, such as GPCRs, ion channels or enzyme-linked receptors (Watson, 1999).

Sex hormones and G-protein-coupled receptors

Regulation of GPCR by steroid hormones is one of the best characterized mechanisms of non-genomic signaling. In fact, the classical estrogen receptors can couple to phospholipase C

(PLC)-β by interacting with a G-protein in osteoblast cells (Le Mellay et al., 1997). This leads to Ca^{2+} mobilization from the endoplasmic reticulum and formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Coupling with G-proteins seems to be restricted to certain isoforms, since, in transfected COS-7 cells, ERα interacts with $G\alpha_i$ but not with $G\alpha_q$ or $G\alpha_s$ (Wyckoff et al., 2001).

Progesterone binds the GPCR for oxytocin (Grazzini et al., 1998), resulting in interference with oxytocin binding, although this action of progesterone seems not to be receptor-specific and a direct involvement of the classical progesterone receptor has not been reported (Burger et al., 1999).

Androgens have been also indicated to interact/modulate GPCRs, through indirect lines of evidence (Heinlein and Chang, 2002). In particular, the ability of androgens to induce a rapid increase in intracellular Ca^{2+} seems to be strictly linked to a GPCR. In T-cells and IC-21 macrophage-like cells, membrane binding of testosterone induced an increase in $[Ca^{2+}]_i$ within seconds (Benten et al., 1999a; Benten et al., 1999b). In IC-21 cells, the Ca^{2+} mobilization is due to release from intracellular Ca^{2+} stores and can be induced by testosterone conjugated to BSA, which retards the free diffusion of the steroid across the plasma membrane. IC-21 cells do not express the classical AR and Ca^{2+} mobilization in these cells is insensitive to the antiandrogens cyproterone acetate and flutamide. However, both in IC-21 and T-cells, the testosterone-induced Ca^{2+} increase is sensitive to pertussis toxin (PTX), an inhibitor of G_i, suggesting that the membrane androgen-binding protein in these cells is either a G protein receptor or its function is closely linked to one (Benten et al., 1999b). A membrane GPCR sensitive to PTX is also involved in androgen-induced increase in $[Ca^{2+}]_i$ in LNCaP prostate cancer cells (Sun et al., 2006), implying that non-genomic actions of testosterone could also be involved in the regulation of cell survival and growth. Interestingly, an increase in $[Ca^{2+}]_i$

induced by testosterone or testosterone:BSA has been observed also in osteoblasts, but this effect was sexually dimorphic, since testosterone was only able to induce Ca^{2+} influx in male primary osteoblasts but not in female-derived cells (Lieberherr and Grosse, 1994).

Therefore, the modulation of GPCRs may possibly explain a variety of cellular actions of sex steroids, such as regulation of the PLC/DAG/IP3 cascade (Le Mellay et al., 1999), intracellular Ca^{2+} mobilization, activation of PKC (Le Mellay et al., 1997), and activation of the adenylate cyclase/protein kinase A pathway (Kelly et al., 1999).

Sex hormones and ion channels

It has been observed in endothelial and smooth muscle cells that sex steroids can also influence cell membrane ion channels and can rapidly modulate transmembrane ion fluxes (White et al., 1995; Barbagallo et al., 2001a; Barbagallo et al., 2001b). However, a direct interaction between sex hormones and ion channels has never been reported and the effect of steroids on ion channels remains still unclear.

Sex hormones and kinase pathways

Sex steroids can also regulate in a rapid fashion several kinase pathways, like the MAPK cascades, several tyrosine kinases and lipid kinases. These signaling machineries are ubiquitous signal-transducers, and their activation is linked to a variety of important cellular events (including gene expression, protein regulation, cell proliferation and survival).

The activation of the MAPK pathways by sex steroids has been characterized in several tissues. Three main cascades are described, the ERK 1/2, the p38 MAPK and c-Jun NH2-terminal kinase (JNK) cascades (Chang and Karin, 2001; Pearson et al., 2001). These pathways are organized in step-modules, where an upstream kinase phosphorylates and

activates the downstream MAPK. The relationship between MAPKs and steroid receptors is complex, since several levels of mutual regulation might exist (Weigel and Zhang, 1998).

Exposure to estrogens leads to rapid activation of the ERK 1/2 in various cell types, like osteoblasts (Endoh et al., 1997), neuroblastoma cells (Watters et al., 1997) and white adipocytes (Dos Santos et al., 2002). This activation may be finely tuned by the coordinated interaction with other signaling cascades. For instance, in adipocytes, PTX and wortmannin reduce the magnitude of MAPK activation by E2 (Dos Santos et al., 2002) suggesting the parallel involvement of the G_i protein/Src family kinase/phosphatidylinositol kinase (PI3K) pathways for the functional activation of MAPK. Although less is known about the regulation of the other MAPK, the p38 cascade is activated by estrogen in endothelial cells (Razandi et al., 2000), whereas estrogens downregulate the activity of JNK in RAW264.7 macrophages (Srivastava et al., 1999).

In breast cancer cells, progesterone plays a relevant role in the regulation of ERK1/2 (Migliaccio et al., 1998). Indeed, the B isoform of the PR stimulates the activated estrogen receptor to recruit the Src/p21ras/ERK pathway, revealing a functional cross-talk between different steroid receptors, which could be relevant for their growth-promoting effects on cancer cells.

Androgens are also able to regulate the MAPK family of protein kinases. In prostate cancer cells, 5α-DHT leads to a rapid and reversible activation of ERK1/2 (Peterziel et al., 1999). Transient transfection assays demonstrated that the androgen receptor-mediated activation of ERK1/2 results in enhanced activity of the transcription factor Elk-1. This action of the androgen receptor differs from its known transcriptional activity, since it is rapid and insensitive to androgen antagonists such as hydroxyflutamide or casodex. In androgen receptor-free macrophages, testosterone acts via a cell membrane receptor to attenuate the

LPS-dependent activation of p38 MAPK (but not of ERK 1/2 and JNK/SAPK), showing a potential signaling cross-talk with the LPS pathway (Guo et al., 2002). On the contrary, 5α-DHT was found to increase the activation of p38 MAPK in male mice after trauma-hemorrhage (Angele et al., 2003).

A prominent feature of sex steroids is also represented by activation of tyrosine kinases. Estrogen treatment of various cell types induces protein tyrosine phosphorylation (Simoncini and Genazzani, 2003). Rapid activation of tyrosine kinases is accomplished also by progesterone, probably via a specific interaction between a polyproline motif in the NH_2-terminal domain of PR with SH3 domains of various cytoplasmic signalling molecules, including c-Src tyrosine kinases (Boonyaratanakornkit et al., 2001).

1.3.1.3. Genomic effects vs non-genomic effects

Based on the evidence of genomic and non-genomic mechanisms by sex hormones, a possible interpretation have been put forward (Simoncini and Genazzani, 2003). Genomic signaling mechanisms may represent the way used by steroid hormones to program target cells, organs and systems for complex functions, providing the single cells and the cell networks with the tools to accomplish these tasks. On the contrary, non-genomic signaling mechanisms may represent a way to rapidly activate the cellular functionalities needed to adapt to dynamic changes in the surrounding milieu. The time of onset of these effects can be extremely rapid, but the modifications of the cell program may be long-lasting anyway and, therefore, altered or differential non-genomic steroid signaling may specifically contribute to determine pathological conditions.

2. AIM OF THE PRESENT INVESTIGATION

5-LO catalyzes the initial steps in the conversion of AA to LTs (Werz, 2002). LTs are potent mediators of inflammatory and allergic reactions, but also may play prominent roles in cardiovascular diseases, cancer, and osteoporosis (Werz and Steinhilber, 2006). Therefore, a number of compounds that interfere with LTs, either with their synthesis (5-LO inhibitors, FLAP inhibitors) or with the respective LT receptors, have been developed. However, for the successful development of drugs interfering with the 5-LO pathway and effective in 5-LO related diseases, the understanding of 5-LO regulation is of utmost importance (Werz and Steinhilber, 2005; Werz and Steinhilber, 2006). In fact, although the biology of 5-LO has been intensively investigated, it is still incompletely understood (Radmark et al., 2007).

The enzymatic activity of 5-LO in cell-free assays is stimulated by Ca^{+2} and Mg^{+2}, phospholipids (e.g. PC), glycerides, ATP, CLP, certain undefined leukocyte proteins and LOOHs with different magnitudes. In intact cells, additional events are of importance in the regulation of 5-LO activity, such as the availability of AA as substrate by $cPLA_2$, the interaction of 5-LO with FLAP, phosphorylations at Ser residues (stimulatory, by ERK1/2 and p38, and inhibitory, by PKA). Moreover, 5-LO product synthesis strictly correlates with subcellular localization of 5-LO, that is a mobile enzyme in cells. In a generally accepted model, 5-LO occurs in resting cells as a soluble enzyme, either in the cytosol or in the nucleus, depending on the cell type (Werz and Steinhilber, 2006). In particular, 5-LO has been reported to be cytosolic in resting neutrophils or peritoneal macrophages, and intranuclear in dendritic cells or in alveolar macrophages. Although it has been shown that the nuclear import is driven by three distinct NLS, the mechanisms involved in NLS activation in different cell types are

still unclear (Jones et al., 2003). Upon cell stimulation, 5-LO comigrates with $cPLA_2$ to the nuclear envelope, in a Ca^{+2}- and phosphorylation-dependent manner. Here, $cPLA_2$ liberates AA from phospholipids, which is then transferred by the membrane-bound FLAP to 5-LO for metabolism. Interestingly, the locus of 5-LO in resting cells depends on the cell type and seems to determine the compartment where 5-LO redistributes upon stimulation. Thus, for example, in activated neutrophils and peritoneal macrophages, cytoplasmic 5-LO has been found to associate with the endoplasmic reticulum and the outer nuclear membrane, whereas the intranuclear 5-LO (e.g. in dendritic cells or in alveolar macrophages) translocates to the inner membrane of the nuclear envelope. Of interest, the capacity for LT generation is higher when 5-LO translocates to the nuclear membrane than to the endoplasmic reticulum (Luo et al., 2003).

Up to date, many reports have confirmed these findings, and it is accepted that the capacity of cellular product synthesis from endogenous substrate in response to an agonist (i.e. ionophore or fMLP) depends on 5-LO translocation to the nuclear envelope. However, in all the animals studies performed, female but never male animals were used and, when human cells were analysed, the sex of the donor was never specified or taken into account. Also, inconsistent patterns of 5-LO translocation were reported when PMNL from several donors, whose gender was not reported, were analyzed (Boden et al., 2000). Moreover, the sex of the subject wherefrom cell lines have been originally established and the effects of sex hormones present in fetal calf serum, that is generally added as a supplement to cell cultures, have been neglected when studying 5-LO cell biology in cell lines. Together, no study has ever considered that the gender, the sex hormone status of the donor, or sex hormones themselves may influence subcellular localization, translocation and biological activity of 5-LO. However, such interrelations are conceivable because several experimental evidences and

clinical data suggest a possible relation between gender and 5-LO. In fact, it was shown that the differences in the survival rate on male and female mice, in an experimental model of autoimmune disease, was abolished after knock-out of 5-LO gene, implying differences in 5-LO product synthesis or distinct actions of 5-LO products in male and female animals (Goulet et al., 1999). Also, as consequence of the target disruption of the LTB_4 receptor 1 (BLT_1) in mice, a reduced survival in males but a higher survival in females were observed, in a model of PAF-induced anaphylaxis (Haribabu et al., 2000). Moreover, many data have shown a differential incidence in human, as well as in animal models, of LT-mediated diseases, such as rheumatoid arthritis, osteoporosis, Alzheimer disease or asthma, for all of which females have a significant higher risk as compared to males (Wingard, 1984; Osman, 2003). Indeed, although other LT-related diseases, like atherosclerosis and cardiovascular diseases, have a higher incidence in males, a protective action of testosterone was demonstrated (English et al., 2000; Malkin et al., 2003). Interestingly, sex hormones were shown to have profound effects on the immune response and inflammation (Obendorf and Patchev, 2004; Bouman et al., 2005), acting through both genomic and non-genomic mechanisms to regulate protein expression and signal pathways, that could consequently regulate 5-LO cellular biology.

In the light of these facts, the aim of the present investigation was to evaluate if 5-LO is regulated in a gender-dependent manner and if sex-hormones influence the 5-LO pathway.

To this purpose, primary human PMNL have been used as experimental model, since they are primary governors of inflammatory processes and the major 5-LO product synthesizing cells in humans. In order to avoid or reduce the occurrence of a possible loss of the *in vivo* cellular state of PMNL by the removal from their normal plasma environment, where they are in contact with the sex hormones, the isolation procedure from peripheral blood was strictly performed at 4° C (Jethwaney et al., 2007). Under these conditions, PMNL have been shown

to retain the effects of treatments carried out in the blood. Moreover, in order to confirm the biological relevance of the results obtained in cellular models, whole blood assays were also performed.

3. MATERIALS AND METHODS

3.1. Materials

Nycoprep was from PAA Laboratories (Linz, Austria). Ca^{2+}-ionophore A23187, ionomycin, AA, LPS, fMLP, prostaglandin B_1, margaric acid, 1-(dimethylaminopropyl)-3-ethylcarbodiimide (EDC), ATP and EDTA were from Sigma (Deisenhofen, Germany). 5α-DHT, testosterone, testosterone 3-(O-carboxy-methyl)oxime:BSA (testosterone:BSA; 20 mol steroid per mol BSA), 17β-estradiol, and progesterone were also purchased from Sigma and were dissolved in EtOH, so that the final concentration of ethanol never exceeded 0.05 %.

Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) was from BIOMOL Research Labs Inc. (Plymouth Meeting, PA, USA). Adenosine deaminase (Ada, bovine intestine), Fura-2/AM, U0126, PD98059, SB203580, and PTX were from Calbiochem (Bad Soden, Germany). Cyproterone acetate and flutamide were from LKT Laboratories Inc. (St. Paul, MN, USA). BAPTA/AM was from Alexis (Grünberg, Germany). MK-886 and BWA4C were generous gifts by Dr. A. W. Ford-Hutchinson, Merck-Frosst (Canada) and by Dr. L. G. Garland (Wellcome Res. Laboratories), respectively. High performance liquid chromatography (HPLC) solvents were from Merck (Darmstadt, Germany).

3.2. Isolation of human PMNL

Human PMNL were freshly isolated from leukocyte concentrates obtained at the Blood Center, University Hospital (Tübingen, Germany). Venous blood was taken from healthy adult

donors, differentiated on the base of the sex, and leukocyte concentrates were prepared by centrifugation at 4,000*g*/20 minutes/20° C. The blood was then diluted 1:1 with phosphate-buffered saline (PBS) and then 4:5 with ice-cold 5 % dextran in PBS, for 30 minutes. After dextran sedimentation PMNL were immediately isolated by centrifugation at 4° C on Nycoprep cushions and hypotonic lysis of erythrocytes, as described previously (Werz et al., 2002b). Cells (purity > 96-97 %) were finally resuspended to the indicate density in ice-cold PBS plus 1 mg/mL glucose (PG buffer), or alternatively in ice-cold PBS plus 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) as specified.

3.3. Determination of 5-lipoxygenase product formation

For assays in intact cells, human PMNL (5×10^6) freshly isolated from whole blood or buffy coats of male and female donors were finally resuspended in 1 mL ice-cold PGC buffer. The reaction was started by addition of ionophore A23187 (2.5 µM) and by AA (20 µM), either used alone or in combination. After 10 minutes at 37° C, the reaction was stopped with 1 mL of methanol and 30 µL of 1 N HCl, 200 ng prostaglandin B_1 and 500 µL of PBS were added.

In the experiments in which PMNL were stimulated with fMLP, cells (2×10^7) were resuspended in 1 mL ice-cold PGC buffer and primed with 1 µg/mL LPS at 37° C for 10 minutes. Ada (0.3 U/mL) was added for additional 20 minutes of preincubation. Cells were then stimulated with fMLP (1 µM) for 5 minutes (Krump et al., 1997) and the reaction was stopped as described above.

In the experiments in which the effect of hormones on 5-LO product formation was evaluated, the cells were preincubated for 30 minutes with the indicated sex steroids prior to the addition of the stimulus. In some experiments, the treatment with 5α-DHT for 30 minutes was

performed in the blood, prior to the isolation of the cells.

Formed 5-LO metabolites were extracted and analyzed by HPLC as described (Werz and Steinhilber, 1996). In detail, after centrifugation (800*g*, 10 minutes, room temperature) the samples were applied to C-18 solid-phase extraction columns (100 mg; IST, Mid Glamorgan, United Kingdom), preconditioned with 1 mL methanol and 1 mL water. The columns were washed with 1 mL water and 1 mL water/methanol (75/25, vol/vol) and 5-LO metabolites were eluted with 300 µL methanol. The extract was then diluted with 120 µL water, and 100 µL diluted extract were analyzed by HPLC using a C-18 Radial-Pak column (Waters) eluted with methanol/water/acetic acid 75/25/0.1 (vol/vol/vol) at a flow rate of 1.2 mL/min. Amounts of different metabolites were determined by peak area integration. 5-LO product formation was expressed as ng of 5-LO products per 10^6 cells. 5-LO products included LTB_4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-DiHETE), 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE), and 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE). 5-HETE and 5-HpETE coelute as one major peak, and the integration of this peak represented both eicosanoids. Cys-LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined.

For assays in cell free systems, human PMNL (5×10^6) freshly isolated from whole blood or buffy coats of male and female donors were finally resuspended in 1 mL ice-cold PBS plus 1 mM EDTA. They were sonicated on ice (5×10 seconds) with a cell disruptor B15 Branson sonifier (output: 6; duty cycle: 40 %) and 1 mM ATP was added. In some experiments, sonified samples were then ultracentrifuged at 100,000*g*, 4° C, for 1 h in an ultracentrifuge (Beckman Instruments) and the resulting supernatant (S100) was used for the evaluation of 5-LO product formation. Samples (1 mL homogenates or S100) were then pre-warmed for 30

seconds at 37° C and 2 mM $CaCl_2$ and AA at the indicated concentration (5, 20 and 80 µM) were added to start 5-LO product formation. As indicated, some experiments were performed in the absence of Ca^{+2}. The reaction was stopped after 10 minutes at 37° C by addition of 1 mL ice-cold methanol, and then 30 µL of 1 N HCl, 200 ng prostaglandin B_1 and 500 µL of PBS were added. The formed metabolites were extracted and analyzed by HPLC as described for intact cells.

3.4. Measurement of AA release

Human PMNL (5×10^7) freshly isolated from whole blood or buffy coats of male and female donors were finally resuspended in 1 mL ice-cold PGC buffer. They were pre-warmed at 37° C for 10 minutes and then the 12-LO inhibitor CDC (10 µM) and the 5-LO inhibitor BWA4C (1 µM) were added to avoid the conversion of liberated AA to LO metabolites. After 10 minutes, the reaction was started by addition of 2.5 µM ionophore A23187 and the samples were incubated for 5 minutes at 37° C. The reaction was stopped with 2 mL of methanol and 60 µL of 1 N HCl and 2 mL of PBS were added together with 60 µg of margaric acid, used as internal standard.

In the experiments in which PMNL were stimulated with fMLP, cells (5×10^7) were resuspended in 1 mL ice-cold PGC buffer and pre-warmed at 37° C for 10 minutes. The 12-LO inhibitor CDC (10 µM) and the 5-LO inhibitor BWA4C (1 µM) were added together with 1 µg/mL LPS at 37° C for 10 minutes. Ada (0.3 U/mL) was added for additional 20 minutes of preincubation. Cells were then stimulated with fMLP (1 µM) for 5 minutes and the reaction was stopped as described above.

In the experiments in which the effect of hormones on ionophore-induced AA release was

evaluated, cells were preincubated for 30 minutes with the indicated sex steroids prior to the addition of the stimulus.

Released AA, as well as the margaric acid used as standard, was then extracted, derivatized with 2,4-dimethoxyanilin and analyzed by HPLC. In detail, after centrifugation (800*g*, 10 minutes, room temperature) the samples were applied to C-18 solid-phase extraction columns (100 mg; IST, Mid Glamorgan, United Kingdom), preconditioned with 1 mL methanol and 1 mL PBS containing 0.03 N HCl. The columns were washed with 1 mL water and 1 mL water/methanol (75/25, vol/vol) and AA was eluted with 300 µL methanol. The extracts were derivatized by adding 37.5 µL 2,4-dimethoxyanilin (0.02 M) in water, and 75 µL of a solution 50/50 of 0.125 M EDC in ethanol and 1.5 % pyridine in ethanol. The reaction was performed at 37° C for 1 hour. Then, 100 µL derivatized extract were analyzed by HPLC using a C-18 Radial-Pak colimn (Waters) eluted as follows:

- *0-20 minutes:* a) methanol/water/acetic acid 75/25/0.1 (vol/vol/vol) (buffer A) at a flow rate of 1.4 mL/min as linear gradient from 100 % to 0 %;

  b) methanol/acetic acid 100/0.1 (vol/vol) (buffer B) at a flow rate of 1.4 mL/min as linear gradient from 0 % to 100 %;
- *20-23 minutes:* 100 % buffer B at a flow rate of 1.4 mL/min;
- *23-35.5 minutes:* 100 % buffer A at a flow rate of 1.4 mL/min.

Released AA was detected at 272 nm and determined by peak area integration. Liberated AA was calculated as percentage of the peak of the derivatized standard (margaric acid) and is expressed as percentage of the increase induced by stimulation, compared to non-stimulated cells.

3.5. Determination of 5-lipoxygenase protein expression

Human PMNL (3×10^7) freshly isolated from whole blood or buffy coats of male and female donors, were finally resuspended in 1 mL ice-cold PBS plus 1 mM EDTA and sonicated (5×10 seconds) with a cell disruptor B15 Branson sonifier (output: 6; duty cycle: 40 %). Total cell lysates were then centrifuged at 12,000*g* for 15 minutes at 4 ° C. The supernatant was collected and mixed 1:1 with 2x ice cold sodium dodecyl sulphate (SDS)-polyacryamide gel electrophoresis (PAGE) sample loading buffer (SDS-b), heated for 6 minutes at 95° C, and analysed for 5-LO protein by SDS-PAGE and immunoblotting. Total cell lysates were also obtained by resuspending freshly isolated PMNL (3×10^7) in lysis buffer (50 mM Tris, pH 7.4, 1 % Nonidet P-40 (NP40), 0.01 % SDS) containing complete protease inhibitor cocktail for 10 minutes at 4° C. The lysates were then centrifuged at 12,000*g* for 15 minutes at 4° C and the supernatants were mixed with gel loading buffer (50 mM Tris, 10 % SDS, 10 % glycerol, 10 % 2-mercaptoethanol, 2 mg/mL of bromophenol) in a ratio of 1:1, heated for 6 minutes at 95° C, and analysed by SDS-PAGE.

3.6. Subcellular fractionation by mild detergent lysis

Subcellular localization of 5-LO, CLP, FLAP and pERK1/2 was investigated by cell fractionation as described previously (Werz et al., 2001b). In detail, human PMNL (3×10^7), freshly isolated from whole blood or buffy coats of male and female donors, were resuspended in 1 mL of cold PGC buffer. A23187 Ca^{+2}-ionophore (2.5 µM) was added as indicated and the samples were further incubated for 10 minutes, and then chilled on ice for 5 minutes to stop the reaction.

In the experiments in which PMNL were stimulated with fMLP, cells (3×10^7) were

resuspended in 1 mL PGC buffer, primed with 1 µg/mL LPS at 37° C for 10 minutes and 0.3 U/mL Ada were added for additional 20 minutes of preincubation. Cells were then stimulated with fMLP (1 µM) for 5 minutes and the reaction was stopped on ice.

In the experiments in which the effects of hormones, MAPK inhibitors or Ca^{+2} chelators were evaluated, the cells were incubated at 37° C with the indicated compounds for the indicated times and the reaction was stopped on ice.

In the experiments in which the effects of human plasma were analysed, the cells (3×10^7) were incubated in autologous plasma for 30 minutes at 37° C. The reaction was stopped on ice, and the cells were washed twice with ice-cold PBS prior to lysis, in order to minimize contamination of plasma proteins in the samples.

After 5 min on ice, the samples were centrifuged at 200*g*, 5 minutes, 4° C, and the pellets were then resuspended in 300 µL ice-cold 0.1 % NP40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1 % NP40, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 60 µg/mL soybean trypsin inhibitor, and 10 µg/mL leupeptin). The samples were vortexed (3 x 5 seconds), kept on ice for 10 minutes, and centrifuged (1,000*g*, 10 minutes, 4° C). Resultant supernatants (non-nuclear fractions) were transferred to a new tube, and the pellets (nuclear fractions) were resuspended in 300 µL ice-cold relaxation buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 µg/mL soybean trypsin inhibitor, and 10 µg/mL leupeptin). Both nuclear and non-nuclear fractions were centrifuged again (1,000*g*, 10 minutes, 4° C) for further purification. Lysis of cells and integrity of nuclei were confirmed by light microscopy with trypan blue exclusion. Nuclei in relaxation buffer were disrupted by sonication on ice (3 x 5 seconds) with a cell disruptor B15 Branson sonifier (output: 3; duty cycle: 40 %). Aliquots of nuclear and non-nuclear fractions were immediately mixed with the same volume of SDS-b, heated for 6

minutes at 95° C, and analyzed for 5-LO, CLP, FLAP and pERK protein by SDS-PAGE and Western blotting. Lamin B, an ubiquitous protein exclusively present in the nuclear membrane, was used as a marker to indicate the correct fractionation (not shown).

3.7. Subcellular fractionation by sonication and ultracentrifugation

Subcellular localization of 5-LO, $cPLA_2$, CLP and FLAP in the soluble and membrane-associated fraction was investigated by sonication and ultracentrifugation (Pouliot et al., 1996). In brief, human PMNL (3×10^7) freshly isolated from whole blood or buffy coats of male and female donors were resuspended in 1 mL ice-cold PGC buffer and activated as described in **3.6.**. The reaction was stopped on ice, the samples were centrifuged at 200*g*, 5 minutes, 4° C, and the pellets were then resuspended in 150 µL ice-cold relaxation buffer (see **3.6.**). PMNL were sonicated on ice (5×10 seconds) with a cell disruptor B15 Branson sonifier (output: 6; duty cycle: 40 %). Cell disruption was confirmed by light microscopy with trypan blue exclusion. Sonicates were centrifuged at 100,000*g*, 70 minutes, 4° C in an ultracentrifuge (Beckman Instruments). The 100,000*g* supernatant was referred as the soluble fraction (S100); the corresponding pellets were resuspended in 150 µL ice-cold relaxation buffer and were referred as the membrane-associated fraction (P100). Aliquots of S100 and P100 fractions were immediately mixed with the same volume of SDS-b, heated for 6 min at 95° C, and analyzed for 5-LO, $cPLA_2$, CLP and FLAP protein by SDS-PAGE and Western blotting.

3.8. Determination of MAPK activation

Human PMNL (1×10^7) freshly isolated from whole blood or buffy coats of male and female

donors were resuspended in 100 µL ice-cold PGC buffer. After addition of the indicated stimuli, samples were incubated at 37° C and the reaction was stopped after the indicated times by addition of 100 µL ice-cold SDS-b, vortexed, and heated 6 minutes at 95° C. In the experiments in which the effects of human plasma were analysed, the cells (1×10^7) were incubated in 100 µL plasma for the indicated times at 37° C. The reaction was stopped on ice and then the cells were washed twice with ice-cold PBS prior to lysis, in order to minimize contamination of plasma proteins in the samples.

Total cell lysates (20 µL) were analyzed for ERK1/2, phosphorylated MAPK and phosphorylated Elk-1 by SDS-PAGE and immunoblotting.

3.9. SDS-PAGE and Western Blotting

Total cell lysates (20 µL) and aliquots of nuclear and non-nuclear fractions (25 µL) were mixed with 4 µl glycerol/0.1 % bromophenolblue (1:1, vol/vol) and analyzed by SDS-PAGE using a Mini Protean system (Bio-Rad) on a 10 % gel (5-LO, ERK1/2, pERK1/2, p-p38 MAPK, pElk-1), 6 % gel ($cPLA_2$) or 20 % gel (CLP, FLAP). Correct loading of the gel and transfer of proteins was confirmed by Ponceau S-staining. After electroblot to nitrocellulose membrane (GE Healthcare), membranes were blocked with 5 % BSA in 50 mM Tris/HCl, pH 7.4 and 100 mM NaCl (TBS) for 1 hour at room temperature. Membranes were then washed and incubated overnight at 4° C with primary antibodies.

5-LO antibody (affinity purified anti-5-LO antiserum on a 5-LO column, 1551, AK7), and polyclonal anti-human CLP antiserum, raised in chickens against amino acids 116–130 of human CLP, were kindly provided by Dr. Olof Rådmark (Karolinska Institute, Stockholm, Sweden). Polyclonal anti-FLAP antiserum was provided by Dr A. Hatzelmann (Konstanz,

Germany). $cPLA_2$ antibody was from Santa Cruz Biotechnology. Antibodies against ERK1/2 and phosphospecific antibodies recognizing pERK1/2 (Thr202/Tyr204), p-p38 MAPK (Thr180/Tyr182) and pElk-1 (Ser383) were from Cell Signaling Technology. The antibodies were used as 1:1,000 dilution in 0.1 % Tween 20/TBS containing 5 % BSA.

After the incubation with the primary antibodies, membranes were washed with TBS and incubated with 1:1,000 dilution of alkaline phosphatase-conjugated IgGs (Sigma) for 3 hours at room temperature. After washing with TBS and TBS plus 0.1 % Tween 20, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

In some experiments, an ECL Plex Western blotting system was used. In detail, after SDS-PAGE, the proteins were electroblotted to Hybond ECL nitrocellulose membranes (GE Healthcare), blocked with 2 % ECL advance blocking agent (GE-Healthcare) in TBS plus 0.1 % Tween 20, for 1 hour at room temperature and then incubated with primary antibodies, overnight at 4° C. Multiple detection was performed by incubating the membranes in solutions containing dilutes of antibodies of different origin (for example: anti-pERK1/2, mouse, and antip-p38 MAPK, rabbit). The membranes were then washed in TBS plus 0.1 % Tween 20 and incubated for 1 hour at room temperature with ECL Plex CyDye conjugated secondary antibodies (Cy5-conjugated anti-rabbit and Cy3-conjugated anti-mouse, GE Healthcare) diluted 1:2,500 in TBS. After washing, the membrane were dried at 37° C, for 1 hour in the dark, and then scanned in the Cy3 (Excitation Filter: 540 nm; Emission Filter: 595 nm) and in the Cy5 (Excitation Filter: 635 nm; Emission Filter: 680 nm) channels by an Ettan DIGE imaging system (GE-Healthcare). Densitometry was performed with ImageQuant TL image analysis software (GE Healthcare); relative intensities were calculated as percentage of the strongest band in the corresponding membrane.

3.10. Indirect Immunofluorescence Microscopy

Human PMNL (1.5×10^6), freshly isolated from whole blood or buffy coats of male and female donors, were resuspended in 1 mL ice-cold PGC buffer. Cells were then centrifuged at 30*g* for 1 minute onto poly-L-lysine (MW 150,000-300,000; Sigma-Aldrich)-coated glass coverslips in the wells of a 12-well plate.

In some experiments, cells were activated by addition of 2.5 µM ionophore A23187, for 3 minutes at 37° C.

In the experiments in which PMNL were stimulated with fMLP, cells (1.5×10^6) were resuspended in 1 mL ice-cold PGC buffer and primed with 1 µg/mL LPS at 37° C for 10 minutes. Ada (0.3 U/mL) was added for additional 20 minutes of preincubation. Cells were then centrifuged onto poly-L-lysine as reported above and stimulated with fMLP (1 µM) for 5 minutes at 37° C. In the experiments in which the effects of hormones or MAPK inhibitors were evaluated, cells (1.5×10^6) were incubated at 37° C with the indicated compounds for the indicated times prior to cytospin onto the glass coverslips.

Cells were fixed in methanol (-20° C, 30 minutes) and permeabilized with 0.1 % Tween 20 in Dulbecco's PBS (room temperature, 10 minutes), followed by 3 washing steps with PBS. Samples were blocked with 10 % non-immune goat serum (Invitrogen) for 10 minutes at room temperature and then washed again twice with PBS.

5-LO staining was performed by incubating the coverslips with the anti-5-LO serum (1551, AK-7) for 1 hour at room temperature. The coverslips were then washed 10 times with PBS, incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, diluted 1:1,500 in PBS) for 10 minutes at room temperature in the dark, and washed 10 times with PBS.

Double staining was performed by incubating the coverslips with rabbit anti-5-LO serum together with either mouse monoclonal anti-lamin B (Abcam, 1/3 in PBS; nuclear envelope

marker) or mouse monoclonal anti-1D3 (Abcam, 1/5 in PBS; endoplasmic reticulum marker (Vaux et al., 1990)) antibody, for 1 hour at room temperature. The coverslips were then washed 10 times with PBS, incubated with a mixture of Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, 1/1500 in PBS) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, 1/800 in PBS) for 10 minutes at room temperature in the dark, and washed 10 times with PBS.

The DNA was stained with 0.1 µg/ml diamidino-2-phenylindole (DAPI) in PBS for 3 minutes at room temperature 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 using a 100X oil-immersion objective.

3.11. Spectruofluorimetric Ca^{+2} imaging

Human PMNL, freshly isolated from whole blood or buffy coats of male and female donors, were resuspended in ice-cold PG (5×10^7 cells/mL), and incubated with 2 µM Fura-2/AM (Molecular Probes) for 30 min at 37° C in the dark. After washing, cells were resuspended in PBS (1×10^7 cells/mL) and transferred into a thermally controlled (37° C) fluorometer cuvette in a spectrofluorometer (Aminco-Bowman series 2; Thermo Electron Corporation, Waltham, MA) with continuous stirring. Two minutes prior to stimulation, 1 mM $CaCl_2$ was added. In some experiments, as indicated, the recalcification of the medium was performed 30 seconds after the addition of the stimuli. The fluorescence emission at 510 nm was measured after excitation at 340 nm (Ca^{+2}-bound Fura-2) and 380 nm (free Fura-2), respectively. The $[Ca^{+2}]_i$ was computed from the ratio of 340:380 nm fluorescence values according to Grynkiewicz et al. (Grynkiewicz et al., 1985). Maximal fluorescence (F_{max}) was obtained by lysing the cells

with 1 % Triton X-100 and minimal fluorescence (F_{min}) by chelating Ca^{+2} with 10 mM EDTA. In some experiments, the area under the cytosolic Ca^{+2} concentration curve was calculated for 100 seconds after recalcification of the medium as an index of Ca^{+2}-mobilisation (Itagaki et al., 2002).

3.12. Preparation of human plasma

For the isolation of human plasma, fresh blood was collected by the Blood Center, University Hospital (Tübingen, Germany) in heparinized tubes (S-Monovetten Sarstedt, 13.1628, 16 I.E. Heparin/mL blood) by venipuncture from fasted (12 hours) male and female volunteers, with consent. The blood was centrifuged at 600*g* for 10 minutes at 4° C. The plasma (supernatant) was then transferred into a different tube and centrifuged again (800*g*, 10 minutes, 4° C) for further purification. The resulting supernatant was analysed to confirm the absence of cellular contaminations and was stored at -80° C till required for use.

3.13. Determination of 5-lipoxygenase activity in whole blood

Freshly drawn venous blood was collected at 9:00 a.m. by the Blood Center, University Hospital (Tübingen, Germany) in heparinized tubes as described in **3.12.**. The subjects had no apparent inflammatory conditions and had not taken any anti-inflammatory drugs or hormones for at least ten days prior to blood collection. The blood was divided in 2 mL aliquots in glass vials and stimulated with either Ca^{+2}-ionophore A23187 (30 µM) or with A23187 (30 µM) + AA (100 µM), for 10 minutes at 37° C. The stimulation was performed within 15 minutes after blood withdrawal.

In experiments evaluating the effect of 5α-DHT on agonist-induced 5-LO product formation, the blood was preincubated with 100 nM 5α-DHT or vehicle (0.05 % ethanol) at 37° C for 30 minutes. In control experiments, the blood was pre-incubated 10 minutes at 37° C with the 5-LO inhibitor BWA4C (3 µM), prior to ionophore addition.

After the stimulation, the reaction was stopped on ice, and the samples were centrifuged at 600*g*, 10 minutes, 4° C. Aliquots of plasma (500 µL of supernatant) were then mixed with 2,000 µL of methanol and 200 ng prostaglandin B_1 were added as internal standard. The samples were put at -20° C for 2 hours and then centrifuged again (600*g*, 15 minutes, 4° C). The supernatants were collected and diluted with 2,500 µL PBS + 75 µL HCl 1N. Formed 5-LO metabolites were extracted and analyzed by HPLC as described in **3.3.**.

For assays in blood homogenates, freshly drawn venous blood was collected in heparinized tubes (see above) and was put on ice for 5 minutes after addition of EDTA 1 mM. The blood was then sonicated on ice (5 × 10 seconds) with a cell disruptor B15 Branson sonifier (output: 6; duty cycle: 40 %) and the cell disruption was checked by light microscopy. ATP was added (1 mM), samples (2 mL of blood) were pre-warmed for 30 seconds at 37° C and 2 mM $CaCl_2$, and 100 µM AA were added to start 5-LO product formation. The reaction was stopped after 10 minutes at 37° C by addition of 8 mL ice-cold methanol and 200 ng prostaglandin B_1 were added as internal standard. The samples were put at -20° C for 2 hours and then centrifuged (600*g*, 15 minutes, 4° C). The supernatants were collected and diluted with 6,000 µL PBS + 180 µL HCl 1N. Formed 5-LO metabolites were then extracted and analyzed by HPLC as described in **3.3.**.

3.14. Statistical analysis

The results are expressed as mean ± standard error (SE) of the mean of *n* observations, where *n* represents the number of experiments performed on different days. Duplicates were used for the various treatment conditions. In the Western Blot or immunofluorescence experiments, the figures shown are representative of at least three experiments.

Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) for independent or correlated samples followed by Tukey HSD post-hoc tests. Where appropriate, Student's *t* test for paired observations was applied. A p value < 0.05 (*), < 0.01 (**) or < 0.001 (***) was considered significant.

4. RESULTS

4.1. Gender-dependent activity and subcellular localisation of 5-LO in human PMNL

4.1.1. 5-LO activity from endogenous AA is significantly lower in intact PMNL from males than from females

Freshly isolated human PMNL from male and female donors were incubated for 10 minutes at 37° C with Ca^{+2}-ionophore A23187 (2.5 µM), AA (20 µM) or both, to induce 5-LO product synthesis.

When cells were stimulated with Ca^{+2}-ionophore alone, 5-LO product formation was 40 % lower in cells from males than from females **(Fig. 14A)**, with high levels of significance ($p < 0.001$). In particular, 5-LO products were 48.04 ± 2.06 ng/10^6 cells in males ($n = 14$) vs 78.16 ± 3.8 ng/10^6 cells in females ($n = 14$).

In contrast, no significant difference was observed between the genders when exogenous AA was added, with or without ionophore (**Fig. 14A**). Thus, the levels of 5-LO products observed in male and female PMNL were 31.7 ± 1.7 and 31.2 ± 1.5 ng/10^6 cells, respectively, after AA-stimulation ($n = 4$, $p > 0.05$), and 165.7 ± 15.5 and 164.2 ± 16.5 ng/10^6 cells, respectively, after stimulation with Ca^{+2}-ionophore plus AA stimulation ($n = 14$, $p > 0.05$).

To evaluate whether the difference in 5-LO activity from endogenous AA was observed also after more physiologic stimulation than Ca^{+2}-ionophore, cells were primed with 1 µg/ml LPS (30 minutes, 37° C) and stimulated with 1 µM fMLP (5 minutes, 37° C). In order to mimic

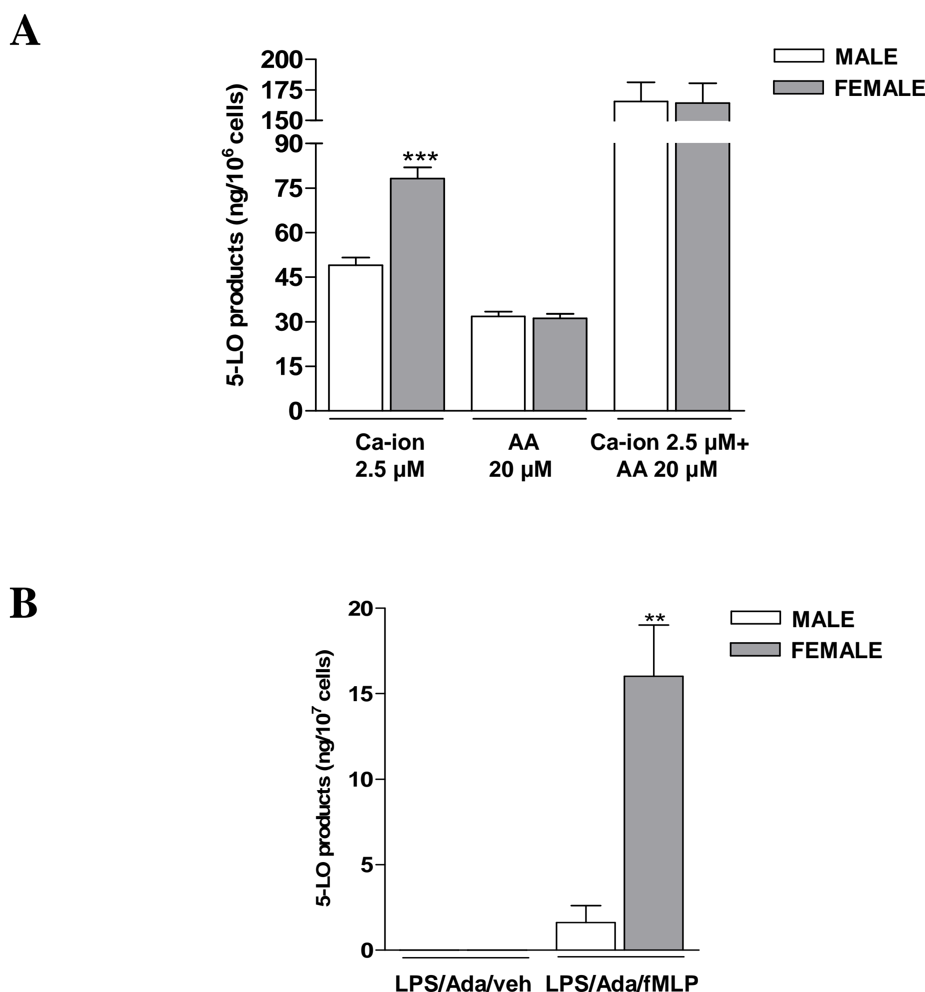


Fig. 14: 5-LO product formation from endogenous AA is significantly lower in intact PMNL from males than from females. *(A)* Human PMNL from males and females were resuspended in PGC buffer (5×10^6/mL), stimulated with Ca^{+2}-ionophore A23187 (2.5 µM) and AA (20 µM) for 10 min at 37° C, and 5-LO products formed were determined. Values are given as mean + SE, n = 14, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: ***$p < 0.001$ vs correspondent treatment in PMNL from males. ***(B)*** Human PMNL from males and females were resuspended in PGC buffer (2×10^7/mL) and pre-incubated with 1 µg/mL LPS at 37° C for 10 min, followed by Ada (0.3 U/mL) for 20 min. Cells were then incubated with vehicle (0.1 % DMSO) or stimulated with 1 µM fMLP for 5 minutes and 5-LO products formed were determined. Values are given as mean + SE, n = 5, duplicates; data were analysed by Student´s t test: **$p < 0.01$ vs corresponding treatment in PMNL from males. *Abbreviations:* AA, arachidonic acid; Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; DMSO, dimethyl sulfoxide; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

physiologic conditions, adenosine deaminase (Ada, 0.3 U/mL) was added to the incubations for 20 minutes prior to fMLP. In fact, it is well established that adenosine accumulates in leukocyte suspensions as a consequence of the extracellular breakdown of ATP (van Waeg and Van den Berghe, 1991) and this accumulation reaches concentrations (25-400 nM after 15-30 minutes of incubation (Krump et al., 1997)) that exert suppressive effects on PMNL functions and 5-LO activity. Adenosine interacts with the A2a receptor that increases the intracellular cAMP concentration leading to PKA-activation and downregulation of 5-LO via phosphorylation at Ser523 (Flamand et al., 2002) (see *1.2.6.2*). As shown in **Fig. 14B**, fMLP-induced 5-LO product formation was 10 fold lower in PMNL from males than from females ($n = 5$, $p < 0.01$), confirming a prominent difference in 5-LO activity from endogenous substrate. In particular, 5-LO products levels were 1.6 ± 1 and 15.9 ± 3 ng/10^7 cells in PMNL males and females, respectively. In resting cells, the amount of 5-LO products was lower than the detection limits of the assay.

4.1.2. AA release is not significantly different between PMNL from males and females

Since the difference in 5-LO product synthesis from endogenous substrate was abolished when AA was exogenously added to the cells, AA liberation was evaluated, in order to assess a possible unequal substrate availability in PMNL from males and females. The increase in the release of AA induced by Ca^{+2}-ionophore or by LPS/Ada/fMLP in PMNL was not significantly different between the genders (Ca^{+2}-ionophore: 245 ± 40 % in male and 246 ± 40 % in female, $n = 4$; fMLP/Ada/LPS: 160 ± 2 % in male and 165 ± 13 % in female, $n = 2$; data are percentage of the increase induced by stimulation, compared to unstimulated control) **(Fig. 15)**. These data indicate that the differential 5-LO product synthesis observed in male and female PMNL was not related to differential AA supply.

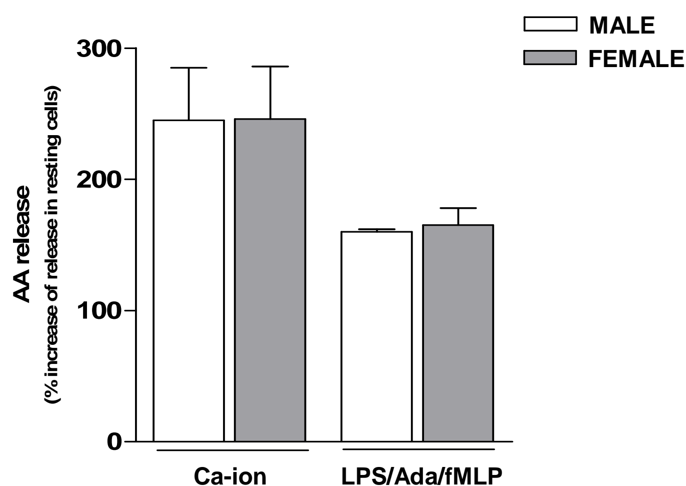


Fig. 15: AA release is not significantly different between PMNL from males and females
Human PMNL from males and females were resuspended in PGC buffer (5×10^7/mL), pre-warmed at 37° C for 10 min and then the 12-LO inhibitor CDC (10 µM) and the 5-LO inhibitor BWA4C (1 µM) were added. Cells were stimulated with 2.5 µM Ca^{+2}-ionophore for 5 min at 37° C. In the experiments in which PMNL were stimulated with fMLP, 1 µg/mL LPS was added together with CDC and BWA4C at 37° C for 10 min. Cells were then pre-incubated with Ada (0.3 U/mL) for additional 20 min and stimulated with fMLP (1 µM) for 5 min. Released AA was then derivatized and analysed by HPLC. Values are given as mean + SE of % increase compared to unstimulated cells, n = 2-4, duplicates; data were analysed by Student´s t test, $p >$ 0.05 (male vs female). *Abbreviations:* AA, arachidonic acid; Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

4.1.3. 5-LO protein expression and the amount of catalytically active 5-LO are not significantly different between PMNL from males and females

No significant difference between PMNL from males and females was observed also in 5-LO protein expression, as evaluated by WB of whole cell lysates prepared by two different methods of lysis (namely, 1 % NP40 or sonication) **(Fig. 16A)**.

Moreover, 5-LO activity was also evaluated in cell free assays. As shown in **Fig. 16B,** 5-LO product synthesis in both homogenates and S100 fractions of PMNL was not significantly different between the genders, at various concentrations of Ca^{++} and/or AA.

These data demonstrate that the amounts of both 5-LO protein (WB) and catalytically active (cell free assays) enzyme are essentially the same in PMNL from both genders.

4.1.4. 5-LO subcellular localization is different in PMNL from males and females

To determine whether the gender-related difference in 5-LO activity in PMNL was accompanied by a differential pattern of enzyme distribution, 5-LO subcellular localization was evaluated. To this purpose, three different techniques have been utilised:

a. Mild detergent (0.1 % NP40) lysis of the plasma membrane, yielding a nuclear (Nuc) and a non-nuclear (Non-N) fraction, that are analysed by WB (see **3.6.**);
b. Sonication of the cells and subsequent preparation of soluble (S100) and membrane (P100) fractions by 100,000*g* centrifugation, that are analysed by WB (see **3.7.**);
c. Indirect immunofluorescence (IF), allowing “in cell” colocalization studies of 5-LO subcellular distribution (see **3.10.**).

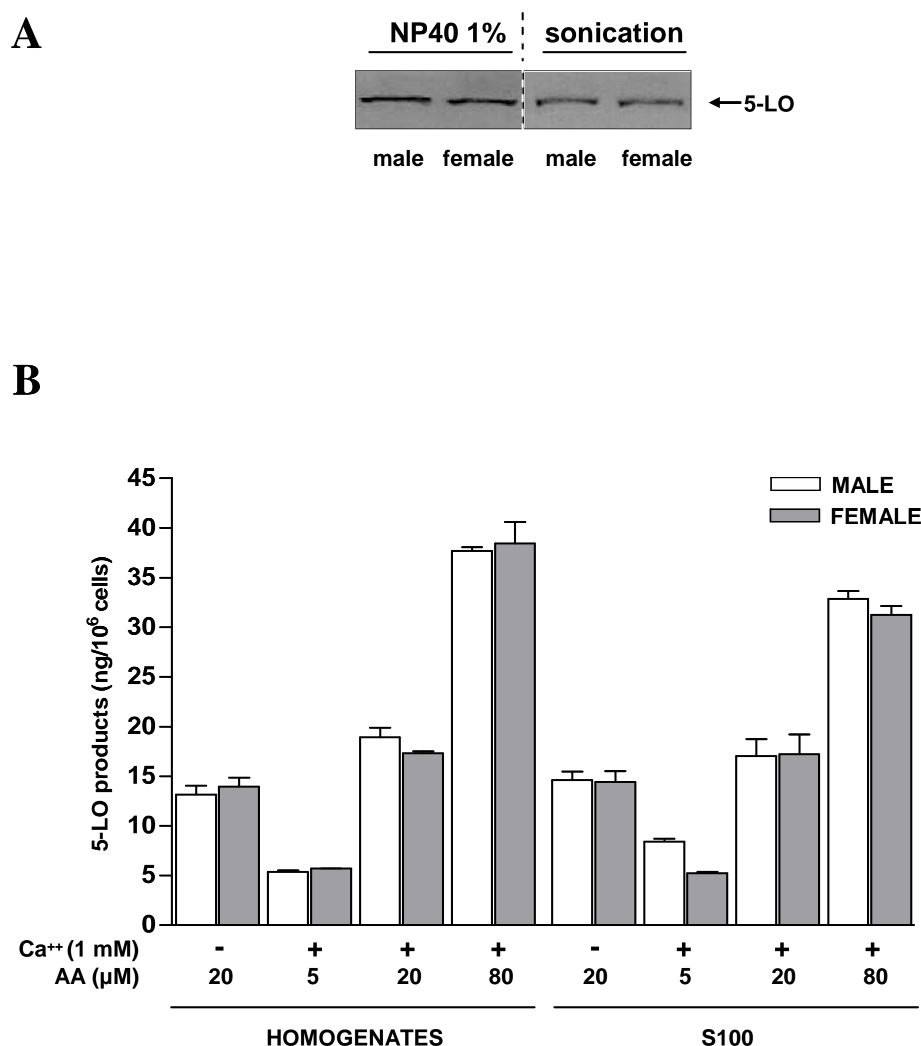


Fig. 16: 5-LO protein expression and the amount of catalytically active 5-LO are not significantly different between PMNL from males and females. ***(A) 5-LO protein expression.*** Human PMNL (3×10^7) from males and females were lysed by NP40 (1 %) buffer or by sonication. Total cell lysates were then analysed by WB. The results shown are representative of at least 2 independent experiments. ***(B) 5-LO activity in homogenates and S100 fractions.*** Aliquots of homogenates and S100 fractions of human PMNL from male and female donors, were pre-warmed for 30 sec at 37° C and incubated, in presence or absence of Ca^{+2} (1 mM, final concentration), with AA (5, 20 or 80 µM) for 10 min at 37° C and 5-LO products formed were determined. Values are given as mean + SE, $n = 4$, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test, $p > 0.05$ (male vs female). *Abbreviations:* AA, arachidonic acid; PMNL, polymorphonuclear leukocytes; S100, 100,000*g* supernatant

a. Surprisingly, after mild NP40 fractionation, 5-LO was equally present in both nuclear and non-nuclear fractions in resting PMNL from males, and low or no translocation of the non-nuclear 5-LO was observed after exposure to LPS/Ada/fMLP (1 µg/mL, 0.3 U/mL and 1 µM, respectively) or Ca^{+2}-ionophore (2.5 µM) **(Fig. 17A, left panel)**. On the contrary, a very prominent non-nuclear localization was observed in resting cells from females and 5-LO was essentially found in the nuclear fraction upon treatment with LPS/Ada/fMLP or Ca^{+2}-ionophore **(Fig. 17A, right panel)**.

b. The differential pattern of 5-LO localization between the genders was confirmed also after fractionation by sonication. In fact, 5-LO was distributed in both the membrane-associated (P100) and the soluble (S100) fractions in resting and activated PMNL from males, whereas 5-LO was found in the S100 fraction in resting cells from females, and translocated to the P100 fraction after activation **(Fig. 17B)**. It should be noted that the P100 fraction contains several cell membranes, not only the nuclear membrane.

c. IF analysis of 5-LO in resting PMNL from males revealed a prevalent punctate staining in the region surrounding the nucleus, visualised by DAPI-staining of DNA **(Fig. 18A)**. The small amount of enzyme present in the cytosolic space, also showed a punctuate staining. Costaining studies showed a clear colocalisation of 5-LO with the endoplasmic reticulum marker protein 1D3 **(Fig. 18B)**, present also in the perinuclear region, and a partial superimposition with the nuclear envelope marker protein lamin B **(Fig. 18C)**. Of interest, after activation with Ca^{+2}-ionophore **(Fig. 18D)** or with LPS/Ada/fMLP **(Fig. 18E)**, a similar staining was observed and the localization in the endoplasmic reticulum was still prevalent. On the contrary, in resting cells from females, 5-LO was homogenously and diffusely localized in the cytosol **(Fig. 19A)**, and was clearly away from DNA. Only a marginal overlay with the endoplasmic reticulum marker 1D3 was observed, probably because in PMNL the

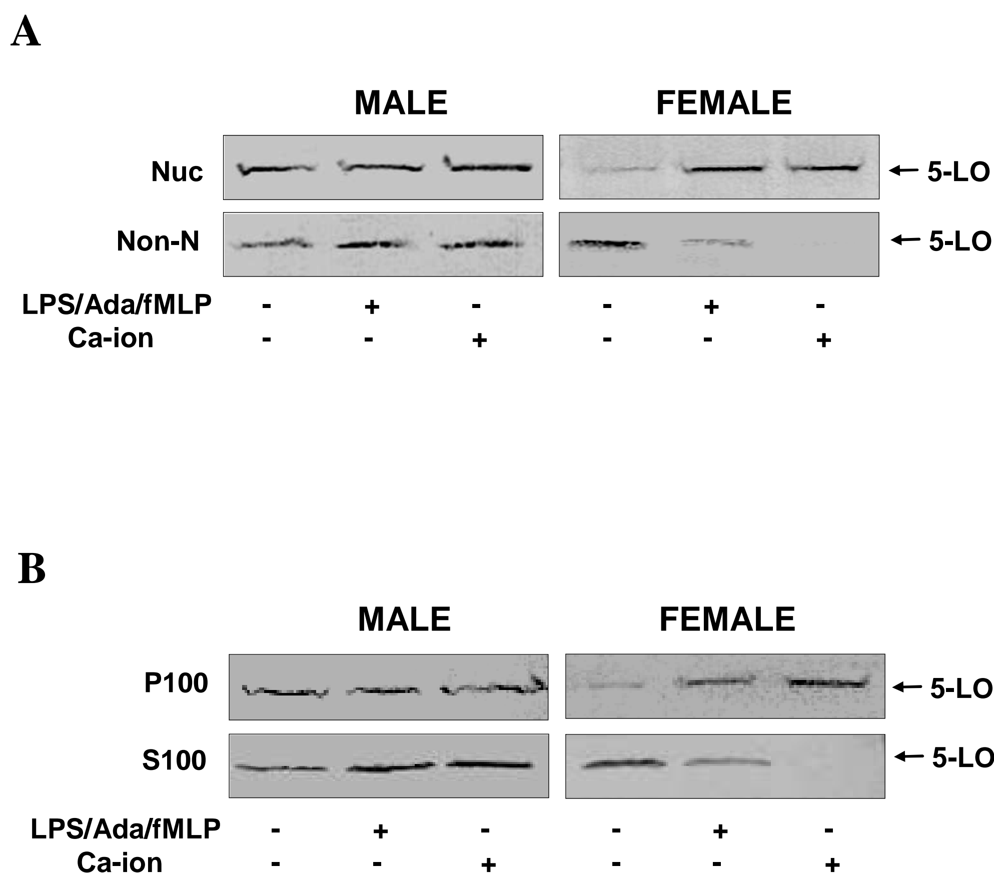


Fig. 17: 5-LO subcellular localization is different in PMNL from males and females. ***(A) 5-LO subcellular localisation by mild NP40 lysis.*** Human PMNL from males and females were resuspended in PGC buffer (3×10^7/mL) and kept on ice or treated with 1 µg/mL LPS (10 min, 37° C) and 0.3 U/mL Ada (20 min, 37° C) and then stimulated with fMLP (1 µM, 5 min, 37° C) or, alternatively, directly stimulated with Ca^{+2}-ionophore (2.5 µM, 5 min, 37° C). After mild detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 9 independent experiments. ***(B) 5-LO subcellular localisation by sonication/ultracentrifugation.*** Human PMNL from males and females were stimulated as in (A). After sonication and ultracentrifugation (100,000*g*, 1 h, 4° C), 5-LO was determined in S100 and P100 fractions by WB. The results shown are representative of 4 independent experiments. *Abbreviations:* Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; P100, membrane-associated fraction; S100, soluble fraction; WB, western blotting.

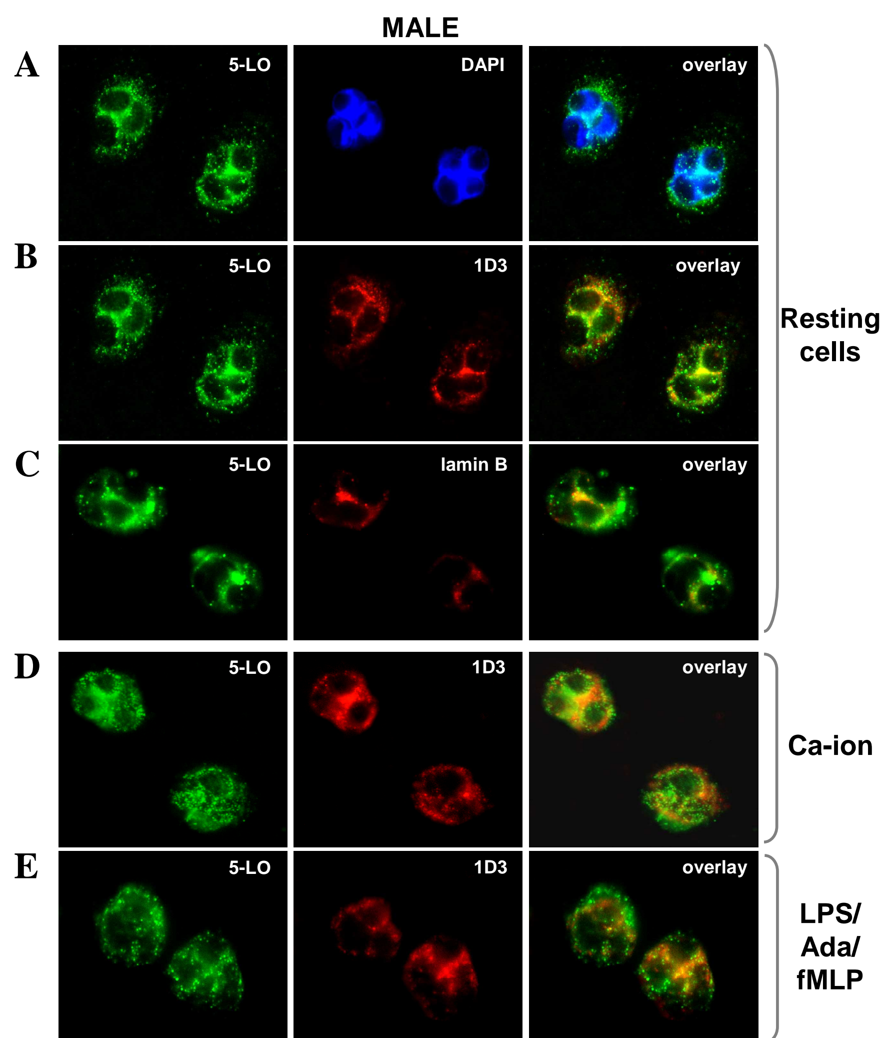


Fig. 18: 5-LO subcellular localisation in PMNL from males by IF microscopy. ***(A-C) Resting cells.*** Human PMNL from males were resuspended in ice-cold PGC buffer (1.5 × 10^6/mL) and cytospun onto poly-L-lysine-coated glass coverslips. Cells were fixed, permeabilized, blocked, and stained for 5-LO with anti-5-LO serum and Alexa Fluor 488 goat anti-rabbit IgG (green) (A-C). The DNA was stained with DAPI (blue) (A), and the samples were double stained for the endoplasmic reticulum marker (1D3, red) (B) or the nuclear envelope marker (lamin B, red) (C), using Alexa Fluor 594 goat anti-mouse IgG as secondary antibody. ***(D) Ca^{+2}-ionophore-activated cells.*** Human PMNL from males were resuspended and cytospun onto coverslips like in (A-C) and activated with Ca^{+2}-ionophore (2.5 µM, 3 min, 37° C). Cells were double stained for 5-LO and 1D3 as described above. ***(E) LPS/Ada/fMLP-activated cells.*** Human PMNL from males were resuspended in PGC buffer (1.5 × 10^6/mL), pre-incubated with 1 µg/mL LPS (10 min, 37° C) and 0.3 U/mL Ada (20 min, 37° C). Cells were then cytospun onto poly-L-lysine-coated glass coverslips and activated with fMLP (1 µM, 5 min, 37° C) and then double stained for 5-LO and 1D3 as described above. Single stainings and overlays are shown being representative of 5-7 independent experiments. *Abbreviations:* Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; DAPI, diamidino-2-phenylindole; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

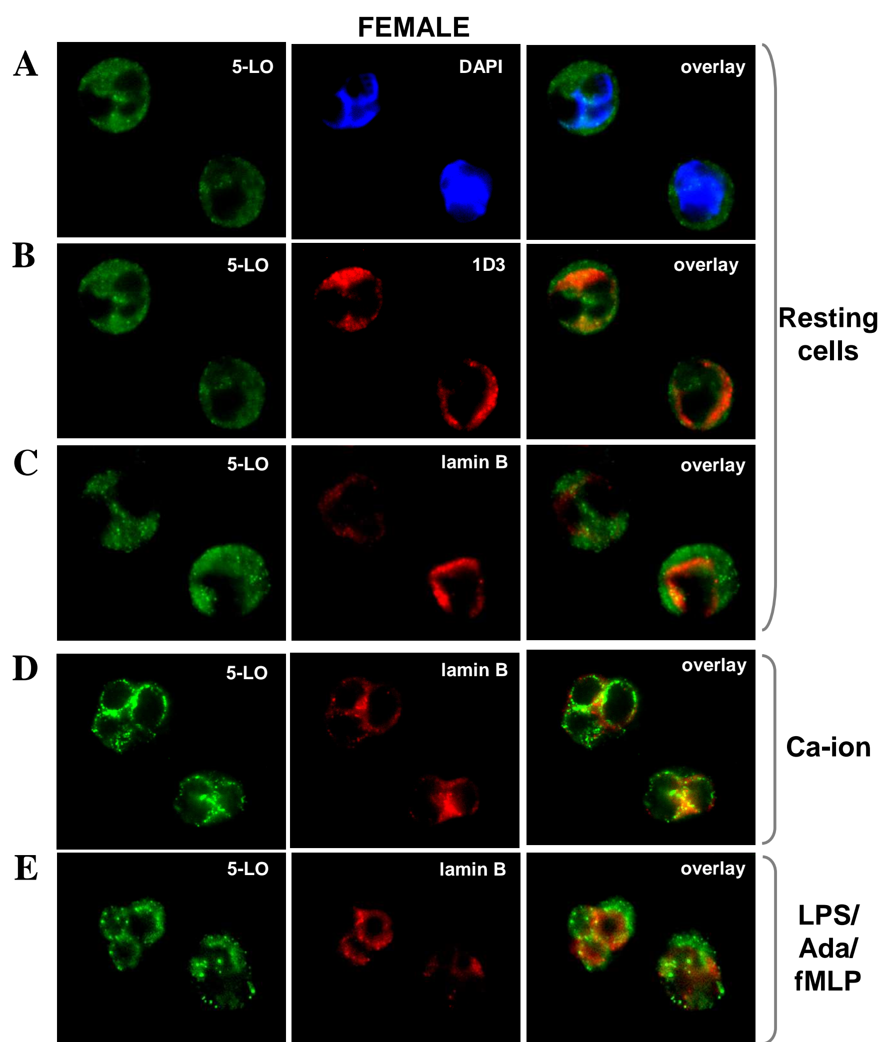


Fig. 19: 5-LO subcellular localisation in PMNL from females by IF microscopy. ***(A-C) Resting cells.*** Human PMNL from females were resuspended in ice-cold PGC buffer (1.5 × 10^6/mL) and cytospun onto poly-L-lysine-coated glass coverslips. Cells were fixed, permeabilized, blocked, and stained for 5-LO with anti-5-LO serum and Alexa Fluor 488 goat anti-rabbit IgG (green) (A-C). The DNA was stained with DAPI (blue) (A), and the samples were double stained for the endoplasmic reticulum marker (1D3, red) (B) or the nuclear envelope marker (lamin B, red) (C), using Alexa Fluor 594 goat anti-mouse IgG as secondary antibody. ***(D) Ca^{+2}-ionophore-activated cells.*** Human PMNL from females were resuspended and cytospun onto coverslips like in (A-C) and activated with Ca^{+2}-ionophore (2.5 µM, 3 min, 37° C). Cells were double stained for 5-LO and lamin as described above. ***(E) LPS/Ada/fMLP-activated cells.*** Human PMNL from females were resuspended in PGC buffer (1.5 × 10^6/mL), pre-incubated with 1 µg/mL LPS (10 min, 37° C) and 0.3 U/mL Ada (20 min, 37° C). Cells were then cytospun onto poly-L-lysine-coated glass coverslips and activated with fMLP (1 µM, 5 min, 37° C) and then double stained for 5-LO and lamin B as descibed above. Single stainings and overlays are shown being representative of 5-7 independent experiments. *Abbreviations:* Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; DAPI, diamidino-2-phenylindole; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

endoplasmic reticulum seems to be spread in the cytosolic space **(Fig. 19B)**. No overlay was observed with the nuclear envelope **(Fig. 19C)**. Note that in Ca^{+2}-ionophore- **(Fig. 19D)** or LPS/Ada/fMLP- **(Fig. 19E)** activated PMNL from females, 5-LO translocated to the nuclear envelope, showing a characteristic granular staining that superimposed with the nuclear envelope marker protein lamin B. No significant difference in 5-LO localisation compared to resting cells was observed when the cells were incubated with the vehicles (0.25 % methanol or 0.1 % DMSO) in the respective experimental conditions (not shown).

4.1.5. The subcellular localization of CLP, but not of $cPLA_2$ and FLAP, correlates to 5-LO

To evaluate if the gender-related difference in subcellular localization was specific for 5-LO or applied also to other proteins involved in LT biosynthesis in intact cells, the subcellular localisation of $cPLA_2$, FLAP and CLP was analysed.

Localisation of $cPLA_2$ has been assessed by WB after sonication of the cells and subsequent 100,000*g* ultracentrifugation (method *b* in **4.1.4.**). The NP40 assay (method *a* in **4.1.4.**), in fact, is not suitable for analysis of $cPLA_2$ redistribution in PMNL (Pouliot et al., 1996). As shown in **Fig. 20A**, $cPLA_2$ was found in the S100 fraction in resting PMNL from both males and females, confirming that $cPLA_2$ is a soluble enzyme in non-activated cells. After stimulation with LPS/Ada/fMLP (1 µg/mL, 0.3 U/mL, and 1 µM, respectively) or with Ca^{+2}-ionophore (2.5 µM), $cPLA_2$ was found in the P100 membrane-associated fraction in both males and females, without any significant difference between the genders.

Also for FLAP no difference between the genders was observed. Thus, FLAP is located in the nuclear (mild NP40 fractionation, method *a* in **4.1.4.**) **(Fig. 20B)** and P100 (P100/S100 fractionation, method *b* in **4.1.4.**) **(Fig. 20C)** fraction in both resting and stimulated PMNL

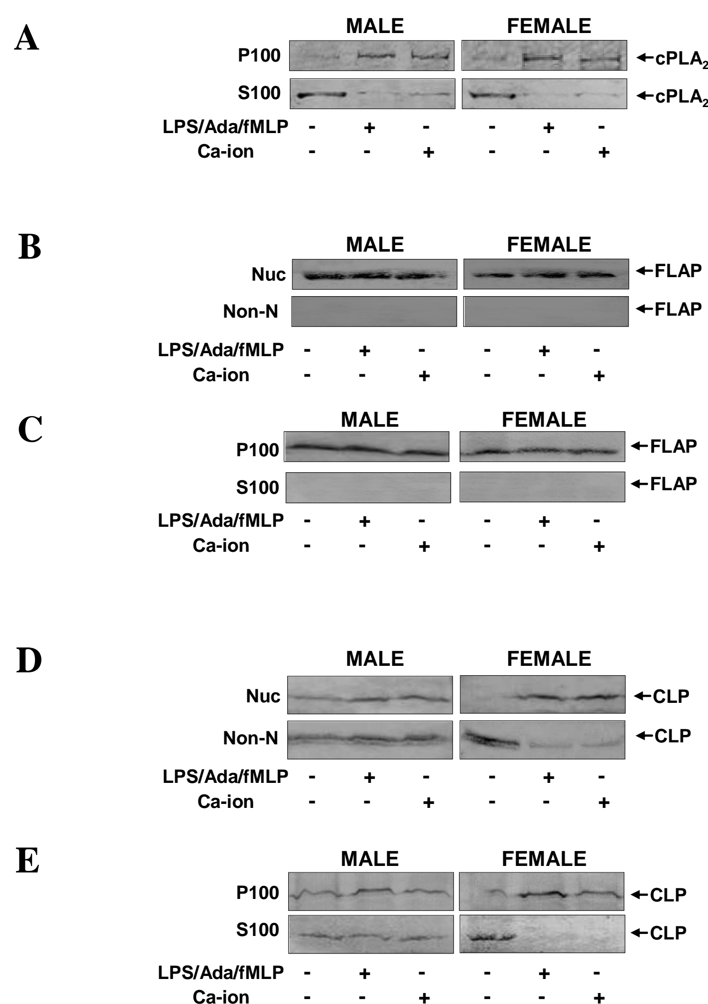


Fig. 20: The subcellular localisation of CLP, but not of $cPLA_2$ and of FLAP, correlates to that of 5-LO. (A,C,E) ***Determination of the subcellular localisation of $cPLA_2$, FLAP and CLP by sonication-ultracentrifugation.*** Human PMNL from males and females were resuspended in PGC buffer (3×10^7/mL) and kept on ice or treated with 1 µg/mL LPS (10 min, 37° C) and 0.3 U/mL Ada (20 min, 37° C) and then stimulated with fMLP (1 µM, 5 min, 37° C) or, alternatively, directly stimulated with Ca^{+2}-ionophore (2.5 µM, 5 min, 37° C). After sonication and ultracentrifugation (100,000*g*, 1 h, 4° C), $cPLA_2$ (A), FLAP (C) and CLP (E) were determined in S100 and P100 fractions by WB. The results shown are representative of 4 independent experiments. ***(B,D) Determination of the subcellular localisation of FLAP and CLP by mild NP40 lysis.*** Human PMNL from males and females were incubated as in (A,C,E). After detergent (0.1 % NP40) lysis and subcellular fractionation, FLAP (B) and CLP (D) were determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 4 independent experiments. *Abbreviations:* Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; CLP, coactosin like protein; $cPLA_2$: cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; P100, membrane-associated fraction; S100, soluble fraction; WB, western blotting.

from males and females. These data confirm the exclusive localization of FLAP in the nuclear membrane-associated locale. Moreover, no difference in the FLAP protein expression levels was observed between the genders.

On the contrary, CLP showed a redistribution pattern similar to 5-LO. Thus, CLP was found both in the nuclear and non-nuclear fraction in resting male PMNL, and only a low amount of protein was found to be enriched in the nuclear fraction after treatment with LPS/Ada/fMLP or Ca^{+2}-ionophore **(Fig. 20D)**. On the contrary, in cells from females, CLP was mainly in the non-nuclear fraction in resting conditions, but was found in the nuclear fraction after treatment with LPS/Ada/fMLP or Ca^{+2}-ionophore **(Fig. 20D)**. Similarly, the same subcellular localization of 5-LO and CLP was found by P100/S100 fractionation (**Fig. 20E**). Thus, CLP was partially soluble-partially pellettable in both resting and activated male PMNL, whereas it was found to be soluble in resting female cells and to translocate after activation to a membranous locus.

4.2. ERK1/2 and Ca^{+2} are the molecular elements involved in the gender-specific regulation of 5-LO in human PMNL

4.2.1. Inhibition of ERK1/2 abolishes the different 5-LO subcellular localization between PMNL from males and females

To determine the molecular machinery responsible for the different 5-LO subcellular localization between the genders, the effect of MAPK inhibition was analysed. In fact, MAPK are, together with Ca^{+2}, the main signalling pathways involved in the regulation of 5-LO subcellular localisation (see *1.2.6.2.*). In particular, both inhibition of ERK1/2, via suppression of their upstream kinases MEK1/2, and of p38 MAPK pathway were analysed.

The MEK1/2 inhibitors U0126 (3 µM) or PD98059 (30 µM), but not the p38 MAPK inhibitor SB203580 (10 µM), induced a prevalent non-nuclear 5-LO distribution in male cells and, notably, conferred the property of 5-LO to translocate to the nucleus after subsequent Ca^{+2}-ionophore activation **(Fig. 21A)**. Therefore, ERK1/2 inhibition, but not p38 MAPK inhibition, causes a 5-LO subcellular localisation pattern in male PMNL as observed in cells from females.

Interestingly, as analysed by IF, treatment of PMNL from males with the MEK1/2 inhibitor U0126 (3 µM) resulted in a redistribution of 5-LO from the perinuclear region to the cytosol, with a homogenous and diffuse staining **(Fig. 21B)**, similar to the staining observed in resting PMNL from females (cfr **Fig. 19A**).

On the contrary, no difference was observed in 5-LO subcellular localisation after MAPK inhibition in female cells **(Fig. 21C)**. Thus, treatment with MAPK inhibitors did not modify the prevalent cytosolic localisation in non-activated cells and did not modify the translocation to the nuclear fraction after Ca^{+2}-ionophore activation.

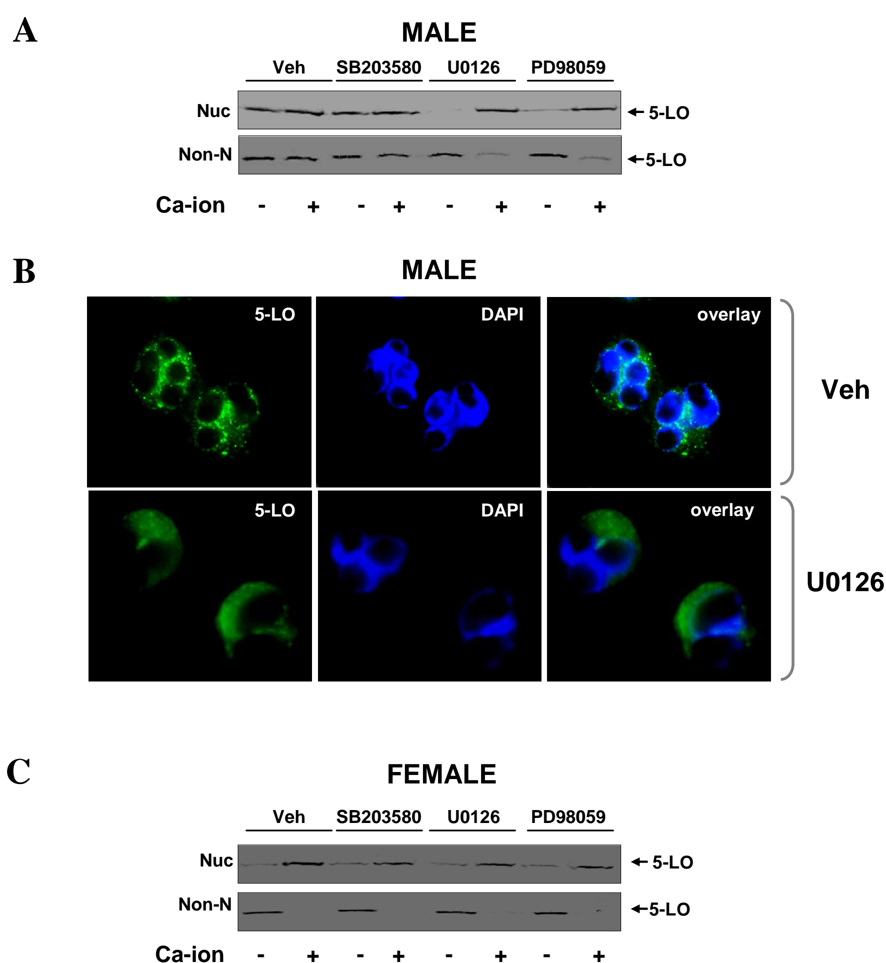


Fig. 21: Inhibition of ERK1/2 abolishes the differential 5-LO subcellular localization pattern between PMNL from males and females. ***Effect of MAPK inhibitors on 5-LO subcellular localisation in PMNL from males and from females determined by mild NP40 lysis.*** Human PMNL from males (A) or females (C) were resuspended in PGC buffer (3 × 10^7/mL) and incubated with vehicle (DMSO, 0.1 %), with the p38 MAPK inhibitor SB203580 (10 µM), or with the MEK1/2 inhibitors U0126 (3 µM) or PD98059 (30 µM) for 15 min at 37° C, before Ca^{+2}-ionophore (2.5 µM, 5 min, 37° C) stimulation. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. ***(B) Effect of U0126 on 5-LO subcellular localisation in PMNL from males by IF mycroscopy.*** Human PMNL from males were resuspended in ice-cold PGC buffer (1.5 × 10^6/mL) and incubated with vehicle (DMSO, 0.1 %), or with U0126 (3 µM, 15 min, 37° C). The incubation was stopped on ice for 5 min and the cells were cytospun onto poly-L-lysine-coated glass coverslips. Cells were fixed, permeabilized, blocked, and stained for 5-LO with anti-5-LO serum and Alexa Fluor 488 goat anti-rabbit IgG (green). The DNA was stained with DAPI (blue). The results shown are representative of 3 independent experiments. *Abbreviations:* Ca-ion, Ca^{+2}-ionophore A23187; DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; IF, indirect immunofluorescence; 5-LO, 5-lipoxygenase; MAPK, mitogen-activated protein kinase; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

4.2.2. ERK1/2, but not p38 MAPK, is constitutively activated in human PMNL from males as compared to females

Since the inhibition of the ERK pathway in PMNL from males yielded a “female type” 5-LO redistribution pattern, it appeared reasonable that PMNL from male might have a higher activation status of ERK as compared to female PMNL. Therefore, it was analysed whether there was a difference in the constitutive activation of ERK1/2 between PMNL from males and females. As shown in **Fig. 22**, male PMNL showed significantly higher levels of phosphorylated ERK1/2. Densitometric analyses of the WB from 5 separate experiments showed that the constitutive phosphorylated ERK1/2 levels observed in resting males were about twice as much as in females (O.D. %: 70.3 ± 13 and 32 ± 7, in male and female, respectively; $n = 5$, $p < 0.05$). No difference was observed in ERK1/2 protein expression (O.D. %: 78.2 ± 3 and 80.0 ± 5.5, in male and female, respectively; $n = 5$, $p > 0.05$) **(Fig. 22)**, indicating that the difference in the amounts of pERK1/2 was related to a different phosphorylation status and not to different protein levels.

No significant difference was observed in p-p38 MAPK levels (O.D. %: 69.3 ± 8.2 and 69.7 ± 6.7, in male and female, respectively; $n = 5$, $p > 0.05$), indicating that the higher pERK1/2 levels in male cells is selective and not widespread to all MAPK pathways.

Phosphorylation of ERK1/2 is considered as an index of their activation (Yoon and Seger, 2006) and activated ERK1/2 is known to translocate to the nucleus (Turjanski et al., 2007), where it phosphorylates several transcription factors (including Elk-1 (Peterziel et al., 1999)). To confirm that the higher levels of pERK1/2 observed in males were accompanied by higher ERK1/2 activity, pERK1/2 nuclear localisation and the phosphorylation of the well-recognized ERK substrate Elk-1 were analysed. Notably, ERK1/2 phosphorylation was

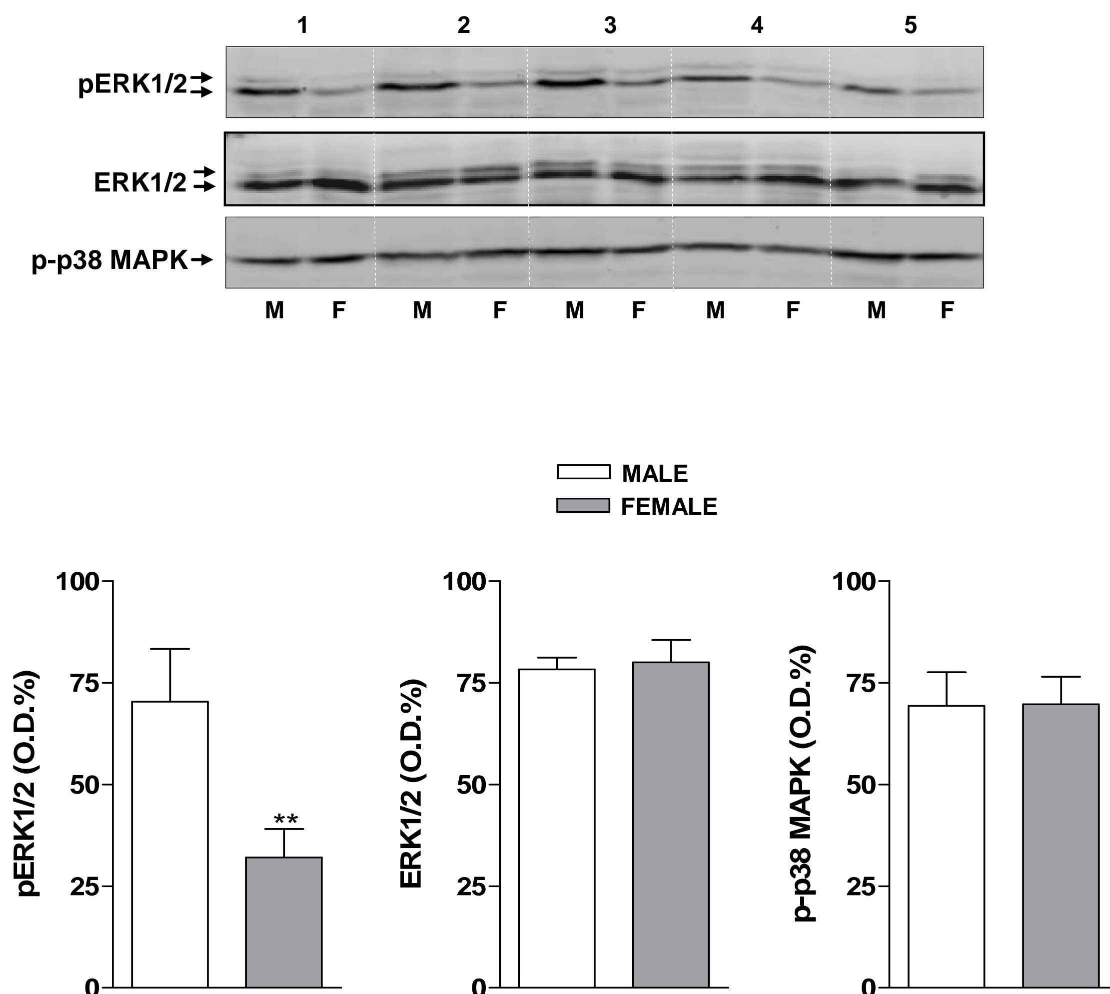


Fig. 22: The levels of phosphorylated ERK1/2 (pERK1/2), but not phosphorylated p38 MAPK (p-p38 MAPK) are constitutively higher in human PMNL from males as compared to females. Human PMNL from males and females were resuspended in PGC buffer (1×10^7/100 µL) and lysed by addition of 100 µL ice-cold SDS-buffer. pERK1/2, ERK1/2 and p-p38 MAPK levels were analysed by WB using an Ettan DIGE imaging system. Densitometry was performed with ImageQuant TL image analysis software. The results shown are from 5 independent experiments and values are given as mean + SE; relative intensities were calculated as percentage of the strongest band in the corresponding membrane; data were analysed by Student's t test: $^{**}p < 0.01$ vs corresponding PMNL from males. *Abbreviations:* ERK, extracellular signal-regulated kinase; F, female; M, male; O.D., optical density; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; SDS, sodium dodecyl sulphate; WB, western blotting.

accompanied by enhanced translocation to the nucleus, since pERK1/2 levels in the nuclear fraction of resting PMNL from male donors were higher than in female **(Fig. 23A)**. Moreover, the levels of pElk-1 in resting PMNL from males were higher than in resting females (O.D. %: 75.2 ± 13 and 48.6 ± 5.9, in male and female, respectively; $n = 5$, $p < 0.05$) and correlated with pERK1/2 levels **(Fig. 23B)**.

4.2.3. Ca^{+2} is required for 5-LO nuclear localisation in resting PMNL from males

Together with MAPK, Ca^{+2} is involved in the regulation of 5-LO subcellular localisation. Interestingly, chelation of intracellular Ca^{+2} by 50 µM BAPTA/AM and of extracellular Ca^{+2} by 1 mM EDTA induced a complete non-nuclear localization of 5-LO in male cells **(Fig. 24A)**. No difference was observed in PMNL from females, where 5-LO was cytosolic in both Ca^{+2}-supplemented and Ca^{+2}-depleted cells **(Fig. 24B)**. These data indicate that the association of 5-LO with the nuclear fraction in male resting PMNL depends on Ca^{+2}.

4.2.4. Intracellular Ca^{+2} concentrations are not significantly different between PMNL from males and females

Because of the Ca^{+2}-dependance of 5-LO nuclear localisation in resting male cells, $[Ca^{+2}]_i$ were investigated as a possible molecular machinery involved in the different 5-LO subcellular localisation between the genders.

No differences were observed in $[Ca^{+2}]_i$ between PMNL from males and females, in resting cells **(Fig. 25A)** or PMNL stimulated with fMLP (1 µM) **(Fig. 25B)** and calcimycin (1 µM) **(Fig. 25C)**, neither in cells activated in Ca^{+2}-containing buffer (PGC Buffer, **Fig. 25**) or cells activated in Ca^{+2}-free buffer (PG Buffer) followed by addition of Ca^{+2} (not shown). Instead of A23187, calcimycin was used, since A23187 interferes with the spectruofluorimetric signal.

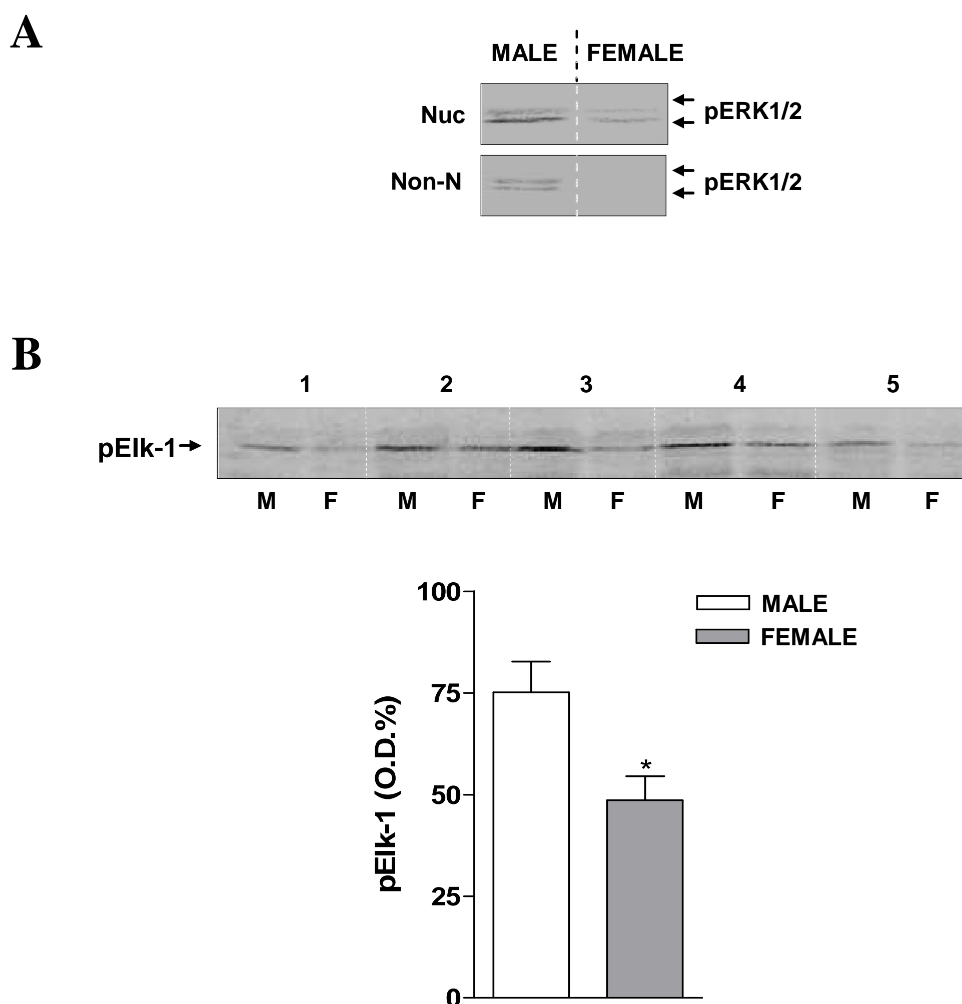


Fig. 23: ERK1/2 activity is constitutively higher in human PMNL from males as compared to females. ***(A) Subcellular localisation of pERK1/2 determined by mild NP40 lysis.*** Human PMNL from males and females were resuspended in PGC buffer (3×10^7/mL) and lysed by 0.1 % NP40. pERK1/2 was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. ***(B) Levels of phosphorylated Elk-1 (pElk-1).*** Human PMNL from males and females were resuspended in PGC buffer (1×10^7/100 µL) and lysed by addition of 100 µL ice-cold SDS-buffer. pElk-1 levels were analysed by WB using an Ettan DIGE imager system. Densitometry was performed with ImageQuant TL image analysis software. The results shown are from 5 independent experiments and values are given as mean + SE; relative intensities were calculated as percentage of the strongest band in the corresponding membrane; data were analysed by Student's t test: $*p < 0.05$ vs corresponding PMNL from males. *Abbreviations:* ERK, extracellular signal-regulated kinase; F, female; M, male; Non-N, non-nuclear fraction; Nuc, nuclear fraction; O.D., optical density; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; SDS, sodium dodecyl sulphate; WB, western blotting.

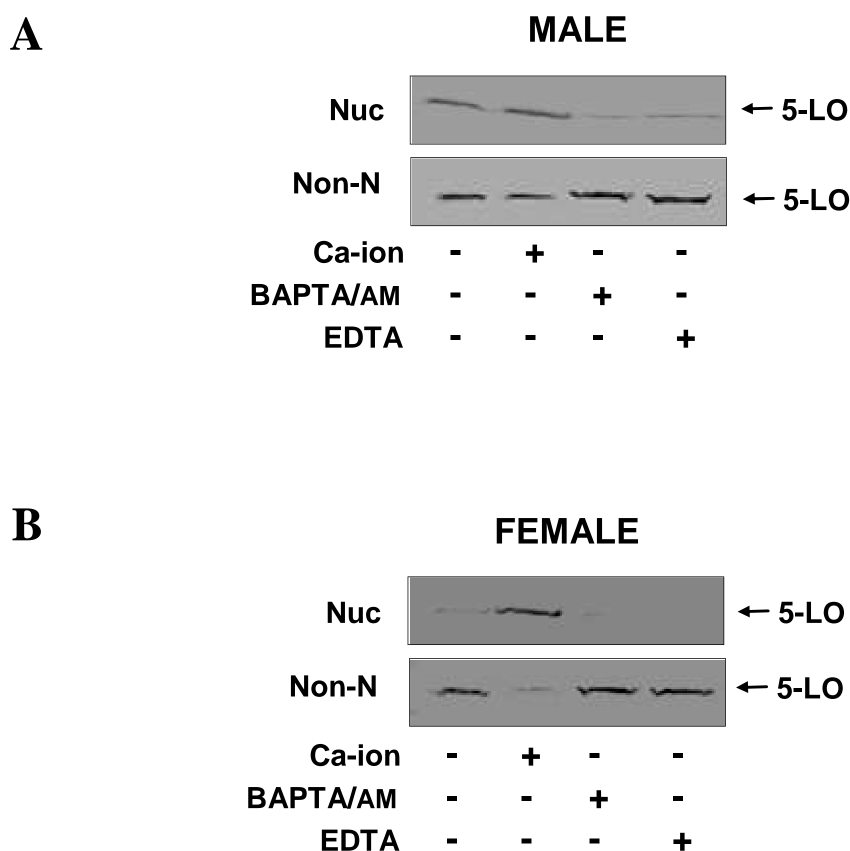


Fig. 24: Ca^{+2} is required for 5-LO nuclear localisation in resting PMNL from males. ***Effect of Ca^{+2} chelators on 5-LO subcellular localisation in PMNL from males and from females by mild NP40 lysis.*** Human PMNL from males (A) and females (B) were resuspended in PGC buffer (3×10^7/mL) and incubated with vehicle (DMSO, 0.1 %) and then rested or stimulated for 5 min at 37° C with 2.5 μM Ca^{+2}-ionophore (as control), or they were incubated with BAPTA/AM (50 μM) for 15 min at 37° C. When the effect of EDTA was evaluated, cells were resuspended in PG buffer (3×10^7/mL) and incubated with 1 mM EDTA for 15 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* BAPTA/AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); Ca-ion, Ca^{+2}-ionophore A23187; EDTA, ethylenediamine-tetraacetic acid; 5-LO, 5-lipoxygenase; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PG, PBS-glucose buffer; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; DMSO, dimethyl sulfoxide; WB, western blotting.

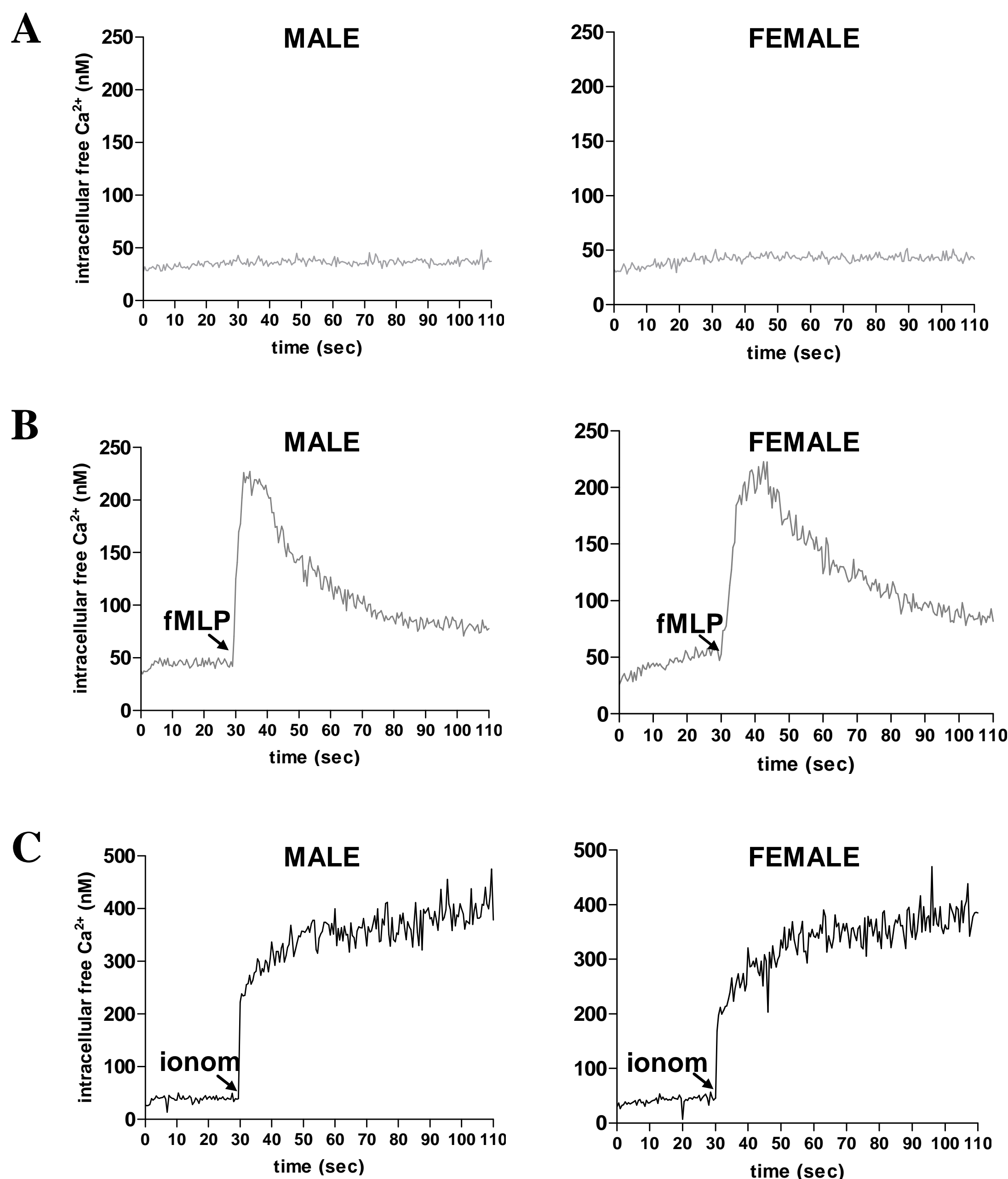


Fig. 25: Intracellular Ca^{+2} concentrations are not significantly different between PMNL from males and females. ***(A) Resting cells.*** Human PMNL from males and females (5×10^7 cells/mL) were loaded with 2 μM Fura-2/AM for 30 min at 37° C. After washing, cells were resuspended in PBS (1×10^7 cells/mL) and the fluorescence was measured two min after 1 mM $CaCl_2$ was added and $[Ca^{+2}]_i$ were calculated. ***(B-C) fMLP- and ionomycin-stimulated cells.*** fMLP or ionomycin (both 1 μM) were added 30 sec after the measurement was started. The curves shown are representative of 6 independent experiments. *Abbreviations:* ionom, ionomycin; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PG, PBS-glucose buffer; PMNL, polymorphonuclear leukocytes.

4.3. Male sex hormones are responsible for the gender-specific subcellular localisation of 5-LO in human PMNL

4.3.1. The male sex hormone 5α-DHT induces 5-LO translocation to the nuclear compartment

To evaluate whether 5-LO subcellular localization is affected by sex hormones, PMNL from males and females were incubated for 30 minutes at 37° C with physiologically-relevant concentrations of 5α-DHT, 17β-estradiol and progesterone and the distribution of 5-LO was analysed by WB in the nuclear and non-nuclear fraction of human PMNL (method *a* in **3.1.4.**). The male sex hormone 5α-DHT (10 nM) induced 5-LO translocation from the non-nuclear to the nuclear compartment **(Fig. 26A-26B)**. Although this effect was more evident in cells from females **(Fig. 26B)**, because of their prevalent 5-LO non-nuclear localisation in the resting state, an increase of 5-LO in the nuclear fraction and a concomitant decrease in the non-nuclear fraction was also observed in PMNL from males **(Fig. 26A)**.

This effect was not achieved by the female sex hormones 17β-estradiol (100 nM) and progesterone (10 μM), either used alone or in combination **(Fig. 26A-26B)**.

Induction of 5-LO nuclear localisation by 5α-DHT suggested that the androgen sex hormone might cause a “male type” 5-LO pattern in cells from females. This hypothesis was confirmed by IF **(Fig. 27A-B)**. In fact, in female PMNL, 10 nM 5α-DHT induced a translocation from the cytosol to membrane structures surrounding the nucleus **(Fig. 27B)**, with a staining similar to that observed in resting male cells (cfr. **Fig. 18A and 27A, resting male cells**). In accordance with the data obtained by subcellular fractionation experiments (NP40 assay), 5α-DHT treatment induced a further increase in the punctuate, perinuclear staining of 5-LO **(Fig. 27A)** also in PMNL from males, probably due to an additive effect.

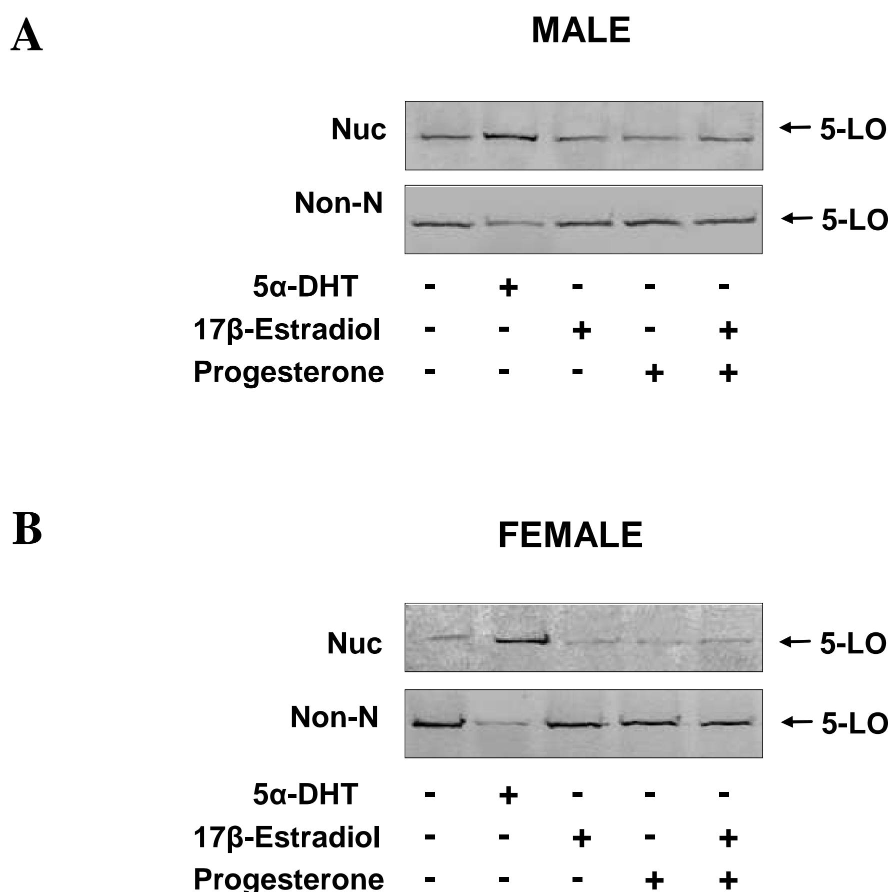


Fig. 26: The male sex hormone 5α-DHT, but not 17β-estradiol and/or progesterone, induces 5-LO translocation to nuclear structures. ***Effect of sex hormones on 5-LO subcellular localisation in PMNL from males and from females by mild NP40 lysis.*** Human PMNL from males (A) and females (B) were resuspended in PGC buffer (3×10^7/mL) and incubated with vehicle (EtOH, 0.05 %) or with the sex hormones 5α-DHT (10 nM), 17β-estradiol (100 nM) and progesterone (10 µM) for 30 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; 5-LO, 5-lipoxygenase; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; WB, western blotting.

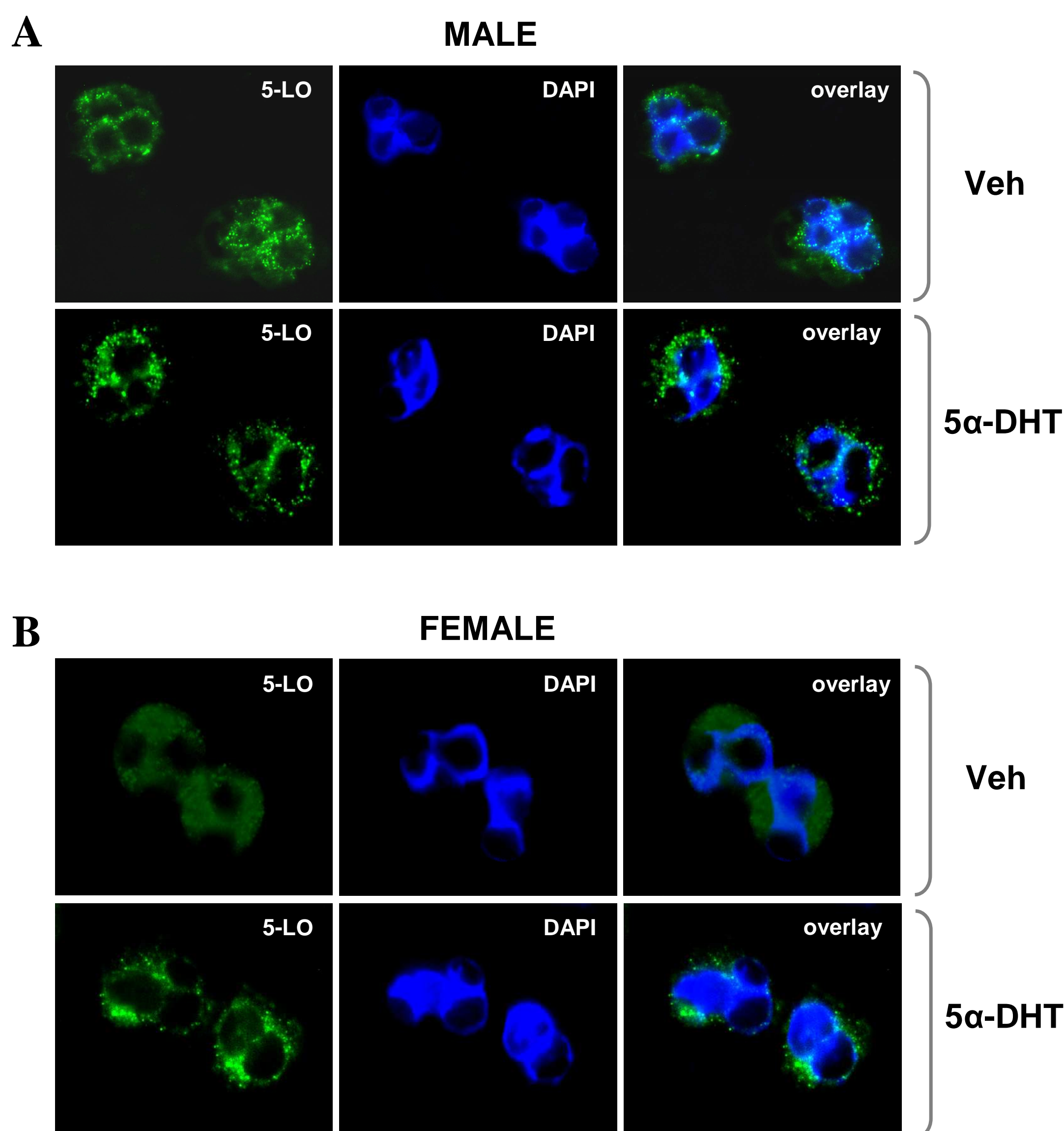


Fig. 27: The male sex hormone 5α-DHT induces 5-LO association with membrane structures surrounding the nucleus. ***Effect of 5α-DHT on 5-LO subcellular localisation in PMNL from males and from females by IF microscopy.*** Human PMNL from males (A) or females (B) were resuspended in PGC buffer (1.5×10^6/mL) and incubated with vehicle (EtOH, 0.05 %) or with 5α-DHT (10 nM), for 30 min at 37° C. The reaction was stopped on ice for 5 min and the cells were then cytospun onto poly-L-lysine-coated glass coverslips. Cells were fixed, permeabilized, blocked, and stained for 5-LO with anti-5-LO serum and Alexa Fluor 488 goat anti-rabbit IgG (green). The DNA was stained with DAPI (blue). Pictures with single stainings and overlays are shown being representative of 5 independent experiments. *Abbreviations:* DAPI, diamidino-2-phenylindole; IF, indirect immunofluorescence; 5-LO, 5-lipoxygenase; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Veh, vehicle.

4.3.2. 5α-DHT induces 5-LO translocation to the nuclear compartment in a rapid and concentration-dependent manner

To characterize the 5α-DHT-induced 5-LO translocation, time course and concentration-response experiments were performed.

5α-DHT induced 5-LO nuclear localisation in a rapid manner **(Fig. 28A-28B)**. In particular, the translocation of 5-LO to the nuclear compartment in PMNL from male donors occurred within 5 and 50 minutes **(Fig. 28A)** and in PMNL from female donors within 5 min **(Fig. 28B)**. Maximum of translocation was reached between 5 and 50 minutes for both genders, and an incubation time of 30 minutes has been therefore chosen for the subsequent experiments. Longer incubation times (up to 16 hours) in presence of 5α-DHT still gave nuclear localisation of 5-LO.

5α-DHT induced the translocation of 5-LO in a concentration-dependent manner **(Fig. 29A-29B)**. To remark, in PMNL from females, 5α-DHT at the concentration of 0.01 nM was already able to induce a partial increase of 5-LO in the nuclear fraction, and the maximal effect was obtained at 10 nM 5α-DHT **(Fig. 29B)**. In cells from males, where 5-LO was already present in the nuclear fraction also in the resting state, a substantial increase in the nuclear fraction was evident only at concentration > 1 nM 5α-DHT **(Fig. 29A)**.

Of interest, the FLAP inhibitor MK886 did not significantly modify 5-LO nuclear localisation in male PMNL and failed to inhibit 5α-DHT induced 5-LO association with the nuclear fraction (not shown), suggesting that nuclear localisation of 5-LO due to 5α-DHT does not depend on FLAP.

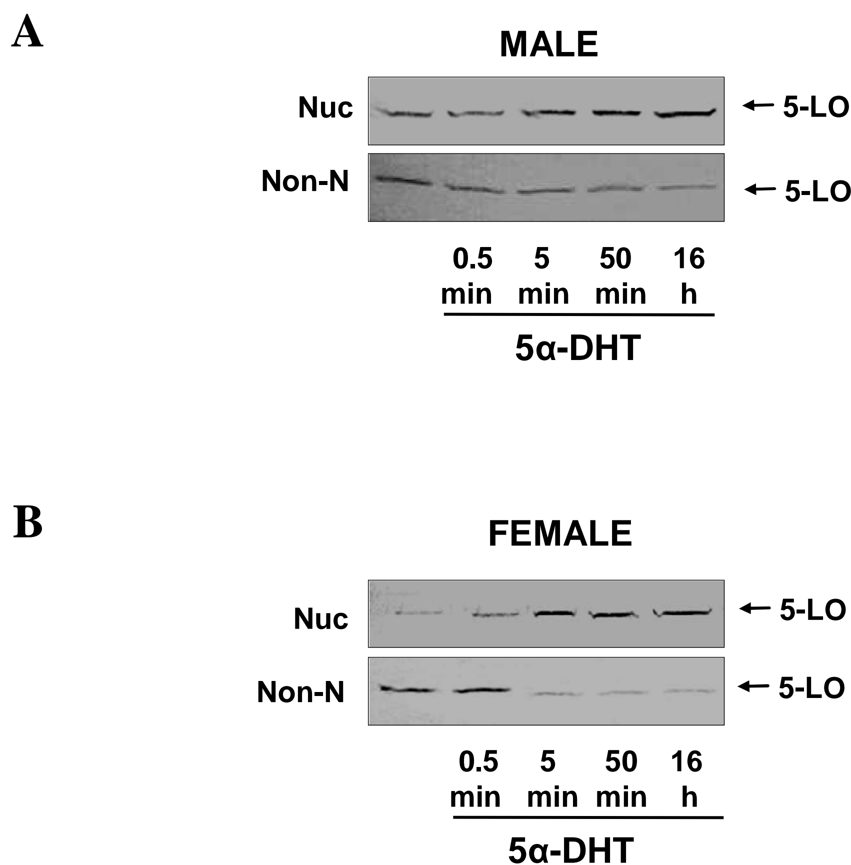


Fig. 28: 5α-DHT induces 5-LO translocation to nuclear structures in a rapid manner. ***Time course of 5α-DHT-induced 5-LO translocation in PMNL from males and from females by mild NP40 lysis.*** Human PMNL from males (A) and females (B) were resuspended in PGC buffer (3×10^7/mL) and incubated with 5α-DHT (10 nM) for the indicated time points at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; 5-LO, 5-lipoxygenase; min, minutes; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; WB, western blotting.

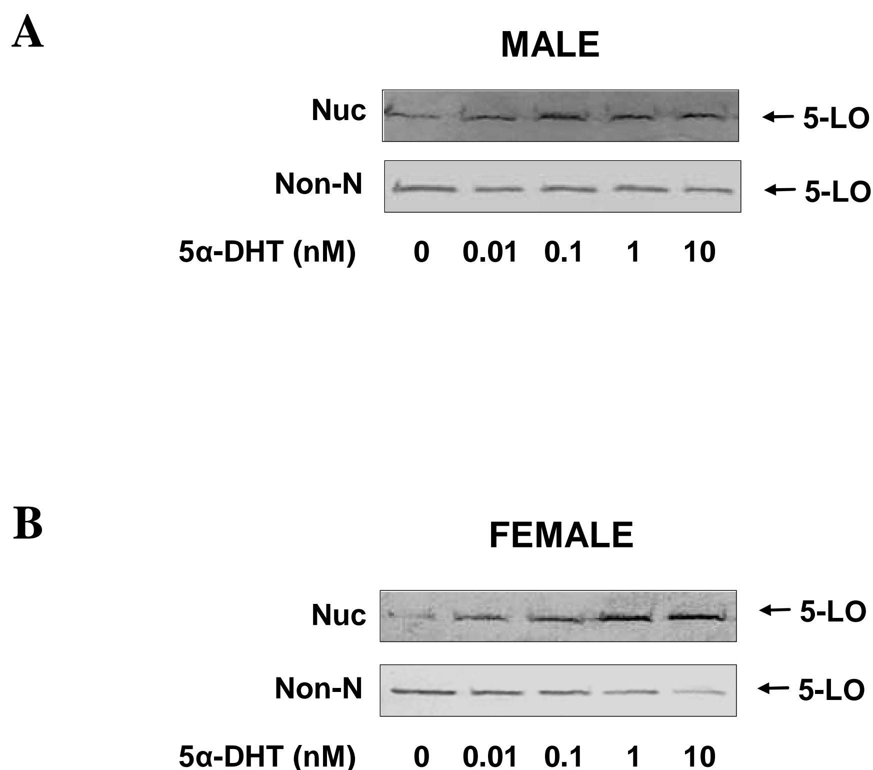


Fig. 29: Concentration-response of 5α-DHT-induced translocation of 5-LO to the nuclear compartment. Human PMNL from males (A) and females (B) were resuspended in PGC buffer (3×10^7/mL) and incubated with 5α-DHT at the indicated concentrations for 30 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; 5-LO, 5-lipoxygenase; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; WB, western blotting.

4.3.3. 5α-DHT-induced translocation of 5-LO is not reversed by antagonists of the classic androgen nuclear–receptor

Since 5α-DHT induced 5-LO translocation in a concentration-dependent manner, a receptor-mediated mechanism could be hypothesized. Sex-steroid effects are known to be mediated either by the classic and well-characterized intracellular steroid receptors, or by novel and still uncharacterized cell-membrane receptors (see **1.3.2.**).

To evaluate the involvement of the intracellular receptor in the effect of 5α-DHT on 5-LO translocation, a pharmacological approach was applied. Thus, PMNL from both male and female donors were stimulated with 5α-DHT in presence of steroidal (cyproterone acetate) or non-steroidal (flutamide) antagonists **(Fig. 30A)** of the classic AR receptor, and 5-LO subcellular localisation was assessed by WB in the nuclear and non-nuclear fraction.

Of interest, neither cyproterone acetate (10 μM) nor flutamide (10 μM) reversed 5α-DHT induced-translocation in PMNL from both males **(Fig. 30B)** and females **(Fig. 30C)**, suggesting that other receptor than the classic AR mediates the effect of 5α-DHT.

4.3.4. Testosterone, testosterone:BSA and human plasma from males induce 5-LO translocation in PMNL from females

To evaluate whether the effect on 5-LO translocation was specific for 5α-DHT or was achieved also by other androgens, human PMNL from males and females were incubated with testosterone (10 nM) and 5-LO subcellular localisation was evaluated by NP40 assay.

As shown in **Fig. 31A**, testosterone modulated 5-LO subcellular redistribution similarly to 5α-DHT. Notably, this effect was achieved also by the non-cell-permeable testosterone conjugated with BSA (testosterone:BSA), suggesting that androgens act via a receptor exposed on the cell surface.

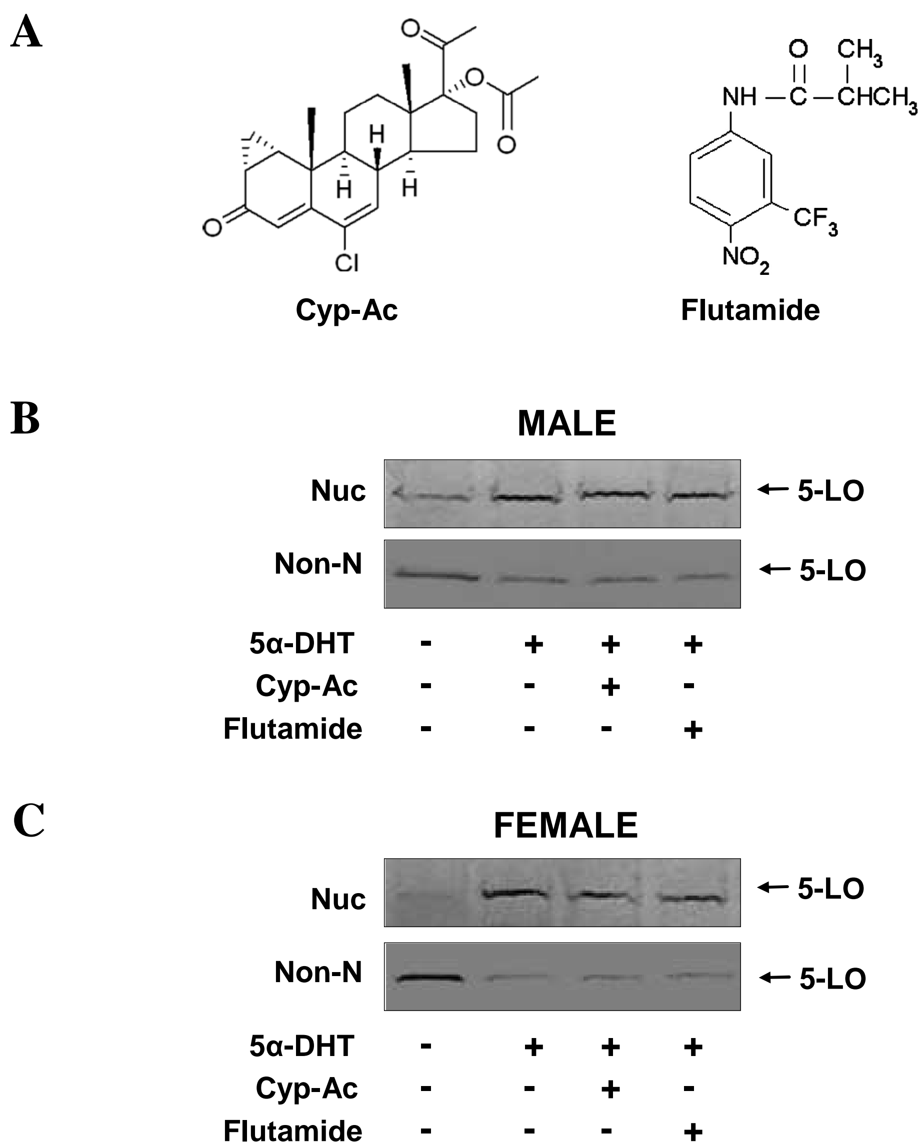


Fig. 30: 5α-DHT-induced 5-LO translocation is not abolished by the antagonists of the classical androgen receptor. ***(A) Molecular structures of Cyp-Ac and Flutamide. (B,C) Effect of Cyp-Ac and Flutamide on 5α-DHT-induced 5-LO translocation in PMNL from males and from females by mild NP40 lysis.*** Human PMNL from males (B) and females (C) were resuspended in PGC buffer (3×10^7/mL) and incubated with vehicle (0.05 % EtOH), Cyp-Ac (10 µM) or flutamide (10 µM) for 30 min and then 5α-DHT (10 nM) was added for additional 30 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; Cyp-Ac, cyproterone acetate; 5-LO, 5-lipoxygenase; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; WB, western blotting.

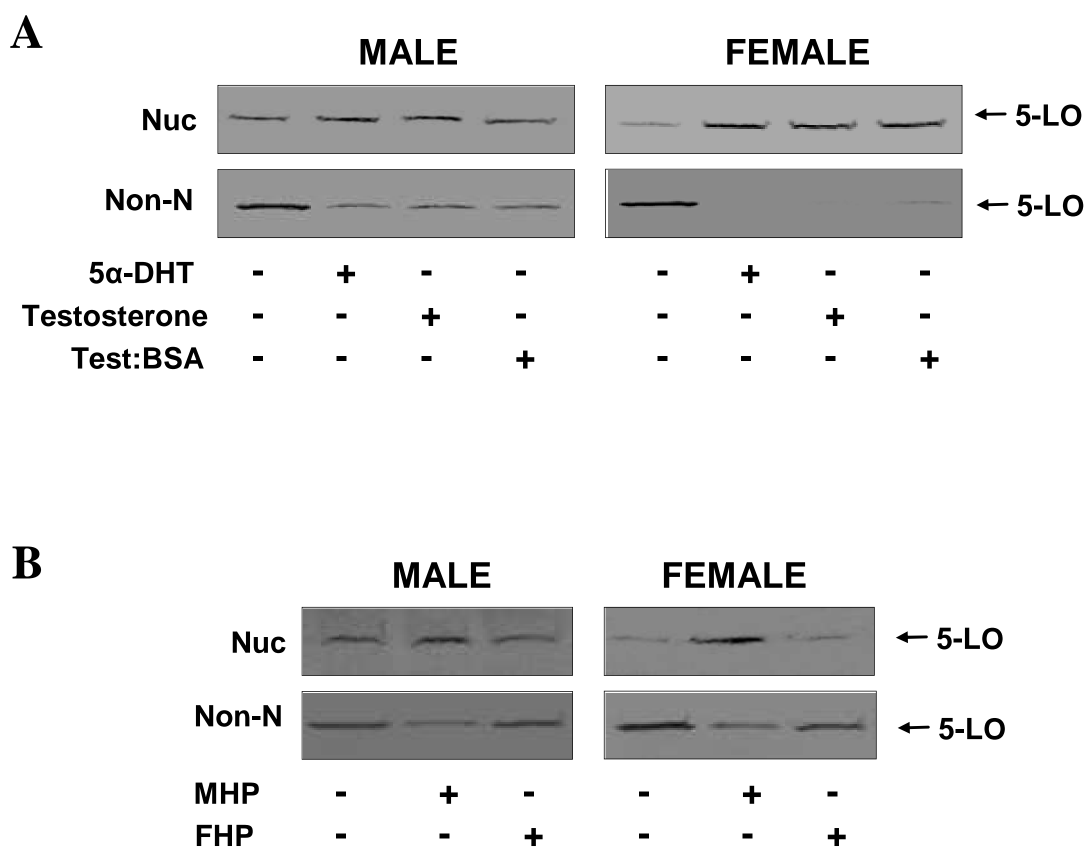


Fig. 31: Testosterone, the non-cell-permeable testosterone:BSA, and human plasma from males induce 5-LO translocation. ***A) Effect of testosterone and Test:BSA on 5-LO subcellular localisation in PMNL from males and females by mild NP40 lysis.*** Human PMNL from males and females were resuspended in PGC buffer (3×10^7/mL) and incubated with vehicle (0.05 % EtOH) or with 5α-DHT (10 nM), testosterone (10 nM) or testosterone:BSA (10 nM for testosterone) for 30 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. ***B) Effect of human plasma on 5-LO subcellular localisation in PMNL from males and females by mild NP40 lysis.*** Human PMNL from males and females were resuspended in male or female human plasma (3×10^7/mL) and incubated for 30 min at 37° C. The reaction was stopped on ice and the cells were washed at 4° C with ice-cold PBS. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; FHP, female human plasma; 5-LO, 5-lipoxygenase; MHP, male human plasma; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Test:BSA, testosterone conjugated with bovine serum albumine; WB, western blotting.

To confirm the biological relevance of these observations, it was evaluated whether also human plasma from males, and therefore the hormones physiologically present in the plasma, could influence 5-LO distribution. As shown in **Fig. 31B**, human plasma from male (MHP) but not from females (FHP), induced nuclear localisation of 5-LO in female PMNL.

4.3.5. Reversibility of 5α-DHT-induced nuclear localisation of 5-LO

Interestingly, 5α-DHT-induced nuclear localisation was found to be reversible when the hormonal stimulus was removed. In fact, after treatment of female PMNL with 5α-DHT and subsequent washing and resting at 37° C, a time-dependent return of the nuclear 5-LO to the non-nuclear fraction was observed **(Fig. 32A)**. Moreover, after such reversal of the hormone effect within 120 minutes, 5-LO was again capable to translocate to the nuclear fraction when Ca^{+2}-ionophore was applied **(Fig. 32B)**.

Similarly, when PMNL isolated from males were incubated at 37° C for 120 minutes (instead of keeping on ice) a prevalent non-nuclear localization of 5-LO was observed and 5-LO translocated to the nuclear fraction after ionophore challenge **(Fig. 32C)**.

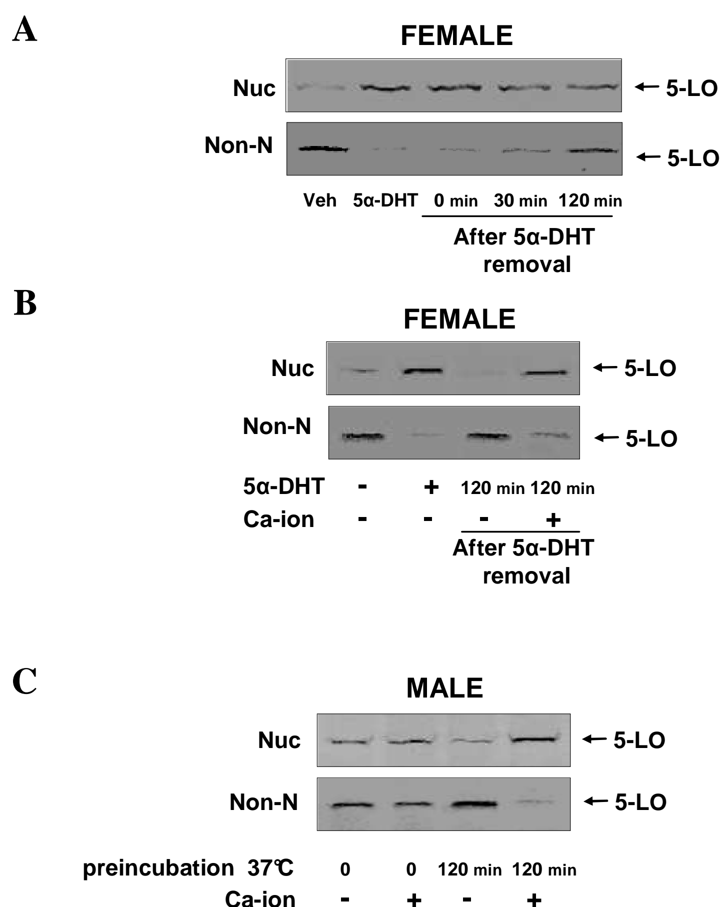


Fig. 32: Reversibility of 5α-DHT-induced nuclear localisation of 5-LO. ***(A) Effect of 5α-DHT removal on 5α-DHT- induced 5-LO nuclear localisation in PMNL from females by mild NP40 lysis.*** Human PMNL from females were resuspended in PGC buffer (3×10^7/mL) and incubated with vehicle (0.05 % EtOH) or with 5α-DHT (10 nM) for 30 min at 37° C. Cells were then centrifuged, washed, and resuspended in PGC buffer (3×10^7/mL) and incubated at 37° C for 0, 30 or 120 min. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. ***(B) Effect of Ca^{+2}-ionophore on 5-LO subcellular localisation in PMNL from females after 5α-DHT removal by mild NP40 lysis.*** Human PMNL from females were treated as in (A). After 5α-DHT removal and incubation at 37° C for 120 min, cells were stimulated with 2.5 µM Ca^{+2}-ionophore for 5 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. ***(C) Effect of resting at 37• C on 5-LO subcellular localisation in PMNL from males by mild NP40 lysis.*** Human PMNL from males were incubated at 37° C for 120 min and stimulated with 2.5 µM Ca^{+2}-ionophore for 5 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; Ca-ion, Ca^{+2}-ionophore A23187; 5-LO, 5-lipoxygenase; min, minutes; Non-N, non-nuclear fraction; Nuc, nuclear fraction; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Veh, vehicle; WB, western blotting.

4.4. ERK1/2 and Ca^{+2} mediate the effect of androgens on 5-LO in human PMNL

4.4.1. Male sex hormones rapidly and moderately activate ERK1/2

To evaluate the molecular machineries involved in the regulation of 5-LO by androgens, the effect of sex steroids on MAPK phosphorylation was evaluated. Addition of 10 nM 5α-DHT rapidly (within 1 to 2.5 minutes) induced phosphorylation of ERK1/2 but not of p38 MAPK in PMNL from both males and females **(Fig. 33A)**. Furthermore, 5α-DHT induced ERK1/2 phosphorylation in a concentration-dependent manner. For female PMNL, 10 pM 5α-DHT were sufficient to induce ERK2 phosphorylation. In PMNL from males, that showed higher basal levels of phosphorylated ERK2 compared to females, maximal stimulation was reached at 1 to 10 nM **(Fig. 33B, upper panel)**, whereas in females 10 nM 5α-DHT were needed **(Fig. 33B, lower panel)**. Notably, the effect of 5α-DHT on ERK2 phosphorylation was only moderate, compared to fMLP (100 nM) **(Fig. 33B)**.

Interestingly, also testosterone and the non-cell-permeable testosterone:BSA (each 10 nM), but not 17β-estradiol (100 nM) or progesterone (10 µM), even when used in combination, increased the levels of phosphorylated ERK2 **(Fig. 34A)**. Note that the phosphorylation state of p38 MAPK was not influenced by sex hormones.

Moreover, male human plasma (MHP), but not female human plasma (FHP), induced ERK1/2 activation in female cells, comparable to the level observed in resting male **(Fig. 34B)**. Hence androgens (or at least male specific factors) present in the human plasma, upregulated ERK1/2 phosphorylation.

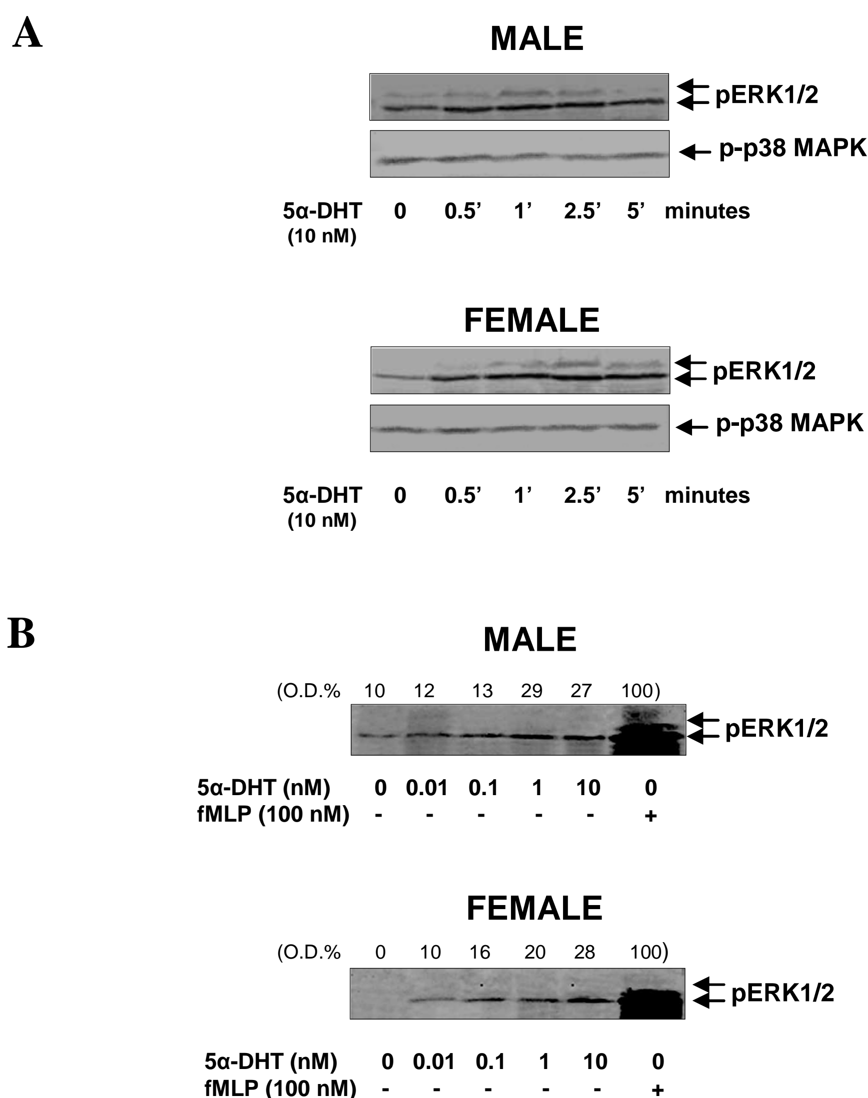


Fig. 33: 5α-DHT rapidly and moderately activates ERK1/2. ***(A) Time course of 5α-DHT-induced MAPK phosphorylation in PMNL from males and females.*** Human PMNL from males and females were resuspended in PGC buffer (1×10^7/100 µL) and incubated with 5α-DHT (10 nM) at 37° C for the indicated periods. Cells were then lysed by addition of 100 µL ice-cold SDS-buffer and pERK1/2 and p-p38 MAPK levels were analysed by WB. ***(B) Concentration-dependency of 5α-DHT-induced ERK1/2 phosphorylation in PMNL from males and females.*** Human PMNL from males and females were resuspended in PGC buffer (1×10^7/100 µL) and incubated with vehicle (0.05 % EtOH), 5α-DHT at the indicated concentrations or fMLP (100 nM) for 1.5 min at 37° C. Cells were then lysed by addition of 100 µL ice-cold SDS-buffer and pERK1/2 levels were analysed by WB using an Ettan DIGE imager system. Densitometry was performed with ImageQuant TL image analysis software. The results shown are representative of 3 similar experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; ERK, extracellular signal-regulated kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; O.D., optical density; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; SDS, sodium dodecyl sulphate; WB, western blotting.

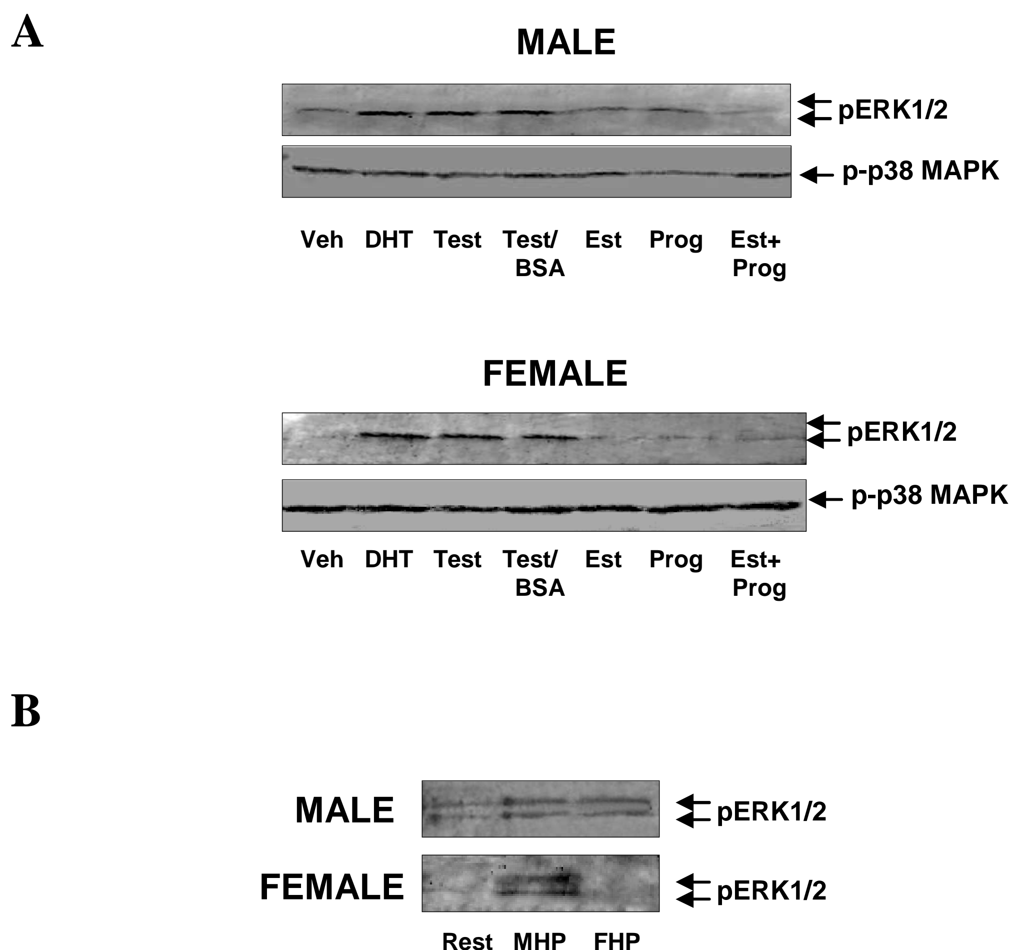


Fig. 34: Testosterone, the non-cell-permeable testosterone:BSA and human plasma from males induce ERK1/2 phosphorylation. ***(A) Effect of sex hormones on MAPK phosphorylation in PMNL from males and females.*** Human PMNL from males and females were resuspended in PGC buffer (1×10^7/100 µL) and incubated with vehicle (0.05 % EtOH), 5α-DHT (10 nM), testosterone (10 nM), testosterone:BSA (10 nM for testosterone), 17β-estradiol (100 nM), progesterone (10 µM), or 17β-estradiol (100 nM) + progesterone (10 µM), at 37° C for 1.5 min. Cells were then lysed by addition of 100 µL ice-cold SDS-buffer and pERK1/2 and p-p38 MAPK levels were analysed by WB. ***(B) Effect of human plasma on ERK1/2 phosphorylation in PMNL from males and females.*** Human PMNL from males and females were resuspended in male or female human plasma (1×10^7/100 µL) and incubated for 5 min at 37° C. The reaction was stopped on ice and the cells were centrifuged at 4° C, washed with ice-cold PBS and lysed by addition of 100 µL ice-cold SDS-buffer. The results shown are representative of 3 similar experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; ERK, extracellular signal-regulated kinase; Est, 17β-estradiol; FHP, female human plasma; MHP, male human plasma; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Prog, progesterone; SDS, sodium dodecyl sulphate; Test, testosterone; Test:BSA, testosterone conjugated with bovine serum albumine; Veh, vehicle; WB, western blotting.

4.4.2. Male sex hormones are involved in the regulation of the intracellular Ca^{+2} homeostasis

To investigate whether also Ca^{+2} is involved in the effect of androgens on 5-LO, PMNL from male and female donors were treated with sex hormones and $[Ca^{+2}]_i$ were determined. In both male and female PMNL, 5α-DHT, testosterone and testosterone:BSA (each 10 nM), rapidly (within 30 seconds) induced Ca^{+2} mobilisation in 4 out of 6 PMNL preparations. Interestingly, the increase in $[Ca^{+2}]_i$ observed in responsive samples was only moderate and slow in comparison to the inflammatory stimulus fMLP (100 nM) **(Fig. 35A)**.

Moreover, 5α-DHT-induced Ca^{+2} mobilisation in responsive PMNL preparations was partially inhibited by preincubation (1 hour, 37° C) with 2 µg/mL PTX, an inhibitor of G_i proteins, suggesting the involvement of a GPCR in the effect of androgens **(Fig. 35B)**.

Furthermore, in both PMNL from male and female donors, 5α-DHT, testosterone and testosterone:BSA (each 10 nM) significantly and moderately increased the area under curve (AUC) regarding elevation of the cytosolic Ca^{+2} concentration for 100 seconds after recalcification of the medium, that is an index of intracellular Ca^{+2} influx **(Fig. 35C)**. This effect was not reversed by the antagonist of the classic intracellular androgen receptor cyproterone acetate (10 µM) (not shown).

No significant effect on $[Ca^{+2}]_i$ was observed by incubating PMNL with the female sex hormones 17β-estradiol (100 nM) or progesterone (10 µM), also not when used in combination (not shown).

Moreover, pre-treatment of PMNL for 30 seconds to 30 minutes with sex-hormones did not significantly modified the Ca^{+2} mobilization induced by subsequent addition of fMLP or ionophore (not shown).

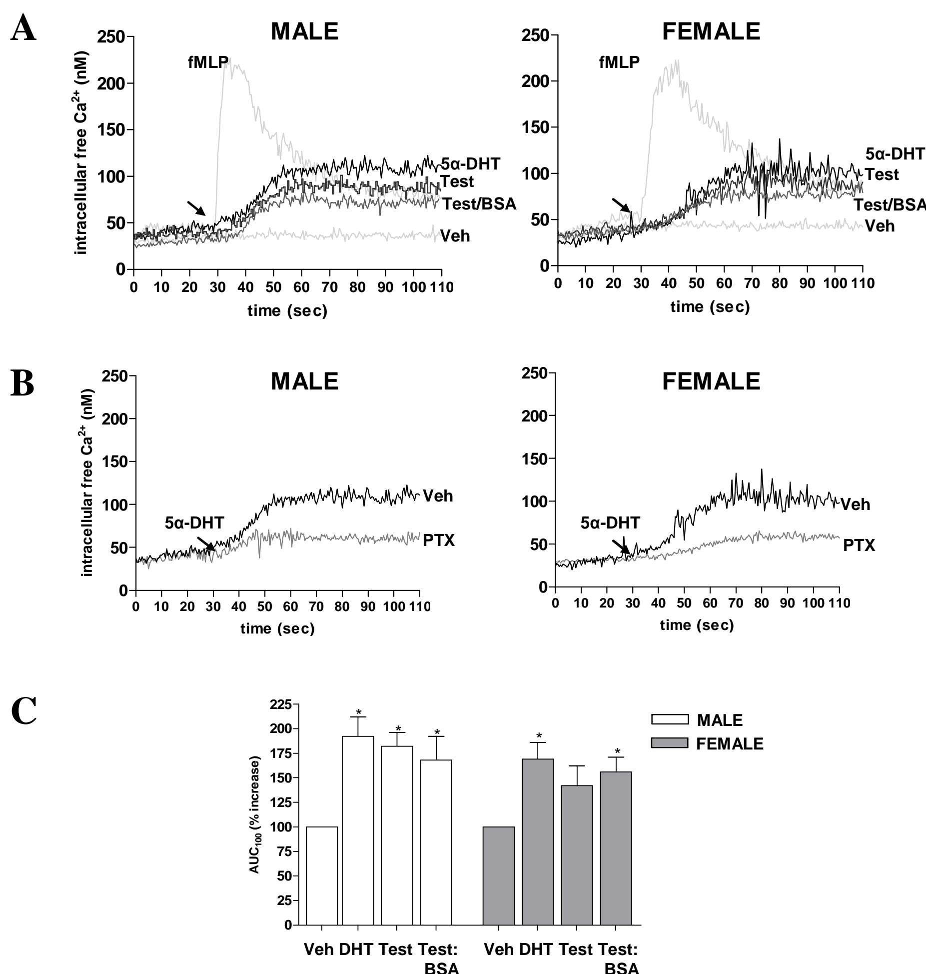


Fig. 35: Male sex hormones regulate intracellular Ca^{+2} levels in PMNL. ***(A) Effect of male sex hormones.*** Human PMNL from males and females (5×10^7 cells/mL) were loaded with 2 μM Fura-2/AM for 30 min at 37° C. After washing, cells were resuspended in PBS (1×10^7 cells/mL) and the fluorescence was measured 2 min after addition of 1 mM $CaCl_2$. Vehicle (0.05 % EtOH), 5α-DHT (10 nM), testosterone (10 nM), testosterone:BSA (10 nM for testosterone), were added 30 sec after the measurement was started. The curves shown are representative of 4 androgen-responsive PMNL preparations out of 6 preparations tested. ***(B) Effect of PTX on 5α-DHT-induced $[Ca^{+2}]_i$.*** Human PMNL from males and females were loaded with Fura-2/AM and resuspended as above, and then they were incubated with vehicle (2 mM Tris, 0.4 mM glycine, 20 mM NaCl, 2 % glycerol, pH 7.5) or 2 μg/mL PTX for 1 h at 37° C. The fluorescence was measured 2 min after the addition of 1 mM $CaCl_2$. 5α-DHT (10 nM) was added 30 sec after the measurement was started. The curves shown are representative of 3 responsive PMNL preparations. ***(C) Calculation of the AUC of $[Ca^{+2}]_i$ in PMNL.*** Human PMNL from males and females were loaded with Fura-2/AM and resuspended as above. Vehicle (0.05 % EtOH), 5α-DHT (10 nM), testosterone (10 nM), testosterone:BSA (10 nM for testosterone), were added and after 200 sec 1 mM $CaCl_2$ was added. The AUC was calculated for 100 sec after recalcification of the medium. Values are given as mean + SE, $n = 3$, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $*p < 0.05$. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; AUC, area under the cytosolic Ca^{+2} concentrations; PG, PBS-glucose buffer; PMNL, polymorphonuclear leukocytes; PTX, pertussis toxin; Test, testosterone; Test:BSA, testosterone conjugated with bovine serum albumine; Veh, vehicle.

4.4.3. ERK1/2 inhibition or Ca^{+2} chelation prevent the effect of 5α-DHT on 5-LO subcellular localisation

To confirm that ERK and Ca^{+2} are the pathways responsible for the androgen-induced 5-LO nuclear localisation, human PMNL were incubated with MAPK inhibitor or Ca^{+2} chelators prior to 5α-DHT treatment and 5-LO distribution was analysed.

Inhibition of ERK1/2 activation, by the MEK1/2 inhibitors U0126 (3 μM) or PD98059 (30 μM), as well as chelation of intracellular (BAPTA/AM, 50 μM) or extracellular (EDTA, 1 mM) Ca^{+2}, prevented 5α-DHT-induced translocation of 5-LO to the nuclear compartment **(Fig. 36A)** in both PMNL from males and females. No significant effect was observed by inhibiting p38 MAPK with SB203580 (10 μM).

Furthermore, the effect of ERK inhibition on 5α-DHT induced-translocation was analysed by IF microscopy. Interestingly, U0126 (3 μM) blocked 5-LO translocation induced by 5α-DHT in PMNL from female donors and, remarkably, re-established the diffuse 5-LO subcellular localisation observed in resting cells **(Fig. 36B)**.

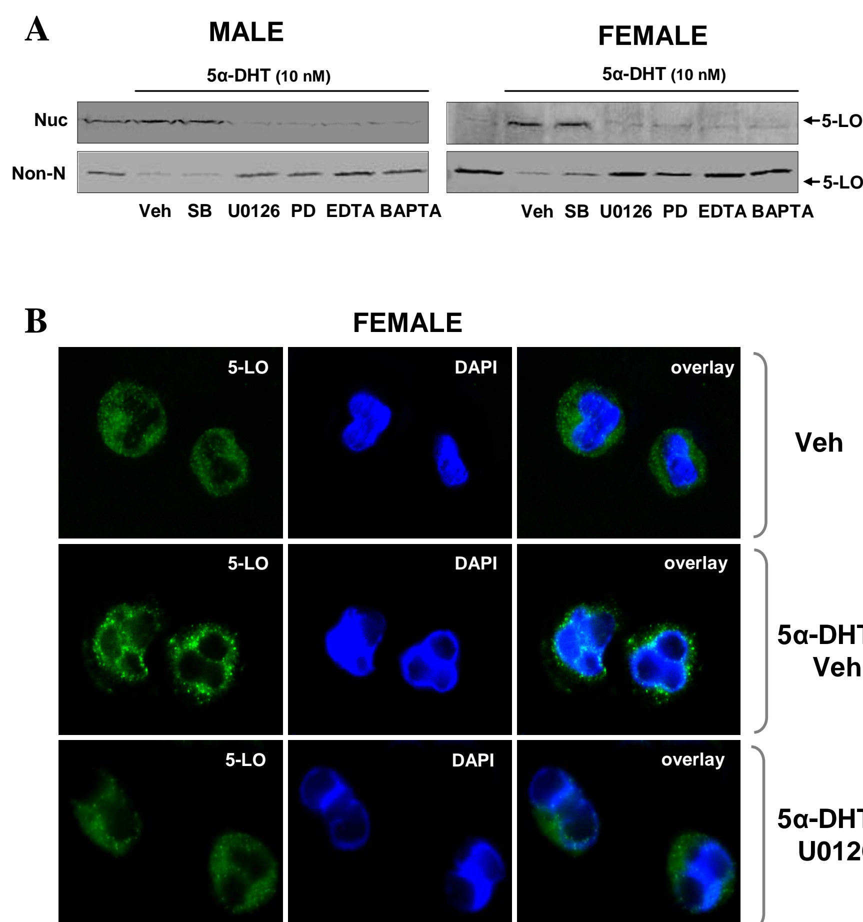


Fig. 36: ERK1/2 inhibition or Ca^{+2} chelation prevent the effect of 5α-DHT on 5-LO subcellular localisation. ***(A) Effect of MAPK inhibitors on 5α-DHT-induced 5-LO nuclear localisation in PMNL: NP40 lysis.*** Human PMNL from males or females were resuspended in PGC buffer (3×10^7/mL) or in PG (EDTA sample) and incubated with vehicle (0.1 % DMSO), with SB203580 (10 µM), U0126 (3 µM) or PD98059 (30 µM), or with BAPTA/AM (50 µM) or EDTA (1 mM) for 15 min at 37° C. 5α-DHT (10 nM) was added for additional 30 min at 37° C. After detergent (0.1 % NP40) lysis and subcellular fractionation, 5-LO was determined in nuclear and non-nuclear fractions by WB. ***(B) Effect of U0126 on 5-LO subcellular localisation in 5α-DHT-treated PMNL from females: IF microscopy.*** Human PMNL from females were resuspended in PGC buffer (1.5×10^6/mL) and incubated with vehicle (0.1 % DMSO), or with U0126 (3 µM) for 15 min, 37° C. 5α-DHT (10 nM) was added for additional 30 min at 37° C. The incubation was stopped on ice for 5 min and the cells were cytospun onto poly-L-lysine-coated glass coverslips. Cells were fixed, permeabilized, blocked, and stained for 5-LO with anti-5-LO serum and Alexa Fluor 488 goat anti-rabbit IgG (green). The DNA was stained with DAPI (blue). The results shown are representative of 3 independent experiments. *Abbreviations:* 5α-DHT, 5α-dihydrotestosterone; BAPTA/AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine-tetraacetic acid; 5-LO, 5-lipoxygenase; MAPK, mitogen-activated protein kinase; PG, PBS-glucose buffer; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Veh, vehicle; WB, western blotting.

4.5. 5α-DHT represses 5-LO activity in human PMNL

4.5.1 5α-DHT downregulates 5-LO activity in ionophore-stimulated PMNL from females

Next, it was evaluated whether the effect of 5α-DHT on 5-LO subcellular localization is connected to changes in the production of 5-LO metabolites.

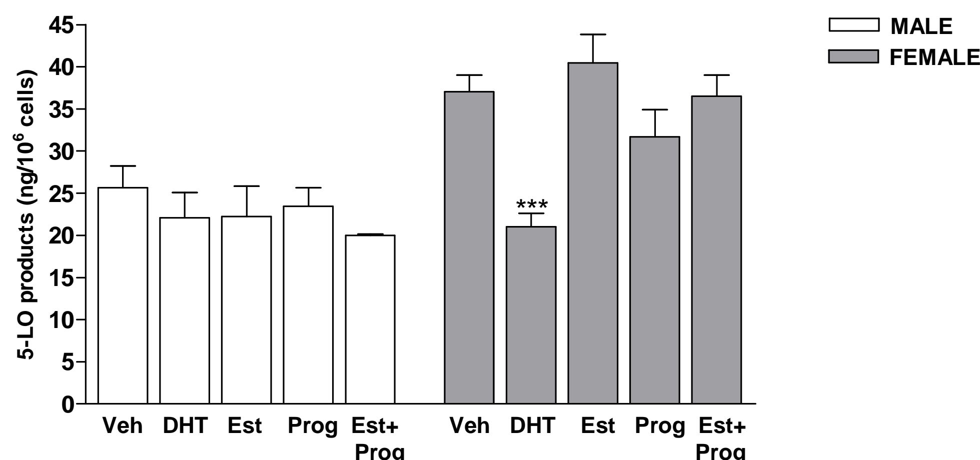
In PMNL isolated from males, preincubation with 10 nM 5α-DHT caused only a slight and not significant decrease of Ca^{+2}-ionophore-induced 5-LO activity **(Fig. 37A)**. In fact, 5-LO product levels were 25.64 ± 2.6 ng/10^6 cells and 22.1 ± 3 ng/10^6 cells ($n = 4$, $p > 0.05$), in vehicle- and 5α-DHT-treated cells, respectively.

On the contrary, in PMNL from females treated with 5α-DHT, 5-LO product synthesis upon Ca^{+2}-ionophore stimulation was about 40 % less than the corresponding control without 5α-DHT **(Fig. 37A)**. 5-LO products formed were 37.04 ± 1.98 ng/10^6 cells and 21.02 ± 1.6 ng/10^6 cells ($n = 4$, $p < 0.001$), in vehicle- and 5α-DHT-treated PMNL, respectively. Of interest, there was no significant difference in ionophore-induced 5-LO activity between vehicle treated males and 5α-DHT treated females (25.64 ± 2.6 ng/10^6 cells cells in vehicle/male and 21.02 ± 1.6 ng/10^6 cells in 5α-DHT/female, $n = 4$, $p > 0.05$), suggesting that 5α-DHT addition to female PMNL abolishes the difference in 5-LO product formation between males and females.

As shown in **(Fig. 37A)**, no significant difference in ionophore-induced 5-LO product synthesis was observed after treatment with the female sex hormones 17β-estradiol (100 nM) and progesterone (10 µM), either used alone or in combination.

Furthermore, 5α-DHT effects were not reversed by the antagonists of the classic intracellular androgen receptor cyproterone acetate (10 µM) and flutamide (10 µM) (not shown).

A



B

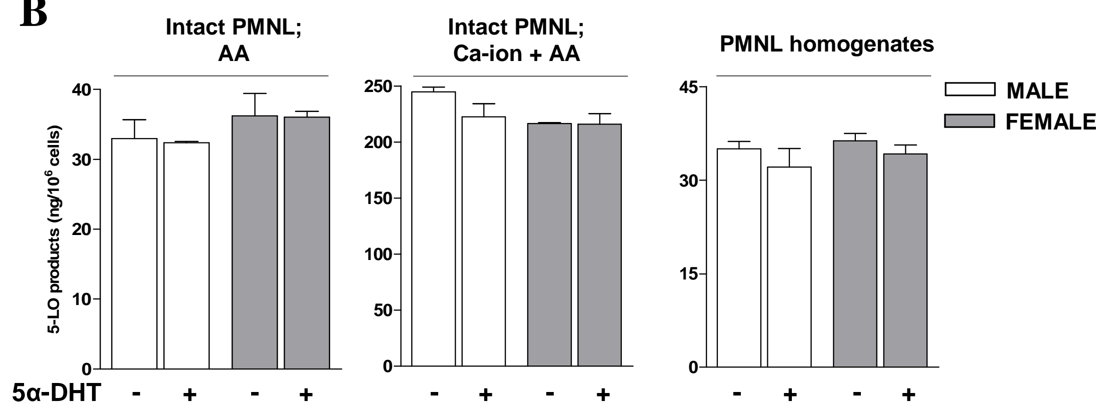


Fig. 37: 5α-DHT downregulates 5-LO activity in ionophore-stimulated intact PMNL from females to the levels in males. ***(A) Effect of sex hormones on 5-LO activity in Ca^{+2}-ionophore-stimulated PMNL.*** Human PMNL from males and females were resuspended in PGC buffer (5×10^6/mL), and incubated with vehicle (0.05 % EtOH), 5α-DHT (10 nM), 17β-estradiol (100 nM), progesterone (10 µM), or 17β-estradiol (100 nM) + progesterone (10 µM), for 30 min at 37° C. Cells were then stimulated with 2.5 µM Ca^{+2}-ionophore for 10 min at 37° C, and 5-LO products formed were determined. Values are given as mean + SE, n = 4, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: ***$p < 0.001$ vs vehicle female. ***(B) Effect of 5α-DHT on 5-LO activity in PMNL stimulated with exogenous AA and in homogenates.*** Human PMNL from males and females were pre-incubated with vehicle (0.05 % EtOH) or 5α-DHT (10 nM) for 30 min at 37° C and then stimulated for 10 min at 37° C with AA (20 µM) (left panel) or Ca^{+2}-ionophore (2.5 µM) + AA (20 µM) (middle panel). The enzyme activity was tested in cell homogenates in presence of Ca^{+2} (1 mM) and AA (20 µM) (right panel). Values are given as mean + SE, n = 3, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $p > 0.05$. *Abbreviations:* AA, arachidonic acid; 5α-DHT, 5α-dihydrotestosterone; Ca-ion, Ca^{+2}-ionophore A23187; Est, 17β-estradiol; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Prog, progesterone; Veh, vehicle.

5α-DHT did not significantly modify 5-LO product synthesis in cells stimulated with exogenous substrate (AA, 20 μM), both in the absence **(Fig. 37B, left panel)** and in presence of 2.5 μM Ca^{+2}-ionophore **(Fig. 37B, central panel)**. Also, in cell homogenates (Ca^{+2} 1mM, AA 20 μM), 5α-DHT did not affect 5-LO product synthesis **(Fig. 37B, right panel)**.

4.5.2. 5α-DHT does not significantly influence ionophore-induced AA release in PMNL from males and females

In order to evaluate whether the inhibitory effect on ionophore-induced 5-LO product synthesis by 5α-DHT was related to a suppression of the release of AA, AA liberation was evaluated in vehicle and 5α-DHT treated PMNL.

5α-DHT did not significantly modify the release of AA induced by 2.5 μM Ca^{+2}-ionophore in both PMNL from males (vehicle: 300 ± 7 %, 5α-DHT: 329 ± 7 % of unstimulated cells; $n = 3$, $p > 0.05$) and females (vehicle: 272 ± 6 %, 5α-DHT: 305 ± 6 % of unstimulated cells; $n = 3$, $p > 0.05$) **(Fig. 38)**. These data show that the reduction in 5-LO product synthesis by 5α-DHT was not related to a suppression of the AA supply.

4.5.3. Pre-treatment of female human blood with 5α-DHT results in a reduction of 5-LO activity in isolated PMNL

Next, it was evaluated whether the treatment of human blood with 5α-DHT affected 5-LO activity in PMNL analysed after subsequent isolation.

To this purpose, blood was incubated with vehicle or with 5α-DHT (used at 100 nM in order to consider the binding to plasma proteins) for 30 minutes at 37° C, and then PMNL were isolated and, therefore, activated to induce 5-LO product synthesis.

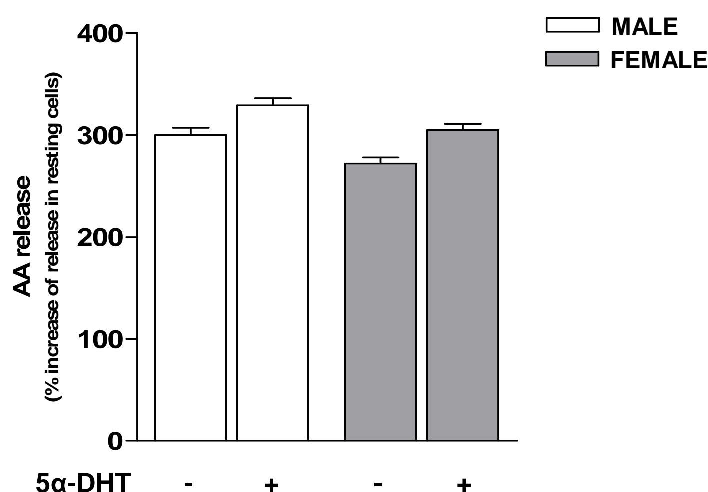


Fig. 38: 5α-DHT does not significantly influence ionophore-induced AA release in PMNL from males and females. Human PMNL from males and females were resuspended in PGC buffer (5×10^7/mL), pre-warmed at 37° C for 10 min and then the 12-LO inhibitor CDC (10 µM) and the 5-LO inhibitor BWA4C (1 µM) were added together with vehicle (0.05 % EtOH) or 5α-DHT (10 nM) for 30 min at 37° C. Cells were then stimulated with 2.5 µM Ca^{+2}-ionophore for 5 min at 37° C. Released AA was then derivatized and analysed by HPLC. Values are given as mean + SE of % increase induced by Ca^{+2}-ionophore compared to unstimulated cells , n = 3, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $p > 0.05$. *Abbreviations:* AA, arachidonic acid; Ada, adenosine deaminase; Ca-ion, Ca^{+2}-ionophore A23187; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes.

Interestingly and similarly to the data shown in **4.5.1.**, no significant difference was observed in Ca^{+2}-ionophore-stimulated PMNL, isolated from vehicle- and 5α-DHT-treated male blood (vehicle: 52.1 ± 4 ng/10^6 cells; 5α-DHT: 42.16 ± 2.6 ng/10^6 cells; $n = 3$, $p > 0.05$; **Fig. 39A**).

On the contrary, in PMNL isolated from 5α-DHT-treated female buffy coats, 5-LO activity was 40 % lower than in the corresponding control (vehicle: 69 ± 3.2 ng/10^6 cells; 5α-DHT: 39.57 ± 1.44 ng/10^6 cells; $n = 3$, $p < 0.001$) **(Fig. 39A)** and, thus, similar to the levels observed in PMNL from males.

These results demonstrate that 5α-DHT is active also in presence of a complex system like whole blood and that this effect in the blood is retained by the cells during the isolation procedure at 4° C.

Notably, such downregulation of ionophore-induced 5-LO product synthesis by 5α-DHT was not observed when exogenous AA was added to the cells **(Fig. 39B, left panel)** or when 5-LO activity was evaluated in PMNL homogenates **(Fig. 39B, right panel)**. Hence, 5α-DHT treatment of the blood did not modify the amount of catalytically active 5-LO.

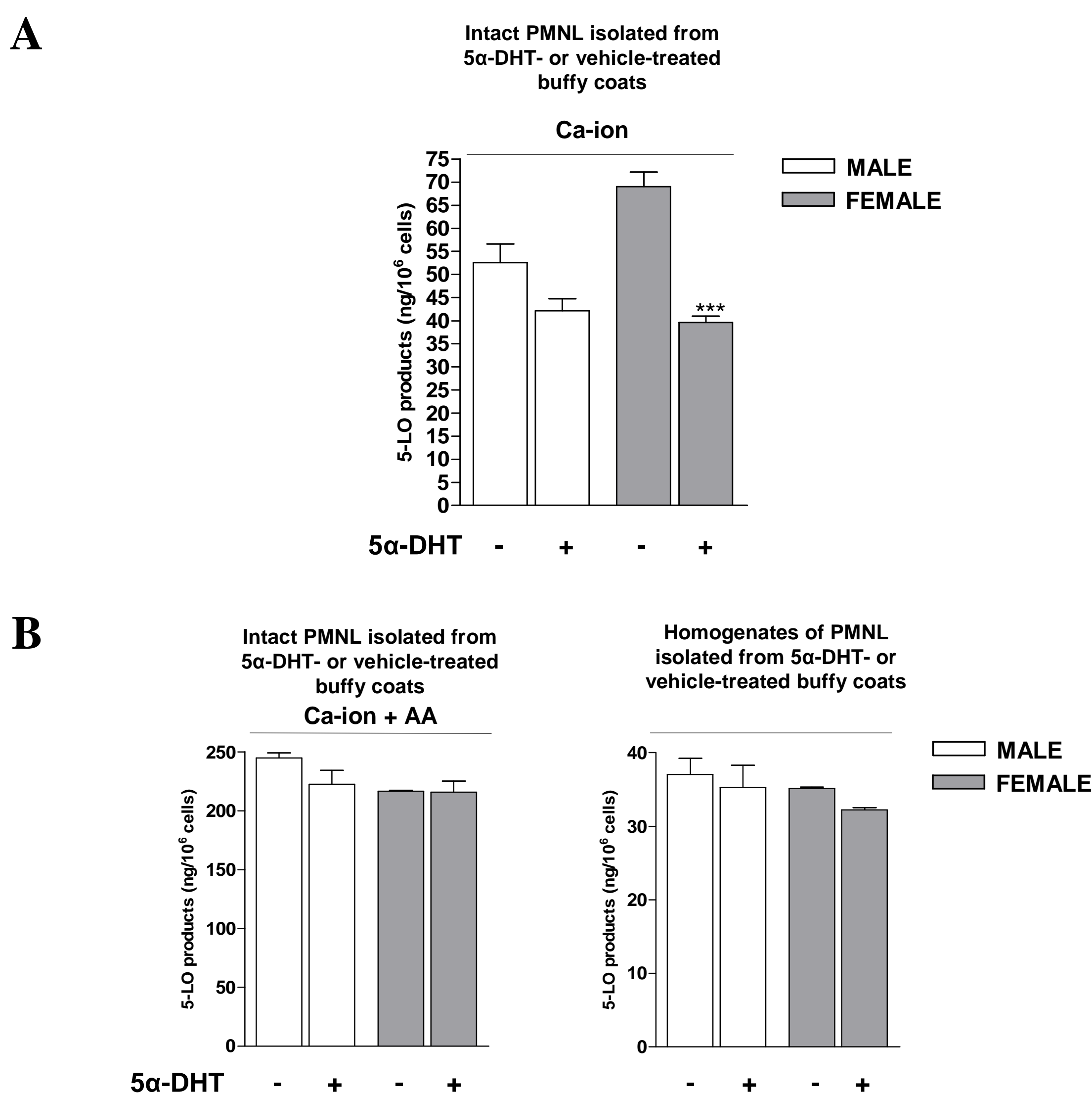


Fig. 39: Treatment of human blood with 5α-DHT results in a reduction of 5-LO activity in subsequently isolated PMNL from females. ***(A) Effect of 5α-DHT treatment on 5-LO activity in isolated PMNL stimulated with Ca^{+2}-ionophore.*** Buffy coat blood from male and female donors was incubated for 30 min at 37° C with 5α-DHT (100 nM). PMNL were then isolated at 4° C, resuspended in PGC buffer (5×10^6/mL), and stimulated with 2.5 µM Ca^{+2}-ionophore for 10 min at 37° C, and 5-LO products formed were determined. Values are given as mean + SE, $n = 3$, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $^{***}p < 0.001$ vs vehicle female. ***(B) Effect of 5α-DHT treatment on 5-LO activity in isolated PMNL stimulated with Ca^{+2}-ionophore plus exogenous AA and in homogenates.*** Human PMNL were isolated from vehicle or 5α-DHT-treated blood from buffy coats as described in (A), then stimulated for 10 min at 37° C with Ca^{+2}-ionophore (2.5 µM) + AA (20 µM) (left panel). The enzymatic activity was analysed in cell homogenates in presence of Ca^{+2} (1 mM) and AA (20 µM) (right panel). Values are given as mean + SE, $n = 3$, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $p > 0.05$. *Abbreviations:* AA, arachidonic acid; 5α-DHT, 5α-dihydrotestosterone; Ca-ion, Ca^{+2}-ionophore A23187; PGC, PBS-glucose-Ca^{+2} buffer; PMNL, polymorphonuclear leukocytes; Veh, vehicle.

4.6. Gender-dependent regulation of 5-LO activity in human whole blood

4.6.1. 5-LO activity from endogenous AA is lower in human whole blood from males than from females: the role of 5α-DHT

To evaluate the physiological relevance of the results obtained in isolated cells, a whole blood assay was performed. Stimulation of blood with Ca^{+2}-ionophore (30 μM) resulted in 5-LO product synthesis in males being 54 % lower than in females **(Fig. 40A)**. Thus, 5-LO product levels were 170.9 ± 2.9 ng/ml plasma in male blood and 372 ± 24 ng/ml plasma in female blood ($n = 6$, $p < 0.001$).

This difference was abolished when female blood was treated with 5α-DHT. Incubation of female blood with 5α-DHT (100 nM, 30 min, 37° C) prior to ionophore addition resulted in a 50 % decrease in 5-LO product synthesis (vehicle: 372 ± 24 ng/mL plasma; 5α-DHT: 181.8 ± 36 ng/mL plasma, $n = 6$, $p < 0.001$), whereas no significant difference was observed in the blood from males (vehicle: 170.9 ± 2.9 ng/ml plasma; 5α-DHT: 169.2 ± 22 ng/mL plasma, $n = 6$, $p > 0.05$) **(Fig. 40A)**. Notably, ionophore-induced 5-LO activity in 5α-DHT-treated female whole blood was not significantly different from that observed in non-treated male blood (5α-DHT/female: 181.8 ± 36 ng/mL plasma, vehicle/male: 170.9 ± 2.9 ng/mL plasma; $n = 6$, $p > 0.05$).

Of interest and in accordance with the data obtained from isolated cells, no difference was observed between the genders when exogenous substrate (AA, 100 μM) was added together with ionophore (5-LO products: 591.2 ± 22 ng/ml plasma in male blood and 542.7 ± 52 ng/ml plasma in female blood; $n = 3$, $p > 0.05$) **(Fig. 40B)**.

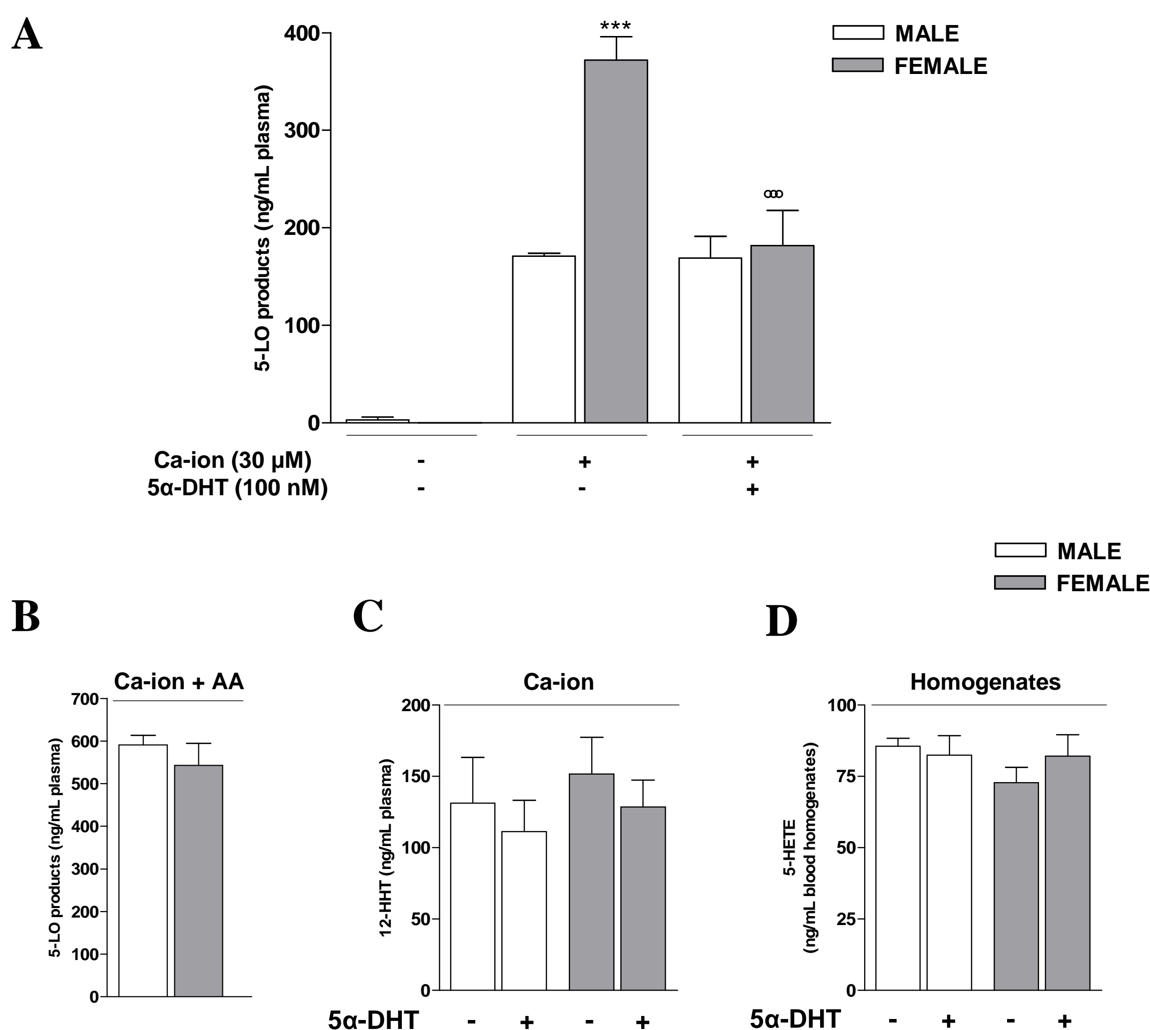


Fig. 40: 5-LO activity from endogenous AA is lower in human whole blood from males than from females: the role of 5α-DHT. ***(A-B) 5-LO activity in untreated and 5α-DHT-treated whole blood from males and females.*** Fresh blood was collected in heparinized tubes from male and female donors. The blood was divided in 2 mL aliquots, preincubated with vehicle (0.05 % EtOH) or 5α-DHT (100 nM) for 30 min at 37° C and stimulated with either Ca^{+2}-ionophore 30 µM (A) or with Ca^{+2}-ionophore 30 µM + AA 100 µM (B), for 10 min at 37° C. Formed 5-LO metabolites were extracted and analyzed by HPLC. Values are given as mean + SE, $n = 6$ (A) and 3 (B), duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: ***$p < 0.001$ vs corresponding male; °°° $p < 0.001$ vs Ca-ion female. ***(C) 12-HHT production in untreated and 5α-DHT-treated whole blood from males and females.*** Samples in A were analysed for the production of the COX metabolite 12-HHT. ***(D) 5-LO activity in homogenates of untreated and 5α-DHT-treated whole blood from males and females***. Fresh blood was incubated as reported in (A-B) and then homogenized by sonication. ATP (1 mM), $CaCl_2$ (1 mM, final concentration) and AA (100 µM) were added and the samples were extracted and analyzed by HPLC. Values are given as mean + SE, $n = 5$, duplicates; data were analysed by ANOVA followed by Tukey-HSD post-hoc test: $p > 0.05$. *Abbreviations:* AA, arachidonic acid; 5α-DHT, 5α-dihydrotestosterone; Ca-ion, Ca^{+2}-ionophore A23187; 5-HETE, hydroxy-6-trans-8,11,14-cis-eicosatetranoic acid; 12-HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid.

Moreover, no difference in the formation of the COX-1 product 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) was observed between the genders or after 5α-DHT treatment in blood treated with ionophore (vehicle/male: 131.34 ± 32 ng/ml plasma; 5α-DHT/male: 111.2 ± 22 ng/ml plasma; vehicle/female: 151.58 ± 26 ng/ml plasma; 5α-DHT/female: 128.6 ± 18.8 ng/ml plasma; $n = 6$, $p > 0.05$) **(Fig. 40C)**. Also, 5-LO activity in whole blood homogenates **(Fig. 40D)**, as evaluated by quantifying 5-HETE production, was not different in males and females (vehicle/male: 85.6 ± 2.76 ng/ml plasma; 5α-DHT/male: 82.5 ± 6.8 ng/ml plasma; vehicle/female: 72.74 ± 5.5 ng/ml plasma; 5α-DHT/female: 82.04 ± 7.5 ng/ml plasma; $n = 5$, $p > 0.05$). These data indicate that the gender-dependent difference might be specific for 5-LO since it did not concern COX. Moreover, the gender-dependent regulation of 5-LO was not related to different amount of catalytically active 5-LO in blood samples from male and female donors, and was operative only in the intact cell environment.

5. DISCUSSION

5-LO catalyzes the first two steps in the conversion of AA to LTs, a group of pro-inflammatory lipids that have been identified as mediators of a variety of inflammatory and allergic reactions, including asthma, rheumatoid arthritis, psoriasis and allergic rhinitis. Moreover, the 5-LO pathway has also been associated with atherosclerosis, osteoporosis and certain types of cancer (e.g., neuroblastoma, prostate and pancreas cancer) (Werz and Steinhilber, 2005). Notably, several of these 5-LO-related diseases display marked higher prevalence in women than in men and a protective effect of androgens in inflammatory conditions has often been reported (see **1.2.9.**). Thus, knowledge of the biological and biochemical processes involved in these gender-related issues would improve the understanding of the pathophysiology of these diseases and could have potential implications for the pharmacological therapy.

In this thesis it is shown for the first time that the subcellular compartmentalization of 5-LO and the efficacy in LT biosynthesis are highly dependent on the gender in humans, due to a differential activation status of ERK. These striking gender-specific differencies are related to variant androgen levels of males and females.

As experimental model, isolated human PMNL have been chosen, since PMNL are characteristic cells present in the acute inflammatory infiltrate and, in line with the function of LTs as mediators of inflammation and immune reactions, the major cell type generating 5-LO products in humans. In order to obtain PMNL for these experiments, a dextran-based method combined with a differential density centrifugation on Nycoprep cushions has been used for

the isolation of PMNL from blood of male and female donors. It must be pointed out that all steps were carried out at 4° C. In fact, this method has been described to avoid priming of cells (Jethwaney et al., 2007) induced, for example, by other preparations (like gelatine-based methods) (Stie and Jesaitis, 2007), and has been used to avoid temperature-dependent functional changes of the cells (Borregaard et al., 1987; Stie and Jesaitis, 2007).

Surprisingly, in isolated human PMNL, the amounts of 5-LO product generated from endogenous AA were 40 % to 90 % lower in males than in females, depending on the stimulus used (ionophore or fMLP, respectively). This difference could be related to varying levels of AA as substrate for 5-LO. However, analysis of the release of AA from membrane phospholipids, showed no differential substrate availability. Also the expression of 5-LO was not different between male and female PMNL. Conclusively, the unequal generation of 5-LO products is apparently related to other regulatory mechanisms concerning 5-LO activity (see ***1.2.6.2.***), strictly linked to the cellular environment. Moreover, when 5-LO product synthesis was evaluated in cell free assays, after disrupting of the cellular context by homogenisation, no difference was observed between the genders.

The results obtained in cell free assays also clearly indicated that the amount of catalytically active 5-LO is essentially equal between the genders. This was confirmed when AA was exogenously supplemented to PMNL. In absence of exogenous AA, 5-LO has to redistribute to a locale (the nuclear membrane) in order to receive liberated AA, provided by the action of $cPLA_2$ on membrane phospholipids (Werz, 2002). In contrast, AA from exogenous sources might be available for metabolism also by cytosolic 5-LO (Sala et al., 1999; Werz, 2002; Luo et al., 2003). Therefore, although the synthesis of 5-LO products from exogenous AA is a valid index and a well-accepted tool for the evaluation of the catalytic activity of 5-LO, it does not allow to discriminate the subcellular locus of metabolism. Upon AA supplementation, 5-

LO product synthesis was not significantly different in intact PMNL from males and females, confirming that the difference between the genders was not due to an unequal 5-LO catalytic activity itself. Instead an unequal capability of 5-LO to access the endogenous substrate, or unequal subcellular compartmentalisation may confer the different capacities of male and female PMNL to generate 5-LO products.

5-LO product synthesis in the cell strictly depends on the subcellular localisation of the enzyme (see ***1.2.6.2.***) (Werz et al., 2001b; Luo et al., 2003). In a generally accepted model for human PMNL, 5-LO occurs as a soluble enzyme in the cytosol of resting cells and translocates to the nuclear membrane or to the endoplasmic reticulum surrounding the nucleus after activation (for review see (Peters-Golden and Brock, 2003)). In association with the nucleus, 5-LO then interacts with FLAP and gets access to AA released by $cPLA_2$. It must be observed that in all the studies addressing the regulation of 5-LO subcellular localisation, the sex of the PMNL donor was never taken into account and inconsistent patterns of 5-LO translocation were reported when PMNL from several donors, whose gender was not reported, were analyzed (Boden et al., 2000). Thus far, there is now concrete explanation for these inconsistencies.

The results of this thesis clearly demonstrates that the long appreciated model of 5-LO subcellular localisation and translocation in human PMNL may apply only to cells from females, but not to those from males. To study the subcellular localisation of 5-LO, different techniques were utilized, that is, (I) mild-detergent (0.1 % NP40) lysis yielding in a nuclear and a non-nuclear (cytosolic) fraction, (II) sonication and separation of a soluble and a particulate (membraneous) fraction, and (III) IF microscopy. Indeed, by using crude

subcellular fractionation methodologies, in female PMNL 5-LO was in the cytosol of resting cells and was detected at the nuclear membrane upon stimulation with ionophore or fMLP, as generally described by others before (Brock et al., 1997; Surette et al., 1998; Werz et al., 2001b). However, resting PMNL from male donors showed a pattern of 5-LO distribution similar to partially activated cells (Brock et al., 1997), namely both nuclear/non nuclear (0.1 % NP40 fractionation) and membrane-associated/soluble (P100/S100 fractionation) association. Of interest, in contrast to female PMNL, no significant change in 5-LO localization in PMNL from males was observed after ionophore or fMLP activation. Upon agonist challenge of PMNL from male donors, 5-LO was still found in both the nuclear and non-nuclear fractions, and in both the membrane-associated and soluble fractions. It has been described that prolonged activation of leukocytes might result in a reduced capability of 5-LO to translocate to the nuclear membrane after a second stimulation (Brock et al., 1998). As consequence of the persistent nuclear association induced by the first stimulus, a reduced ability of generating 5-LO products on re-stimulation has been observed. Notably, the first stimulus does not have necessarily to induce 5-LO product synthesis, since only nuclear persistent association but not 5-LO action is required for the inactivation (Brock et al., 1998). In the light of these observations, the pattern of 5-LO subcellular localisation observed in PMNL from male donors might then be due to constitutive stimulation *in vivo*, resulting in a persistent nuclear localisation (but not necessarily in 5-LO product synthesis). As a consequence, 5-LO would be desensitized in both respects, translocation and product synthesis, when a second and inflammatory stimulus would be applied. It must be pointed out that besides being pro-inflammatory, LTB_4 (the main LT formed in PMNL) is an important agent in the first line of the immune defence of the body. Thus, our findings may have a profound physiological importance: the mechanisms governing the subcellular localisation in male PMNL could

represent a regulatory process culminating in a well-balanced formation of 5-LO metabolites upon exposure to an inflammatory stimulus.

Moreover, as analysed by IF microscopy, 5-LO was mainly located in the perinuclear region of male resting PMNL, showing a prominent colocalisation with the endoplasmic reticulum marker 1D3 (Vaux et al., 1990). In male PMNL only a partial increase of the perinuclear staining was observed after agonist activation. On the contrary, a homogeneously diffuse staining for 5-LO was observed in the cytosol of resting PMNL from female donors, and this 5-LO was detected at the nuclear membrane after activation. Although a partial overlay was also observed with the endoplasmic reticulum marker 1D3, probably because of the close vicinity of the outer nuclear envelope with the rough endoplasmic reticulum (Spector, 2001), activation of PMNL from females resulted in a clear translocation of 5-LO to the nuclear membrane, where 5-LO colocalised with the nuclear lamina in a clustered pattern. Interestingly, the clustering of 5-LO, $cPLA_2$, and the nuclear membrane integral FLAP and LTC_4-synthase has been suggested as a metabolome for LT synthesis (Murphy and Gijon, 2007). Data obtained from the recent resolution of the crystal structure of both FLAP (Ferguson et al., 2007) and LTC_4-synthase (Ago et al., 2007) seemingly confirm this hypothesis. Therefore, the clustered localisation of 5-LO in activated female PMNL may reflect this metabolome implying that the nuclear envelope, but not the endoplasmic reticulum, is the locus where 5-LO is eventually redistributed.

It has been hypothesized that the nuclear envelope could be a better site for 5-LO to access released AA than the perinuclear region (Luo et al., 2003). Since 5-LO localizes at the nuclear membrane in activated female PMNL but in the perinuclear region in males, the difference in the synthesis of 5-LO products could be also related to a different substrate accessibility.

Nuclear translocation of 5-LO has been described to be induced by an increase of $[Ca^{+2}]_i$ and/or by phosphorylation by protein kinases (MK-2/3, on Ser271 and ERK, on Ser663) (see ***1.2.6.2.***). Interestingly, depletion of extra- or intra- cellular Ca^{+2} by the chelators EDTA and BAPTA/AM, respectively, as well as inhibition of ERK1/2 pathway, induced a cytosolic distribution of 5-LO in male PMNL, yielding a "female type" 5-LO pattern. On the contrary, the inhibition of the p38 MAPK pathway did not modify 5-LO subcellular distribution. This implies that both Ca^{+2} and ERK1/2 are required for 5-LO nuclear localization in resting male cells. Although Ca^{+2} was required for membrane binding (cfr. also ***1.2.6.1***), no difference in the $[Ca^{+2}]_i$ was observed between male and female PMNL indicating that the differential regulation of 5-LO between the genders is not due to variant $[Ca^{+2}]_i$. However, the experimental setting for the measurement of the $[Ca^{+2}]_i$ requires the incubation of PMNL for 30 minutes at 37° C in order to load the cells with the dye (see **3.11.**). During this period the gender-specific Ca^{+2} physiology of PMNL may change, so that the *in vivo* condition (sex hormone effects) could partially be lost.

One of the most intriguing findings in this thesis is the observation that ERK1/2 is constitutively activated in human PMNL from males as compared to females which has not been discovered before. Activation of ERK1/2 has been analysed by its dual phosphorylation at Thr202 and Tyr204, which is recognized by a phospho-specific antibody. Thus, the levels of phosphorylated ERK1/2 were significantly higher in PMNL from male donors as compared to females. This variance is not due to unequal amounts of total ERK1/2 protein expression.

Notably, the higher levels of phosphorylated ERK1/2 in PMNL from males were accompanied by an enhanced translocation of ERK1/2 into the nucleus. Moreover, this was accompanied also by an increased kinase activity visualized by higher levels of phosphorylated Elk-1, a well-recognized ERK1/2 substrate (Davis et al., 2000). The finding that male PMNL have a

higher ERK activation status could have several major implications for PMNL functions since ERK1/2 regulate many processes in human PMNL, including expression of proteins, PMNL activation, degranulation, apoptosis (Lu et al., 1993; Burg and Pillinger, 2001). In line with the role of PMNL as critical effector cells in humoral and innate immunity, they contribute to the control of phagocytosis and bacterial killing (Downey et al., 1998; Hii et al., 1999; Raeder et al., 1999; Mansfield et al., 2000; Zhang et al., 2003). The increased activation status of ERK1/2 coincided with the nuclear localisation of 5-LO in PMNL from males. Also pharmacological ERK1/2 inhibition caused depletion of 5-LO from the nuclear loci and 5-LO was redistributed into the cytosol. Hence, the ERK1/2 pathway is likely to be a determinant in the gender-dependent 5-LO regulation. Such speculations are in accordance with the literature (Werz et al., 2001b; Luo et al., 2003), indicating that phosphorylation events are important regulators of 5-LO localisation which as a consequence determine the cellular formation of 5-LO products, rather than modulating 5-LO catalytic activity itself. 5-LO is phosphorylated by ERK1/2 at Ser663 and by the p38 MAPK-regulated MK-2 at Ser271 (Werz et al., 2002a; Werz et al., 2000). If phosphorylation of Ser663 by ERK1/2 mediates the gender-effect remains to be determined.

Ca^{+2} and ERK1/2 are also recognized as activators of $cPLA_2$ (see ***1.2.6.2.***). Based on the fact that ERK1/2 is more active in male PMNL, one may expect that $cPLA_2$ is also regulated in a gender-specific manner. However, no differencies between the genders were observed in $cPLA_2$ subcellular localization or in the release of AA from intact PMNL. How can this be explained? For activation of $cPLA_2$ by phosphorylation the enzyme requires phosphorylation by both ERK1/2 and p38 MAPK at different serine residues (Hazan-Halevy and Levy, 2000; Hazan-Halevy et al., 2000). Furthermore a significant higher threshold of $[Ca^{+2}]_i$ is needed for activation of $cPLA_2$ (350 to 400 nM) than for activation of 5-LO (200 nM) (Fischer et al.,

2005; Schatz-Munding et al., 1991). This observation is in accordance with previous reports (Pruzanski et al., 1988), where no difference in serum PLA_2 activity among subjects of different sex was observed. Moreover, although a difference in PLA_2 activity has been observed in plasma extracts (acid extraction and heating) of males and females (Kuslys et al., 1996), no difference between the genders were found in the same study in PLA_2 activity in crude plasma, crude serum, neutrophils or lymphocytes. It should be noted that PLA_2 activity in plasma extract is an index for group II secretory PLA_2 ($sPLA_2$), but not for $cPLA_2$ activity (Vishwanath et al., 1996), whereas the activity in crude plasma or serum is an index for cellular PLA_2 enzymes, including $cPLA_2$. Therefore, our and previous results clearly indicate that $cPLA_2$ activity and the subsequent AA release is not regulated in a sex-dimorphic manner. Nevertheless, in light of the differential ERK activity between the genders, a sex-related regulation could affect also other enzymes or proteins involved in the modulation of the inflammatory response (e.g. $sPLA_2$). This is supported by the observation of a sex-influence on the activity of antioxidant enzymes of PMNL in healthy subjects (Saraymen et al., 2003). Similarly, a higher activity of myeloperoxidase has been demonstrated in neutrophils from females than from males (Kabutomori et al., 1999).

Interestingly, a gender-dependent regulation was observed also for CLP. In fact, in human PMNL, CLP showed a similar pattern of subcellular distribution of 5-LO. CLP is an F-actin binding protein that was previously found to bind also 5-LO (Provost et al., 2001) and it has been established as a relevant factor for 5-LO product formation (Rakonjac et al., 2006) (see ***1.2.6.1.***). It has been hypothesized that, in intact cells, CLP may form a stable complex with 5-LO and function as a chaperone and/or a scaffold to support 5-LO activity (Rakonjac et al., 2006). Notably, our data showing a similar pattern of distribution between 5-LO and CLP in both PMNL from males and females seem to confirm the idea that CLP is a scaffold protein

for 5-LO in the cells. It must be mentioned that the gender-related difference in 5-LO subcellular localisation involving ERK1/2 may also function via CLP in an indirect manner, which means that CLP is primarily affected and acts as transport vehicle for 5-LO.

In contrast to CLP, no difference was observed between the genders for FLAP expression and subcellular localisation. In accordance with the literature (cfr. ***1.2.6.2.***), FLAP was found only as a nuclear membrane-associated protein of both male and female cells.

To identify the molecular basis responsible for the difference in 5-LO cellular biology between the genders, the effects of sex-hormones on 5-LO subcellular localisation, activity and on the mechanisms involved in 5-LO regulation (i.e. ERK1/2, Ca^{2+}) were analysed. Treatment of female PMNL with physiologically relevant concentrations of androgens (testosterone and its active metabolite 5α-DHT; ≤ 10 nM) resulted in the translocation of 5-LO to the nuclear membrane. This translocation was not reversed by the FLAP inhibitor MK-886, showing that the association with the nuclear membrane is probably not accompanied with a binding to FLAP and, therefore, the apparatus yielding to 5-LO product synthesis in the cells might be not recruited under these conditions. Notably, MK-886 also did not modify 5-LO association to the nuclear fraction in unstimulated male cells and, therefore, 5-LO may not bind to FLAP under these conditions.

In contrast, no effect on 5-LO subcellular localisation was observed after treatment with female sex hormones (17β-estradiol and progesterone; 100 nM and 10 µM, respectively). Of interest, under the IF microscope 5α-DHT-treated PMNL from females showed a granular 5-LO staining in the perinuclear region, similar to that observed in male resting cells. The androgen-induced “male-type” 5-LO pattern in these female PMNL suggests that the gender-related difference might be due the unequal exposition to androgen levels in the blood *in vivo*.

From this point of view, elevated levels of testosterone and its active metabolites in the plasma of males could influence PMNL to induce a persistent association of 5-LO with perinuclear structures, thereby desensitising the enzyme to subsequent inflammatory stimuli. The hypothesis of an interaction between plasma androgens and PMNL was confirmed by the finding that also male human plasma, but not female human plasma, induced an increase of 5-LO in the nuclear fraction. Interestingly, a further increase in the content of 5-LO in the nuclear fraction and in the 5-LO staining in the structure surrounding the nucleus was also observed in 5α-DHT-treated male PMNL. This effect could be most probably ascribed to an additive effect of androgens or to a partial return of the nuclear 5-LO to the cytosol, in PMNL from male donors, during the isolation procedures from the blood where PMNL are separated from the androgens.

Nuclear localization induced by 5α-DHT was found to be reversible which is in favour of the speculations made above. Thus, in PMNL from female donors, the androgen effect was reversed within 2 hours upon removal. At the same time that the androgen effect was reversed, 5-LO recovered the property to translocate from the cytosol to the nuclear membrane after ionophore stimulation. Interestingly, in PMNL from male donors, 5-LO redistributed to the cytosol when PMNL were freshly isolated and incubated at 37° C for 2 hours. Also in this case, 5-LO was able to translocate to the nucleus after Ca^{+2}-ionophore stimulation. Therefore, the nuclear localisation of 5-LO in resting male PMNL shares the same characteristics as 5α-DHT-induced nuclear localisation of 5-LO in PMNL from females. It is interesting that in RBL cells and alveolar macrophages prolonged stimulations with strong stimuli (e.g. Ca^{+2}-ionophore) resulted in an irreversible association of 5-LO with the nuclear envelope (Brock et al., 1998). Therefore, the reversibility of the androgen-induced association of 5-LO with the

nuclear membrane may reflect a role as physiologic and subtle negative modulator of 5-LO function rather than an 5-LO-activating stimulus.

5α-DHT-induced nuclear localisation occurred in a concentration-dependent manner, starting already at 0.01 nM 5α-DHT in female PMNL and peaking at 10 nM. The concentration-dependency and the high effectiveness even at picomolar concentrations strongly suggests that 5α-DHT might act via a high-affinity receptor. Sex hormones have been traditionally considered to bind to and act via the so-called "classic sex-steroid receptors", a class of nuclear steroid receptors that functions as a ligand-activated transcription factors to mediate genomic effects of steroids (see ***1.3.2.1.***). However, sex hormones have also been reported to induce non-genomic effects through other and still uncharacterised plasma membrane-bound receptors (see ***1.3.2.2.***). These non-genomic sex-steroid effects are typically characterized by:

1. their rapid onset of action (within seconds to minutes);
2. insensivity to the antagonists of the classic sex-steroid receptors;
3. the property to be stimulated also by hormones bound to high molecular weight compounds (e.g. BSA), that retard their diffusion across the cell membrane.

Notably, nuclear localization induced by 5α-DHT was:

1. rapid (occurring within 5 minutes);
2. not reversed by antagonists of the classical androgen nuclear receptor, namely cyproterone acetate (steroidal antagonist) and flutamide (non-steroidal antagonist);
3. achieved also by the non-cell permeable testosterone:BSA;

and therefore it showed all the characteristics of a non-genomic, cell-membrane-receptor-mediated effect.

The non-genomic effect of sex hormones (Falkenstein et al., 2000), in particular of androgens (Heinlein and Chang, 2002) are known to mainly affect intracellular signalling, that is,

phosphorylation events and Ca^{+2} signalling (see ***1.3.2.2.***). In fact we found that the androgen hormones 5α-DHT and testosterone, and also testosterone:BSA, induced a fast but moderate increase in pERK1/2 levels, whereas p38 MAPK was not affected. Notably, the effect of 5α-DHT was moderate in comparison to the inflammatory stimulus fMLP, most likely reflecting a basal homeostatic physiological regulation rather than a stimulatory excessive burst. Interestingly, a moderate activation of ERK was also observed by incubating PMNL in male human plasma, confirming the physiological relevance of these findings. Thus, male human plasma induced ERK1/2 phosphorylation in female cells to similar levels that were observed in resting male cells. This effect can be most likely be ascribed to the androgens present in the plasma, since no significant effect was observed by female human plasma. Moreover, ERK1/2 inhibitors suppressed 5α-DHT-induced nuclear translocation of 5-LO, and re-established its diffuse cytosolic staining in female PMNL challenged with 5α-DHT. Together, these data clearly indicate that ERK1/2 are the molecular pathway underlying the regulation of 5-LO by androgens.

5α-DHT-induced 5-LO localization was also inhibited by calcium chelators, confirming the putative role of Ca^{+2} in the membrane-binding of 5-LO (cfr. ***1.2.6.1.***). Moreover we found that androgens influence the cellular Ca^{+2} homeostatis as much as they facilitate Ca^{+2} influx, thereby increasing $[Ca^{2+}]_i$. Although androgens significantly mobilised intracellular Ca^{+2}, the increase in $[Ca^{2+}]_i$, was only moderate in comparison to the inflammatory stimulus fMLP and was not always evident in all PMNL preparation tested. However, this inconsistency could be due to the involvement of multiple independent pathways in the regulation of the Ca^{+2} entry in human neutrophils (Itagaki et al., 2002). One could speculate that the moderate effects of androgens on intracellular Ca^{2+} in human PMNL are sort of a guarantee for sufficient $[Ca^{2+}]_i$ allowing modulation of select homeostatic cellular processes without inducing overall Ca^{+2}-

dependent cellular responses, including, for example degranulation, presentation of adhesion molecules and oxidative burst.

Interestingly, the effect of 5α-DHT on $[Ca^{2+}]_i$ was not blocked by the antagonists of the androgen nuclear receptor, but was partially inhibited by PTX, a G_i protein-coupled receptor inhibitor, suggesting that the cell membrane receptor for androgens could either be a GPCR or could be functionally linked to such one. Moreover, the observations of non-genomic androgen-mediated increases of $[Ca^{2+}]_i$ appear to occur through different mechanisms in different cell types (Heinlein and Chang, 2002), suggesting that Ca^{+2} increase may not be an intrinsic property of the membrane-androgen receptor and supporting the idea of a different GPCR associated to it. A different degree of coupling of these proteins could therefore partially explain the difference in the $[Ca^{2+}]_i$ increase in different cell types or PMNL preparations. Of importance, androgen pre-treatment did not modify the Ca^{+2} influx induced by ionomycin or fMLP, suggesting that male sex hormone guarantee appropriate PMNL activation in response to inflammatory stimuli. This is of great importance since suppression of PMNL function has been associated, for example, with septic complications (Aldridge, 2002).

As major consequence of the androgen effect on 5-LO subcellular localisation, exogenous addition of 5α-DHT induced a decrease of about 40 % in ionophore-induced 5-LO product synthesis in female PMNL, but not in PMNL from males. Notably, the 5-LO product levels in ionophore-activated male PMNL were not significantly different as compared to 5α-DHT-treated female PMNL. These data strongly suggest that androgens may be the responsible factors resulting in sex-related differencies in 5-LO product synthesis. Of interest, the inhibitory effect of 5α-DHT was observed when leukocyte concentrates (buffy coats) from female blood were exposed to the androgen prior isolation of PMNL, implying that 5α-DHT

acts also in the presence of a complex system (i.e. blood) and that its effect was retained by the PMNL under the specific cell isolation condition applied in this study.

In the light of the above reported data, we propose a new model for 5-LO regulation in human PMNL **(Fig. 41A,B)**. Thus, the classical model of a cytosolic 5-LO, which translocates to the nuclear envelope interacting with FLAP after agonist challenge can be considered valid only in PMNL from (normal) female donors **(Fig. 41B)**. On the other hand, we suggest that the androgens present in the plasma of male subjects act on PMNL via a non-genomic mechanism, most probably via a membrane receptor, to constitutively and moderately stimulate ERK1/2 and to slightly elevate the $[Ca^{2+}]_i$ **(Fig. 41A)**. This leads in turn to an association of 5-LO with the endoplasmic reticulum, and to a desensitization of 5-LO and/or to a different capability to access the endogenous substrate, therefore resulting in a reduced 5-LO product synthesis in males compared to females when an inflammatory stimulus is operative.

The biological relevance of these findings and the validity of our model were confirmed by a whole blood assay which is recognised to be a likely predictor of the *in vivo* functions (Chadwick et al., 1992). In whole blood, ionophore-induced 5-LO product synthesis from endogenous AA was 55 % lower in males than in females. This difference was abolished by the treatment of female blood with 5α-DHT and these levels of 5-LO products were not significantly different compared to ionophore-stimulated male blood. Of interest, the addition of 5α-DHT to male blood did not significantly modify ionophore-induced 5-LO product synthesis, suggesting that the concentration of hormones physiologically present in male plasma might be already in the optimum range to regulate 5-LO. Notably, the differences

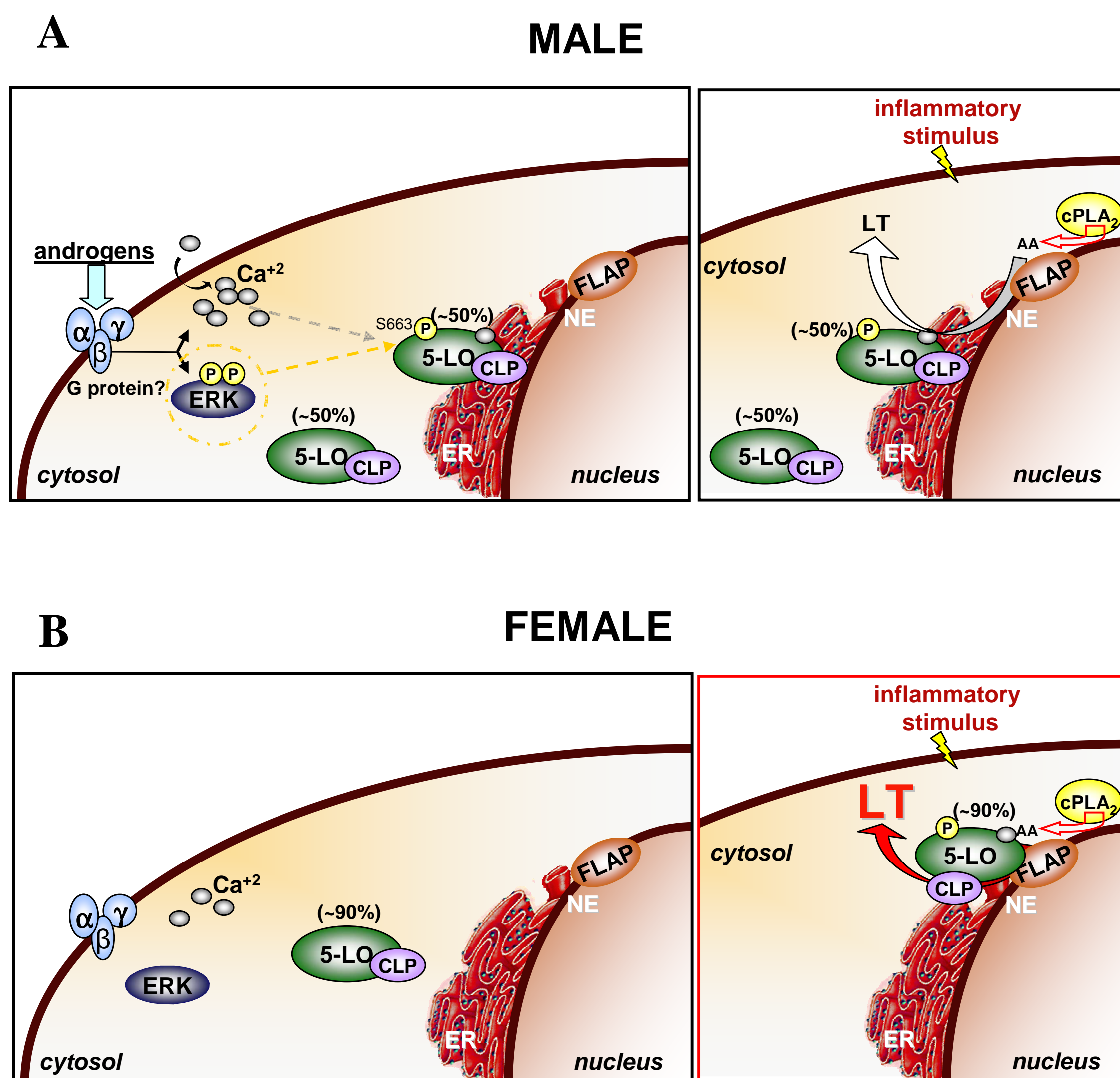


Fig. 41: Proposed models for 5-LO subcellular redistribution and activation in human PMNL from males and females. In PMNL from males **(A)**, androgens present in the plasma act via a non-genomic mechanism, most probably mediated by a membrane receptor, to constitutively and moderately stimulate ERK1/2 and to mobilize Ca^{2+} (left panel). This leads, in turn, to the association of 5-LO (bound to CLP) with the endoplasmic reticulum, where it does not interact with FLAP. This mechanism implies a desensitization of 5-LO and/or a lower capability to access the endogenous substrate in comparison to PMNL from female, in response to an inflammatory agent (right panel). In cells from (normal) female **(B)** donors, 5-LO is cytosolic (left panel) and translocates to the NE, where FLAP is located, upon stimulation (right panel). The overall consequence of the androgen effect is that 5-LO product synthesis in presence of an inflammatory stimulus is significantly reduced in males compared to females. *Abbreviations:* AA, arachidonic acid; ER, endoplasmic reticulum; CLP, coactosin-like protein; 5-LO, 5-lipoxygenase; LT, leukotrienes; NE, nuclear envelope; PMNL, polymorphonuclear leukocytes.

between the genders or after 5α-DHT treatment of female blood were abolished when exogenous AA was added to the blood or when the assay was performed in whole blood homogenates. These results are in complete accordance with the findings made using isolated PMNL and further confirm that the amount of catalytically active 5-LO was not different between the genders. Again, the cellular environment seems absolutely required for the gender-specific 5-LO regulation. Moreover, no difference was evident in the amount of the COX product 12-HHT, suggesting that the sexual dimorphic regulation is not a general phenomenon but instead seems specific for 5-LO.

In summary, the data presented here provide the first evidence for a gender-related regulation of 5-LO and reveal the molecular mechanisms involved. This might be of great importance since the increased synthesis of 5-LO products in females correlate with the higher incidence of 5-LO-related diseases in females observed in several clinical studies (cfr. **1.2.9.**). Moreover, the 5-LO suppressive properties of male sex hormones correlate with the protective effects of androgens observed in diseases where 5-LO plays a critical role (cfr. **1.2.9.**). As an example, the incidence of asthma before puberty is higher in male, and after puberty, when the testosterone levels rise in male subjects, is significantly reduced. For females the incidence does not change around puberty and becomes predominant versus males in the post-puberty phase (see also **Fig. 11**). Furthermore, our results strongly suggest a direct influence of testosterone/5α-dihydrotestosterone on PMNL via regulating ERK activation. Since ERK is known to regulate numerous neutrophil functions, our findings could provide the molecular basis for differential regulation of PMNL biology. It must be observed that in studies addressing the biology and functionality of blood cells the influence of gender/sex hormones has been rarely taken into account. As mentioned above, ERKs regulate numerous functions of

blood cell and, as visualized with 5-LO, the differential activation state of these kinases may have significant consequences on the overall conclusions.

These sex differences and the role of androgens are of more than purely biological interest. The anti-inflammatory effects of testosterone at physiological concentrations raise the prospect of putting this knowledge to therapeutic use, both for male and female patients. In fact, the sex-related difference in 5-LO regulation seems to be dependent only on different plasma levels of androgens between the genders, since androgens induced significant inhibitory effects in 5-LO product synthesis in cells isolated from female subjects and in female whole blood, demonstrating their responsiveness to androgens. However, for an effective therapeutic use, the anti-inflammatory properties of androgens should be separated from their effects on the reproductive system. Interestingly, adrenal androgens, such as dihydroepiandrosterone, are shared between males and females and have fewer virilising effects (van Vollenhoven et al., 1998). Development of drugs by modification of androgens in order to keep their anti-inflammatory effectiveness without the masculinizing consequences could therefore form a strategy of research. To this aim, and in the light of the anti-inflammatory non-genomic effects of androgens observed in this study, the cloning of the respective membrane-associated receptor for androgens could be a step forward to characterise its biological role and to develop selective agonists lacking virilising effects, normally mediated by the classic nuclear androgen receptor.

In any case, an alternative approach could be represented by the so-called “gender tailored therapy”, which means treatment with testosterone of male patients that exert relatively low testosterone levels. Of interest, on this subject, restoring testosterone in male rheumatoid arthritis patients resulted in a clinically significant reduction in the number of affected joints

and minimised the need for non-steroidal anti-inflammatory drugs (Cutolo et al., 1991). Moreover, males with Klinefelter's or Sjogren's syndrome have a reduction in associated inflammatory mediators and antibodies on being treated with replacement of testosterone (Bizzarro et al., 1987).

Notably, despite evidences of sex-based differences in the pathophysiology of diseases certainly imply important underlying differences in physiological function, most basic and clinic research either was performed exclusively in male or female subjects, or included both sexes, but did not differentiate between the genders in the data analysis (Blair, 2007). Recently, the description of sex-differences in drug responses is outlining the idea that the gender is a fundamental variable that cannot be discounted in the evaluation of the pharmacological efficacy of drugs (Martin, 2006; Franconi et al., 2007). Therefore, the discovery of a gender-specific regulation of 5-LO directly implies that in the development of drugs modifying the 5-LO pathway as therapeutic agents, the sex issue must be considered in order to improve their efficacy and optimize medical therapy both in men and women.

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APPENDIX 1: ABBREVIATIONS

AA	arachidonic acid
Ada	adenosine deaminase
AF	activation function domain
AR	androgen receptor
AUC	area under the curve
BAPTA/AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
BSA	bovine serum albumine
$[Ca^{2+}]_i$	intracellular calcium concentration
Ca-ion	calcium-ionophore A23187
CaMK	Ca^{2+} /calmodulin-dependent protein
CLP	coactosin-like protein
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CYP	cytochrome p450
cys-LT	cysteinyl-leukotriene
DAG	diacylglycerol
DBD	DNA binding domain
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
EBV	Epstein Barr Virus
EDTA	ethylenediamine-tetraacetic acid
EPR	electron paramagnetic resonance
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FHP	female human plasma
FLAP	5-lipoxygenase activating protein
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte/macrophage colony-stimulating factor

GPCR	G-protein-coupled receptor
GPx	glutathione peroxidase
HEDH	hydroxyeicosanoid dehydrogenase
HETE	hydroxyeicosatetraenoic acid
12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
HpETE	hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid
HPLC	high performance liquid chromatography
HRE	hormone response element
IF	immunofluorescence
IP3	inositol 1,4,5-trisphosphate
JNK	c-Jun NH2-terminal kinase
LBD	ligand binding domain
LO	lipoxygenase
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MHP	male human plasma
MK	MAPK activating protein kinase
Mnk	MAPK-interacting kinase
NE	nuclear envelope
NES	nuclear export signal
NF-κB	nuclear factor-κB
NIS	nuclear import sequence
NLS	nuclear localization sequence
Non-N	non nuclear fraction
NP40	Nonidet P-40
Nuc	nuclear fraction
OAG	1-oleoyl-2-acetyl-sn-glycerol
O.D.	optical density
PAF	platelet activating factot

PBS	phosphate buffered saline
PC	phosphatidylcholine
PG buffer	PBS-glucose buffer (see materials and methods)
PGC buffer	PBS-glucose-Ca^{+2} buffer (see materials and methods)
PI3K	phosphatidylinositol 3-OH kinase
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipase
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethyl sulfonyl fluoride
PR	progesterone receptor
PS	phosphatidylserine
PTX	pertussis toxin
RBL	rat basophilic leukemia cells
RORα	retinoic acid receptor-related orphan-receptor α
RZRα	retinoid Z receptor alpha
SDS	sodium dodecyl sulphate
SH3	Src homology
SHBG	sex hormone binding globulin
$sPLA_2$	secretory phospholipase A_2
TGFβ	transforming growth factor β
TRAP-1	TGF beta receptor-I-associated protein I
WB	western blot

APPENDIX 2: CURRICULUM VITAE

PERSONAL DATA

Name: **Carlo Pergola**

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EDUCATION

1994 - 1999	Liceo Gymnasium "J. Sannazzaro" of Naples
July, 1999	European High School Diploma awarded with 100/100
1999 – 2004	Pharmaceutical Chemistry and Technology at the University of Naples "Federico II"
July, 2004:	Degree, *cum laude and honorary mention*, in Pharmaceutical Chemistry and Technology at the University of Naples "Federico II" Experimental Thesis in Pharmacology: Role of 5-Lipoxygenase in the Regulation of the Biosynthesis of Prostaglandins
2004 -	Phd in Drug Science, XX cycle, University of Naples "Federico II" Tutor: Prof. Lidia Sautebin (Department of Experimental Pharmacology)
2006 -	Research at the Department of Pharmaceutical Analytics, Institute of Pharmacy, Eberhard-Karls-University, Tübingen. Supervisor: Prof. Dr. Oliver Werz

TIROCINIUM AND PROFESSIONAL QUALIFICATION

2002 – 2003	Tirocinium at "Farmacia Centrale" of Naples
December, 2004	Qualification for the Profession of Pharmacist

ACADEMIC EXPERIENCES

2003 – 2004	Boarder student at Department of Experimental Pharmacology, University of Naples “Federico II”
2005-	Graduate doctoral research fellow of the University of Naples “Federico II”, Department of Experimental Pharmacology

RESEARCH EXPERIENCES ABROAD

May, 2006 -	Department of Pharmaceutical Analytics, Institute of Pharmacy, Eberhard-Karls-University, Tübingen. Supervisor: Prof. Dr. Oliver Werz

CONGRESSES AND SCIENTIFIC MEETINGS

October 10-13, 2007:	"German Pharmaceutic Society (Deutschen Pharmazeutischen Gesellschaft) Annual Meeting 2007"; Erlangen (Germany)
September 20-21, 2007:	"Scientific Days of the Pole for Life Science and Technologies 2007"; Naples (Italy)
September 5-8, 2007:	"EICOSANOX Annual Meeting 2007"; Aigen/Ennstal (Austria)
June 6-9, 2007:	"33rd National Congress of the Italian Pharmacological Society"; Cagliari (Italy)
June 15-16, 2006:	"Scientific Days of the Pole for Life Science and Technologies 2006"; Naples (Italy)
December 3, 2005:	"Natural Antioxidants: current status and perspectives for the future"; Naples (Italy)
June 1-4, 2005:	"32nd National Congress of the Italian Pharmacological Society"; Naples (Italy)
May 26-27, 2005:	"Scientific Days of the Pole for Life Science and Technologies 2005"; Naples (Italy)
June 18, 2004:	"1st Meeting of the Pharmacologists of Regione Campania"; Naples (Italy)
May 20-21, 2004:	"Scientific Days of the Pole for Life Science and Technologies 2004"; Naples (Italy)
April 3, 2003:	Monothematic Day on: "Flavonoids: chemistry, pharmacognosy, pharmacology and therapeutical use"; Naples (Italy)

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