Regulation of platelet-type 12-lipoxygenase activity in platelets with special consideration of gender effects

Regulation der Aktivität der platelet-type 12-Lipoxygenase in Thrombozyten unter besonderer Berücksichtigung geschlechtsspezifischer Einflüsse

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Ulrike Bühring

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Dekan: Prof. Dr. L. Wesemann

1. Berichterstatter: Prof. Dr. O. Werz

2. Berichterstatter: PD Dr. M. Düfer

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List of publications/ presentations

“Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1.” Koeberle A, Siemoneit U, Bühring U, Northoff H, Laufer S, Albrecht W, Werz O. J Pharmacol Exp Ther. Sept 2008.

“Structural Optimization and Biological Evaluation of 2-Substituted 5-Hydroxyindole-3-carboxylates as Potent Inhibitors of Human 5-Lipoxygenase” Karg E-M, Luderer S, Pergola C, Bühring U, Rossi A, Northoff H, Sautebin L, Troschütz R, Werz O, JMedChem, May 2009

“Sulindac sulfide suppresses 5-lipoxygenase at clinically relevant concentrations” Steinbrink S, Pergola C, Buehring U, George S, Metzner J, Fischer A, Haefner A-K, Wisniewska J, Geisslinger G, Werz O, Steinhilber D, Maier T, Cell Mol Life Sci, November 2009

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„12-Lipoxygenase: Expression and Purification“, Bühring U, Poeckel D, Werz O, poster presentation, DphG Jahrestagung 2007, Erlangen

“Novel potent inhibitors of human 5-lipoxygenase: Structural modification of the hitherto existing 5-LO inhibitors of 2-amino-5-hydroxyindole-3-cyrboxylate type”, Karg E-M, Jazzar B, Bühring U, Werz O, Troschütz R, poster presentation, DphG Jahrestagung 2007, Erlangen

„Progesterone regulates 12-Lipoxygenase in human platelets”, Bühring U, Pergola C, Verhoff M, Werz O, oral presentation, DphG- Jahrestagung 2009, Jena

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3. Results

Abbreviations

12-H(P)ETE 12-hydro (pero)xyeicosatetraenoic acid

12-HHT 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid

AA arachidonic acid

[^{3}H]-AA tritium labeled arachidonic acid

ADP/ATP adenosine diphosphate/adenosine triphosphate

ANOVA analysis of variance between groups

BSA bovine serum albumin

$[Ca^{2+}]_i$ intracellular Ca^{2+} concentration

$CaCl_2$ calcium chloride

cAMP/ cGMP cyclic adenosine/guanidine monophosphate

CDC cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate

COX cyclooxygenase

$cPLA_2$ cytosolic phospholipase A_2

DAG diacylglycerol

DHT 5α-dihydrotestosterone

DMSO dimethylsulfoxide

ERK extracellular signal-regulated kinase

Est estradiol

EDTA ethylenediaminetetraacetic acid

FLAP 5-lipoxygenase activating protein

GP glycoprotein

GPCR g-protein coupled receptor

H(P)ETE hydro(per)oxy-eicosatetraenoic acid

HEPES N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC high performance liquid chromatography

IP3 inositoltrisphosphate

LO lipoxygenase

LT leukotriene

L12-LO leukocyte 12-LO

MAPK mitogen-activated protein kinase

MARCKs myristoylated alanine-rich C kinase substrate

Mife mifepristone

MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid)

OD optical density

p12-LO platelet-type 12-lipoxygenase

PAPC 1-Palmitoyl-2-Arachidonyl-sn-Glycero-3-Phosphocholine

PAR protease activated receptor

PBS phosphate-buffered saline

PC phosphatidylcholine

PG buffer PBS plus 1 mg/ml glucose

PGC buffer PG buffer plus $CaCl_2$ 1 mM

PG prostaglandin

PGB_1 prostaglandin B_1

PGE_2 prostaglandin B_2

PGI_2 prostaglandin H_2

PGH_2 prostaglandin H_2

PI3K phosphoinositide 3-kinase

PIP3 phosphatidylinositol (3,4,5)-trisphosphate

PKA protein kinase A

PKB protein kinase B (Akt)

PKC protein kinase C

PLC phospholipase C

PMA phorbol-12-myristate-13-acetate

PMNL polymorphonuclear leukocytes

POG 1-palmitoyl-2-oleoyl-sn-glycerol

PPARγ proliferator peroxysome activated receptor γ

Prog progesterone

PRP platelet rich plasma

PR progesterone receptor

ROS reactive oxygen species

RP reversed phase

RT room temperature

SHBG steroid hormone binding globulin

SDS sodium dodecylsulfate

SDS-LB SDS-loading buffer

SDS-PAGE SDS-polyacrylamide gel electrophoresis

TBS tris-buffered saline

TFA trifluoro acetic acid

TXA_2 thromboxane A_2

TXB_2 thromboxane B_2

vWF van Willebrand factor

WB Western blot

1 Introduction

1.1 Platelet physiology

Platelets are highly specialized blood cells that play central roles in physiologic and pathologic processes of haemostasis, tumour metastasis and wound healing [1]. They originate from the cytoplasm of bone marrow megacaryocytes as anucleated subcellular fragments [2]. Platelets are the smallest of human blood cells (3.6×0.7 µM) with a life-span of 7-10 days [1, 2] and are present in circulation in high numbers ($1.5\text{-}4.5 \times 10^8$/ml) that vary strongly between individuals. Since they are anucleate, platelets are only able to newly synthesize proteins in very confined limits as they have to "bring" all necessary mRNA with them from the beginning, so genomic effects in the classical meaning can not be observed in platelets. [3] For platelets all activating events culminate in aggregation, their main purpose. To fulfil their physiological assignments, platelets have a unique cytoskeleton that enables them to change their shape from discoid (non-activated) to rounded-up with extensive formation of pseudopodia (activated) [1, 4] (Fig. I).

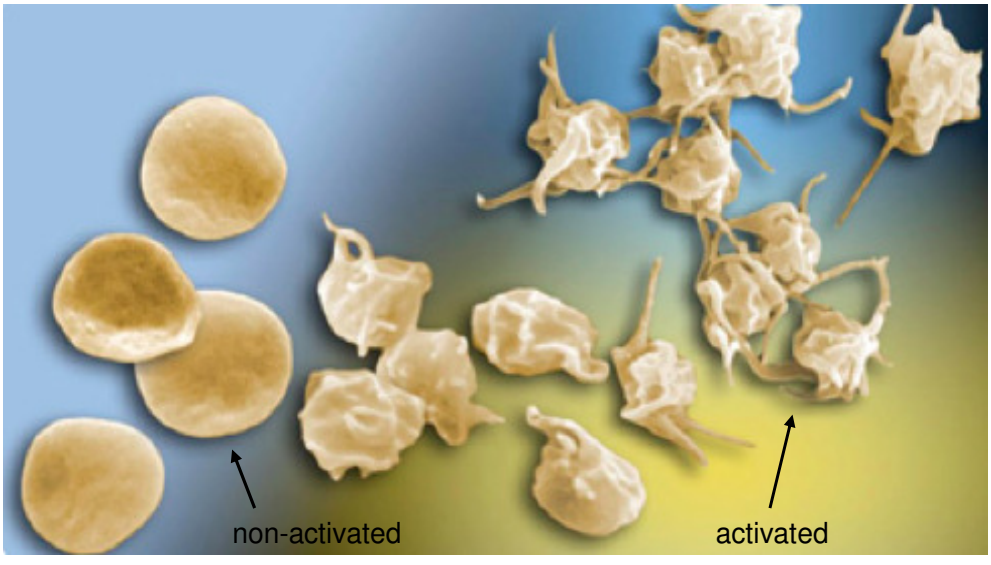

Fig. I *Platelet shape change from discoid shape to formation of pseudopodia*

Shape change is needed for attachment of platelets to the site of injury, where they enact their major function. Shape change and especially all secretory functions of platelets are supported by the open canalicular system,

a dense tubular membrane system that crosses the whole cell and enlarges platelet membrane surface area [2, 4].

The physiological role of platelets in the blood is biased. On one hand they are recruited to vessel wall injuries to create a physical barrier at the site of vessel wall damages to limit blood loss [5]. On the other hand they also attach to sites of ruptured atherosclerotic plaques and trigger heart attacks and strokes [1, 6]. The formation of a haemostatic plug requires platelet-vessel wall (adhesion) and platelet-platelet (aggregation) interactions. The first step in formation of a haemostatic plug is interaction of platelets with van Willebrand Factor (vWF) and specific glycoproteins (GP) on the subendothelial surface. This interaction (adhesion) enables a firm connection of platelets to the injured vessel endothelium by binding to exposed collagen via its main platelet receptors GPIa/IIb and major collagen signalling GPVI receptor [7]. Initial activation and further stimulation by agents from the surrounding environment (i.e. thrombin and thromboxane A_2 (TXA_2)) lead to a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels and subsequently to spreading of platelets and activation of cytosolic phospholipase A_2 ($cPLA_2$), granule secretion (dense and alpha granules) and activation of GPIIb/IIIa (integrin αIIb/βIIIa) culminating in binding of soluble fibrinogen (inside-out signalling) which is essential for platelet bridging and subsequent aggregation [1, 8, 9]. Substances secreted from dense granules include secondary agonists like adenosine diphosphosphate (ADP) and serotonin that play important roles in additional autocrine/ paracrine platelet activation and haemostatic plug growth [1] as they recruit more platelets to the initial plug. The α-granules release large adhesive proteins, mitogenic factors, coagulation factors as well as glycoproteins such as P-selectin (CD62) and CD40 ligand [1]. The latter is well recognized to induce platelet binding to monocytes and neutrophils. The central receptor in platelet aggregation is the GPIIb/IIIa (integrin αIIb/βIIIa). Cross linking of two GPIIb/IIIa molecules on activated platelets results in a firm connection through fibrinogen bridges, moreover, immobilized fibrinogen bound on stimulated platelets serves as an adhesive substrate for resting platelets leading to amplification of primary aggregation. [1]. Increase in $[Ca^{2+}]_i$ promotes

efficient thrombin generation and cleavage of fibrinogen to fibrin to form a stable plug. All major events involved in aggregation including major agonists and their receptors are depicted in Fig. II. [10].

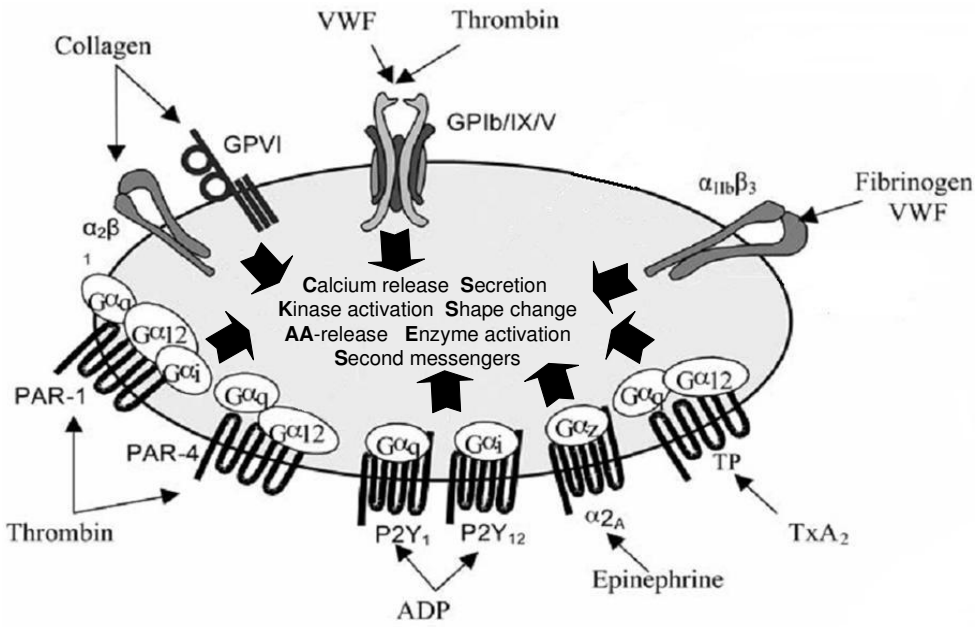

Fig. II *Intracellular events and signal transduction in platelets after initial activation by different platelet agonists ([10] modified)*

Due to activation of platelets also enzymes of the arachidonic acid (AA) cascade are activated. In platelets these enzymes are cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-1 (COX-1), platelet-type 12-lipoxygenase (p12-LO) and in a wider angle also thromboxane synthase (TX synthase). At least $cPLA_2$ and COX-1 are predominately localized at the membranes of the dense tubular system [1]. In due course $cPLA_2$, COX-1 and TX synthase are generating signalling molecules like TXA_2. Also other AA metabolites as COX-1 metabolite 12*(S)*-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) or p12-LO product 12*(S)*-hydroxyeicosatetraenoic acid (12-HETE) are generated after platelet activation. The role of these latter molecules for the events of platelet aggregation has not yet been clearly identified. So far, they are mostly used as markers to evaluate COX-1 and p12-LO activity in stimulated platelets.

1.2 Platelet intracellular kinase signalling cascades

Most signalling events in platelet activation are mediated by rise of $[Ca^{2+}]_i$ levels [11]. Activation of different kinase cascades and the formation of second messenger molecules are dependent on $[Ca^{2+}]_i$. As platelet intracellular signalling can be initiated by a wide variety of platelet agonists including thrombin, TXA_2, ADP and epinephrine only pathways induced by thrombin and collagen will be discussed here in detail. Thrombin and collagen are considered to be strong platelet agonists as opposed to ADP and serotonin [12]. Soluble agonists as thrombin activate specific G-protein coupled receptors (GPCRs) that in turn activate their own specific signalling kinases in platelets. When thrombin binds to its platelet surface GPCRs, also known as protease activated receptors (PAR) 1/4 [13], it induces a conformational change of its receptors leading to activation of three major kinase pathways (Fig. III).

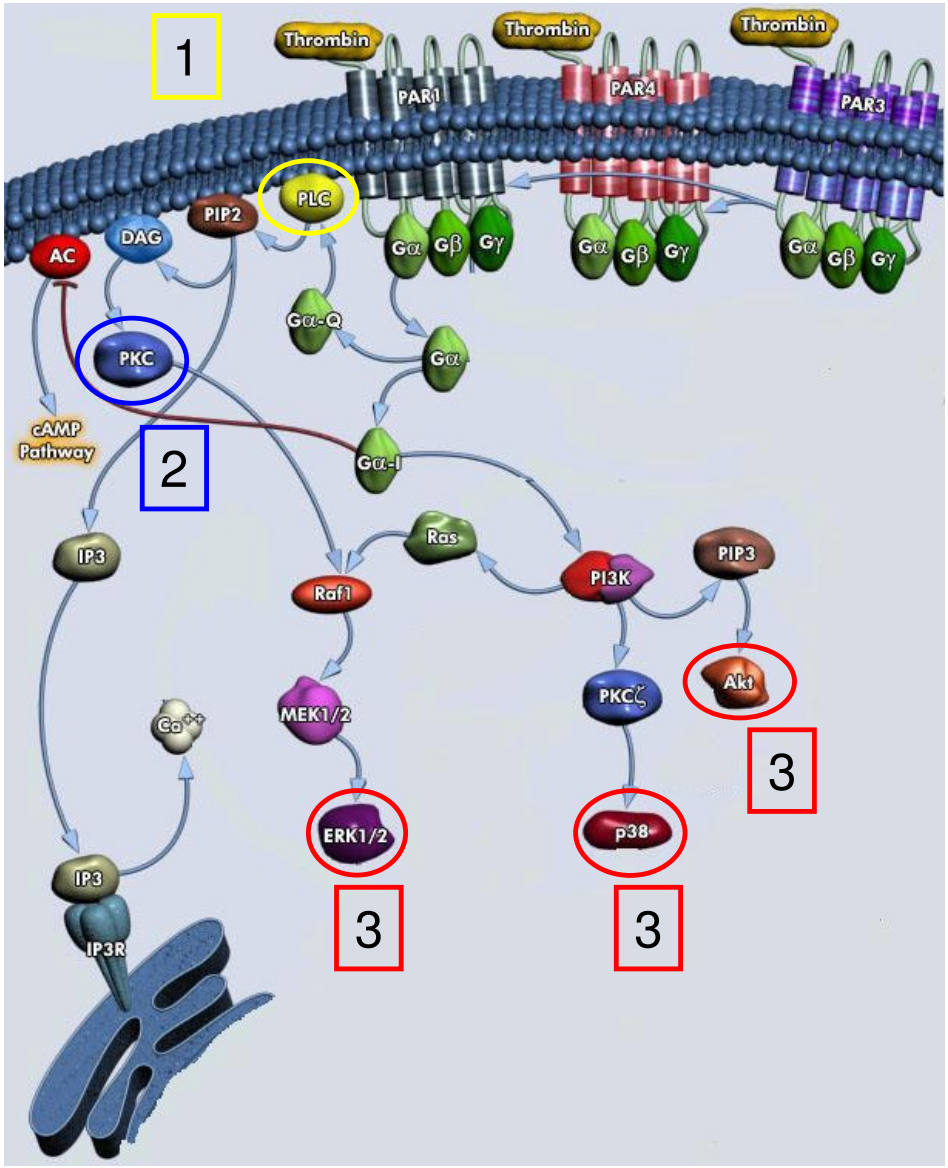

Fig. III *Kinase signalling after thrombin stimulation of platelets (https://www1.qiagen.com/Geneglobe/PathwayView.aspx?pathwayID=434 modified)*

First (1), phospholipase C (PLC) is activated and hydrolyses phosphatidylinositol-(4,5)-bisphosphate liberating inositol trisphosphate (IP3) and diacylglyerol (DAG). IP3 binds to its receptor and induces $[Ca^{2+}]_i$ mobilisation from the endoplasmatic reticulum increasing $[Ca^{2+}]_i$ levels. DAG activates protein kinase C (PKC) which in turn activates its target enzymes or kinases by phosphorylation [13]. Increase in $[Ca^{2+}]_i$ and activation of PKC [14, 15] lead to activation of $cPLA_2$, liberation of AA and TXA_2 formation [5, 13]. To study PKC activation, the phosphorylation status of its main target, myristoylated alanine-rich C kinase substrate (MARCKs), also can be examined [16] (not displayed in Fig. III).

Second (2), the phosphoinositide 3 kinase (PI3K) pathway is activated leading to a prolonged activation of integrin $\alpha_{IIb}/\beta_{IIIa}$ and stabilized platelet aggregates [13]. The third (3) route of signal kinases activated are mitogen-activated protein kinase (MAPK) signalling pathways downstream of PI3K. p38 MAPK, which is involved in $[Ca^{2+}]_i$ -dependent activation of $cPLA_2$ [14], is activated by thrombin signalling as well as the extracellular signal-regulated protein kinases (ERK1/2). However, the functional outcome of ERK1/2 activation in platelets is so far not fully understood [13]. But since maximum phosphorylation of ERK1/2 occurs approximately 2 to 3 minutes after cell stimulation those kinases have probably only a secondary involvement late in aggregation events [13]. Protein kinase B (PKB), also known as Akt, is also activated in platelets by thrombin stimulation and plays an important role in platelet thrombus formation and stabilisation [17, 18]. All these events lead to prominent platelet aggregation [5].
Collagen also activates PKC and PI3K and these, as in thrombin signalling, generate second messengers IP3, DAG and PIP3 [5]. Not all signalling proteins and interaction partners are displayed in this simple overview (Fig. IV).

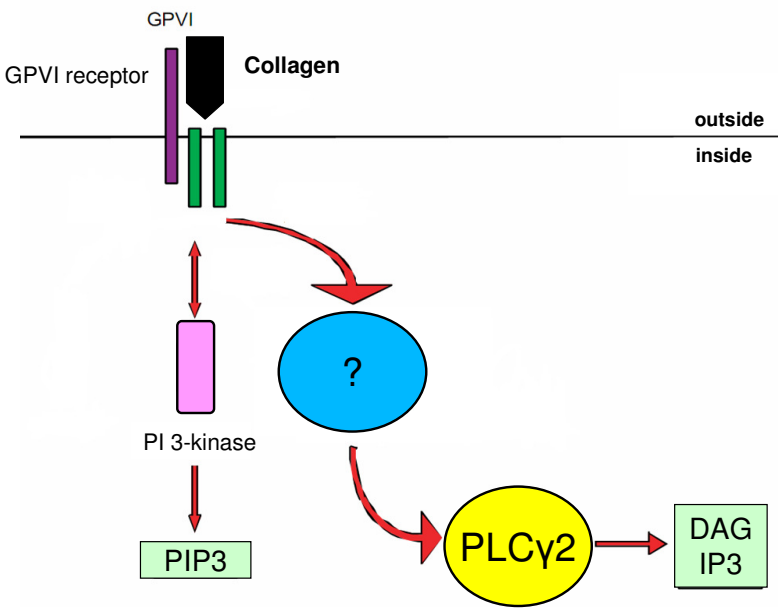

Fig. IV *Collagen-induced kinase signalling ([5] modified)*

How other kinases such as p38 MAPK and ERKs are activated is not quite clear as collagen signalling in platelets seems to be very diverse and not all pathways have been elucidated completely. Nevertheless, collagen-induced

activation of kinases takes different pathways than kinase activation by thrombin as could be proved for PKC activation where two different regulatory mechanisms could be identified [19] and also different isoforms of PKC are activated (Fig. III, Fig. IV). All of the kinases that are activated when platelets are stimulated with thrombin or collagen promote platelet activation. Platelet activation always implicates activation of residing enzymes like $cPLA_2$, COX-1 and p12-LO.

As platelets are involved in the genesis of many cardiovascular disorders platelet inhibition bears a tremendous significance for treatment of these diseases. Negative regulation or platelet inhibition is essential to prevent uncontrolled thrombosis. In this regard the role of nitric oxide and prostacyclin (prostaglandin I_2, PGI_2) are well established. These mediators regulate platelet function by increasing intracellular concentrations of cyclic nucleotides cGMP/cAMP via guanyl and adenyl cyclase activation and thereby decrease platelet activity [20]. Antiplatelet agents such as aspirin suppressing TXA_2 formation and abciximab blocking GPIIb/IIIa receptor signalling are used to impair platelet activation in cardiovascular disease [21]. One prominent pathway down regulating platelet activity and thereby activity of platelet enzymes involves increase of intracellular cAMP and activation of cAMP dependent protein kinase A (PKA). Phosphorylation of PKA target proteins occurs at serine and threonine residues [22] and leads to their inhibition in most cases. PKA involvement in inhibition of platelet adhesion and shape change could already be established [23, 24]. PKA also seems to be involved in regulation of $[Ca^{2+}]_i$ mobilisation [25]. A negative regulation of platelet intracellular enzymes as p12-LO via PKA pathways seems likely as PKA is strongly expressed in platelets [25] and has mostly inhibitory functions. To activate PKA by increasing cAMP levels prostaglandin E_2 (PGE_2) can be used although no specific PGE_2 receptor has been identified on platelets and PGE_2 also seems to induce different reactions depending on the applied concentration [26]. However, a rise in cAMP levels could be induced by 5 µM PGE_2 addition to rat and pig platelets [27] and proved to be an efficient tool to elucidate PKA functions and cAMP properties in platelet regulation.

A second route leading to platelet inhibition seems to be provided by protein tyrosine phosphatase (PTP) activity that are apparently essential for platelets to avoid activation [28]. Inhibition of PTP caused platelet aggregation, secretion and TXA_2 formation [13], yet a precise role for PTP in platelets has to be established.

1.3 Cytosolic phospholipase A2 ($cPLA_2$)

Most platelet functions including aggregatory events depend on AA metabolism as some important signalling molecules as TXA_2 are derived from AA. To start the synthesis of prostaglandins and thromboxanes, the most important AA metabolites synthesised in platelets, AA has to be released from the cell membrane phospholipids as starting point of the AA cascade (Fig. V).

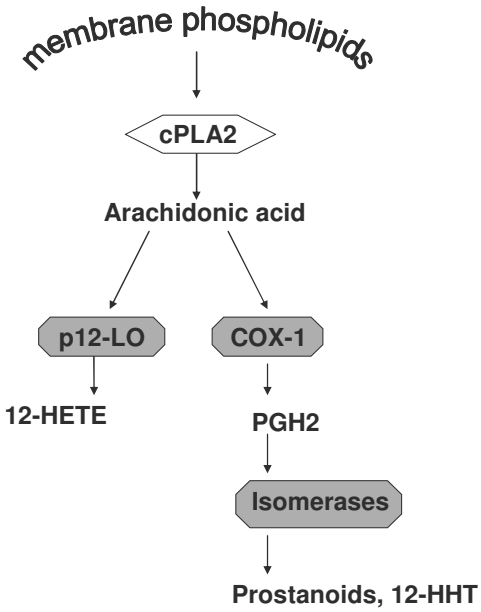

Fig. V *Arachidonic acid pathways: main metabolic activities in platelets*

To release AA, human platelets contain significant amounts of cytosolic $cPLA_2$ [29] that is thought to be the main phospholipase providing AA for aggregatory events [30]. The enzyme has a molecular weight of 95 kDa and is found in the cytosolic fraction of platelets. To access its substrate, preferentially arachidonate containing phospholipids, at the plasma membrane $cPLA_2$ has to bind Ca^{2+} to be able to access the plasma membrane [30-32]. $cPLA_2$ activity is strongly $[Ca^{2+}]_i$ -dependent and rises approximately 10-fold as $[Ca^{2+}]_i$ increases from levels in resting cells (< 100

nM) to levels measured in activated cells (300 nM). If only little $[Ca^{2+}]_i$ is present, only a very low $cPLA_2$ activity can be observed [33]. Inhibition of $cPLA_2$ is followed by inhibition of major platelet functions accompanied and in part caused by reduction of activity of down stream enzymes dependent on AA release as COX-1 or p12-LO [34].

1.4 Cyclooxygenase-1 (COX-1)

After liberation of AA from the membrane, AA is metabolised via different pathways. One pathway runs via p12-LO metabolism and will be discussed in detail (1.5.2). The other pathway in platelets involves COX-1. Metabolites from the COX-derived precursor PGH_2 such as TXA_2 and prostacyclin (PGI_2) exert important functions in platelet aggregation and regulation of vessel wall tone [26]. COX-1-derived arachidonate metabolites are outlined in Fig. VI, platelet specific products are indicated (red squares).

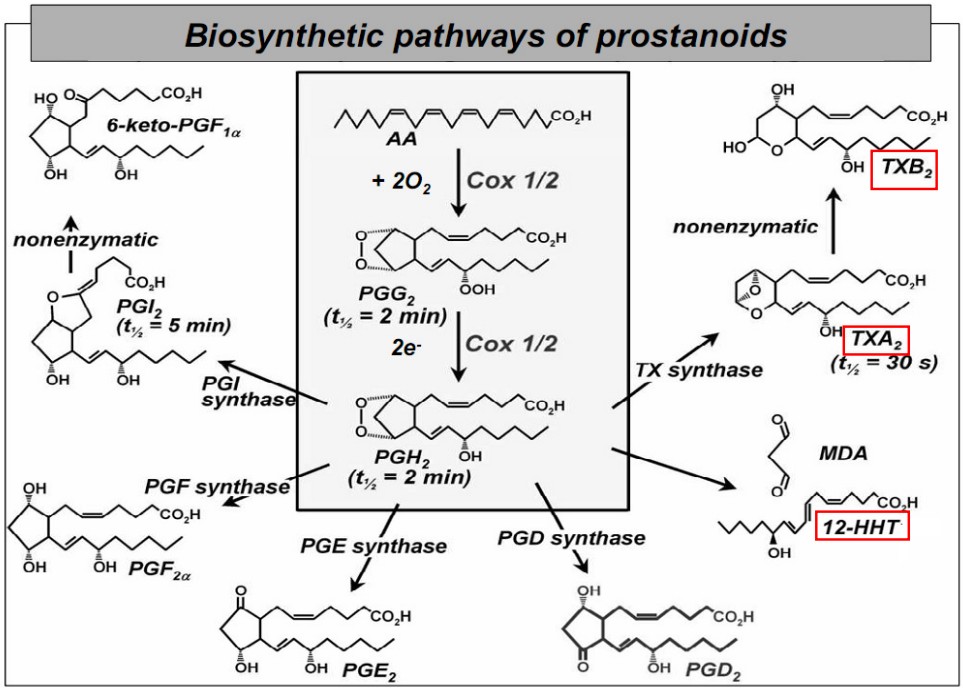

Fig. VI *Formation of prostanoids derived from cyclooxygenase pathways*

COX-1 is constitutively expressed in many tissues such as blood vessel wall, platelets and renal tubulus cells [35] and performs mostly house-keeping functions for vascular haemostasis, gastric mucosal integrity and renal blood flow. It exists as homodimer with a molecular weight of approximately 70 kDa [36, 37]. COX-1 is a bifunctional enzyme with both cyclooxygenase and peroxidase functions as displayed in Fig. VI. COX-1 metabolizing activity ceases after a couple of minutes in a suicide inactivation mechanism

[38, 39]. Only unbound fatty acids such as free AA can be used as a substrate by COX-1 [40].

Besides substrate supply also the intracellular redox state of the cell is important for COX activity and therefore platelet functions. The intracellular redox state of platelets is controlled by endogenous generation of reactive oxygen species (ROS) after platelet activation by thrombin or collagen [41]. ROS generation after platelet activation was first observed in 1977 by Marcus et al [42] and platelet-derived ROS seem to play an important role in aggregation and recruitment of platelets to an already existing haemostatic plug as well as in overall modification of platelet functions [41, 43]. Any inhibitory substance that is able to lower intracellular hydroperoxide concentrations beneath 10 nM by inhibiting ROS generation potently inhibits COX-1 activity [38]. The oldest route commonly known to inhibit COX-1 activity and therefore TXA_2 synthesis and platelet activation uses irreversible acetylation of COX-1 in platelets by low dose acetylsalicylic acid (ASS). As platelets are anucelate cells with only a small capability for *de novo* protein synthesis [3], the COX-1 inhibition persists for the life-span of the platelets as COX-1 protein cannot be renewed [36]. This acetylation effect is used in long-time preventive therapy of cardiovascular disease.

For studies not directly investigating COX-1 its products can be used as a convenient control activity of other platelet enzymes as p12-LO to check, for instance, selectivity of investigated effects on these enzymes. In this context the stable metabolites TXB_2 and 12-HHT are often measured as parameters for COX-1 activities as they can easily be analyzed by ELISA (enzyme linked immunosorbent assay) and HPLC (high performance liquid chromatography), respectively.

In conclusion, platelets would not be able to fulfil their important functions in haemostatic events without the endowment with $cPLA_2$ and COX-1. However, these two enzymes are also important players in platelet dysfunctional properties as cardiovascular disease and as such in the focus of antiplatelet therapy.

1.5 Platelet-type 12-lipoxygenase: Occurrence and (patho-) physiology

1.5.1 Human 12-lipoxygenases

In 1974 a new enzyme in human platelets metabolizing arachidonic acid was identified by M. Hamberg and B. Samuelson [44]. This enzyme was characterized as a 12-lipoxygenase (LO) and was the first LO to be identified in mammalian tissues. So far, only plant LOs were known [45] and it was believed that "there was no lipoxygenase in animal tissues" [46]. Later on, other 12-LOs were found to be expressed and catalytically active in many different mammalian tissues, i.e. in porcine leukocytes [47], bovine platelets [48] and mouse epidermis cells [49]. Until today, functions and catalytic properties of the different LOs that were identified until now are studied intensely. Lipoxygenases (E.C.1.13.11.) are a family of non-heme iron containing dioxygenase enzymes that introduce molecular oxygen into unsaturated fatty acids such as AA or linoleic acid [46, 50]. The individual LOs are named after the oxygenation position at which they introduce oxygen into their respective substrate. So far, 5-, 8-, 12- and 15-LOs have been identified in mammals [45, 51-53]. In human, 5 different LOs have been identified [54], namely 5*(S)*-, 12*(S)*- and 12*(R)*- and two distinct 15*(S)*-LOs (Fig. VII).

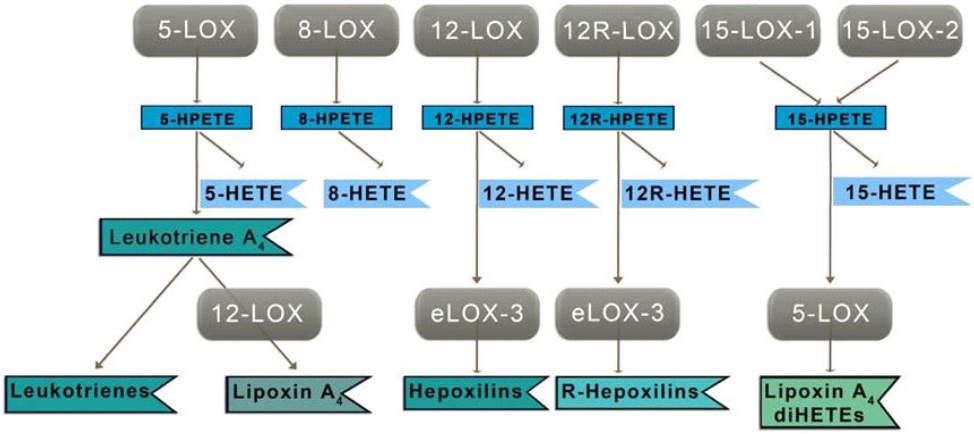

Fig. VII *Human LOs and their metabolic products*

3D X-ray structures are so far only available for soybean 1-LO and rabbit reticulocyte 15-LO [55, 56]. Molecular modelling comparisons of proposed amino acid sequences of the different non-crystallized LOs showed that the catalytical domain is very well conserved in all animal and plant LOs [55,

56]. Together, plant and animal LOs show a huge diversity of proteins (Fig. VIII [57]).

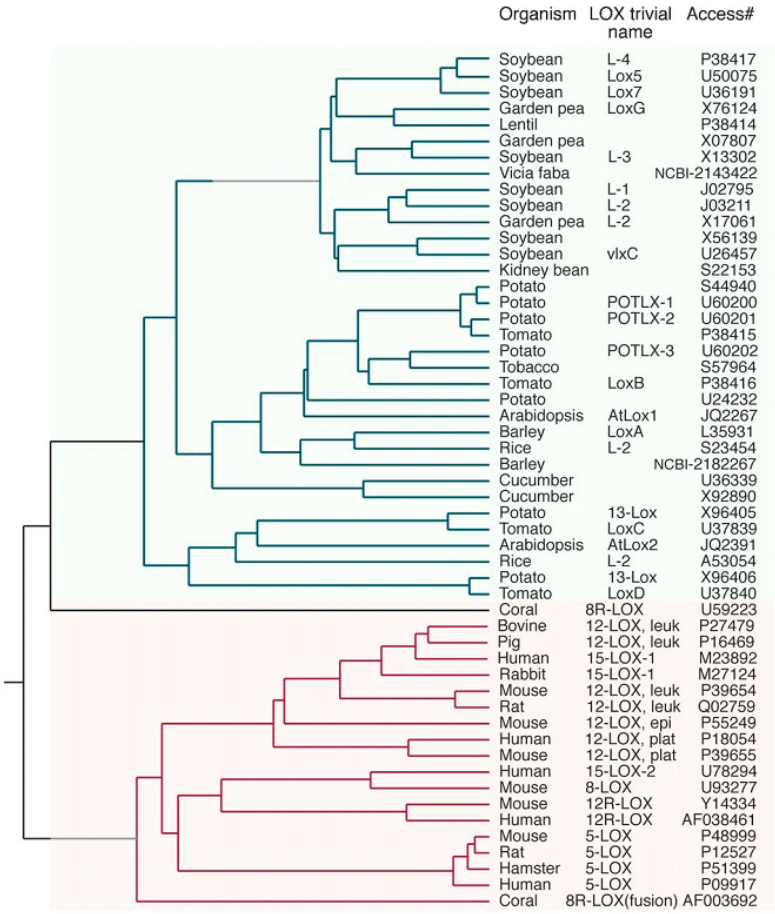

Fig. VIII *Phylogenetic tree of LOs [57]*

The main oxygenation site for 12-LOs is the C12 of AA or other fatty acids that can be used as substrate. However, as 12-LOs differ strongly with respect to homology in amino acid sequence, substrate preferences, dependence on cofactors and inactivation mechanisms [50] a more detailed characterization of the individual 12-LOs is necessary. The group of 12-LOs contains three isoforms, p12-LO and its isoforms leukocyte- and epidermis-type12-LO [50, 57, 58]. As the leukocyte 12-LO show more homologies in amino acid sequence to the human and rabbit 15-LOs and also create considerable amounts of a 15-oxydized product [50], they have been more correctly classified as 12/15-LOs. The platelet- and leukocyte isoforms share 57-66% amino acid homology [46] deduced from their cDNA composition. The epidermis-type 12-LO stands a little outside the group as it is the only 12-LO that produces 12*(R)*-HETE instead of the *(S)*-isomer. In this context it fits that murine epidermis-type 12-LO that also turns out 12*(R)*-HETE as

metabolite shows a 54% homology in amino acid structure to this recently identified 12*(R)*-LO in humans [59], although other studies propose a 84% homology [60]. Antibodies raised against the different isoforms show no cross reactivity [61] which further strengthens the differences between 12-LO isoforms. 12-LOs are very widely distributed in many cell types and tissues [46, 50, 62]. To name a few, p12-LO is expressed in human, ovine, rat and murine platelets [62, 63], human and murine epidermis cells [62, 64] and prostate [65] and skin cancer cells [66]. Leukocyte 12-LOs can be found in mouse macrophages, rat and rabbit leukocytes [50], whereas the epidermis type 12-LO so far could only be identified in mouse tissues [50]. Human 12*(R)*-LO was found to be expressed in human skin [67] aggravating its affiliation with murine epidermis-12-LO and making it the "human epidermis 12-LO". Simply considering such a variety of enzymes it is not surprising that substrate specificities and formed products also vary between the different isoenzymes [46, 50].

Leukocyte 12-LOs are able to metabolise AA as well as C18 and C22 fatty acids, and even phospholipids and esterified fatty acids can serve as a substrate [46, 68, 69]. In contrast, p12-LO are only able to metabolise C20-fatty acids [48, 69], in humans only arachidonic acid is metabolised. The epidermis isoforms preferably use esters of the respective fatty acids as substrate rather than the free non-esterified acids [50, 58]. The main metabolite of all 12-LOs consists of 12*(S)*-hydroxy-5,8,10,14-eicosatetraenoic acid (12*(S)*-HETE) [50] except for the epidermis 12*(R)*-LO and was first found in human and bovine platelets [44, 70]. Leukocyte isoforms also produce the equivalent 15*(S)*-HETE in a fixed ratio to 12*(S)*-HETE which was established in porcine leukocytes at 9:1 (12-:15-HETE) [68]. The exact numbers of the ratio between the two metabolites depends on the enzyme. This double outturn of metabolites is specific for leukocyte 12-LO, hence as mentioned the naming of the enzyme group as 12/15-LO.

1.5.2 Platelet-type 12-lipoxygenase (p12-LO)

p12-LO is only able to produce one metabolite, 12*(S)*-HETE, from its - in human - only substrate, arachidonic acid. Only this metabolite could be identified during own studies, although a 15-LO activity was proposed for

platelets as well [71]. Structures of the main 12/15-LO metabolites and a short overview of the possible metabolism pathways are given in Fig. IX [46].

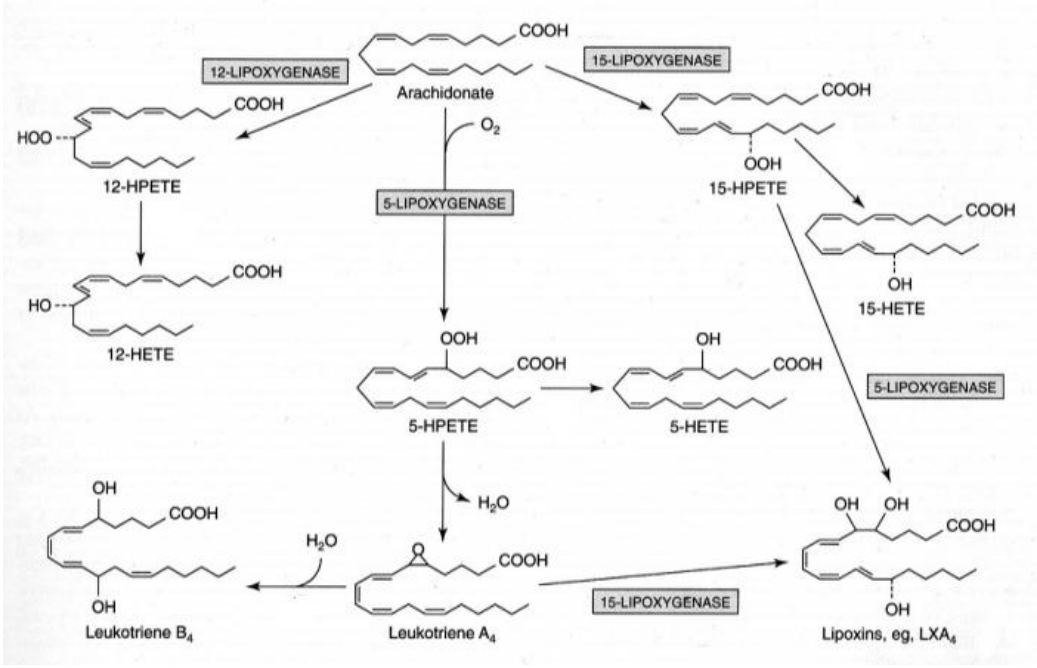

Fig. IX *Metabolism of arachidonic acid by different LOs*
(www.benbest.com/health/Lipoxygenase.jpg)

The peroxidized products (HPETEs) are metabolised to their stable products by peroxidase activity. Metabolites used as parameters for activity determination are the “HETEs”. Also duration of metabolic activity varies between the isoforms. In fact, catalytical activity of leukocyte 12-LOs ceases after a few minutes due to suicide inactivation of the enzyme [46, 58, 62, 72] in contrast to the p12-LO isoform which is active over long time periods [73]. The enzyme reaction catalyzed by p12-LO proceeds almost linearly over 30 minutes [48, 74] and p12-LO keeps producing 12-HETE over even longer periods up to 115 minutes [73]. Even without stimulation a basal spontaneous p12-LO activity in intact cells can be measured [75, 76]. 12-LOs have been studied extensively in pathophysiological contexts. Leukocyte 12/15-LO activity has been connected to many different disorders including genesis of atherosclerotic events in connection to their ability to oxidize LDL [77, 78] and diabetic disorders [79]. Epidermis-type 12-LO involvement in development of psoriasis in human has been established as its metabolite was found to be enriched in psoriatic skin [67]. p12-LO was the first mammalian LO to be identified [44] and has been extensively

studied. Still, only little is known about p12-LO activation, regulation of its activity and its physiological relevance. Its genetic locus has been precisely mapped to chromosome 17p13 [80] with a size of 13-17 kb, about twice as large as the genes for its leukocyte and epidermis isoforms. p12-LO is recognized as protein in platelet homogenates by specific antibodies as a protein of approx. 72 kDa. Its exact secondary structure can only be proposed along the crystallized structure of soybean 1-LO and rabbit reticulocyte 15-LOs [55, 56, 81], as no X-ray crystallized structure has yet been analyzed for p12-LO. A calculated structure was proposed by Aleem et al [82] and is depicted in Fig. X**.**

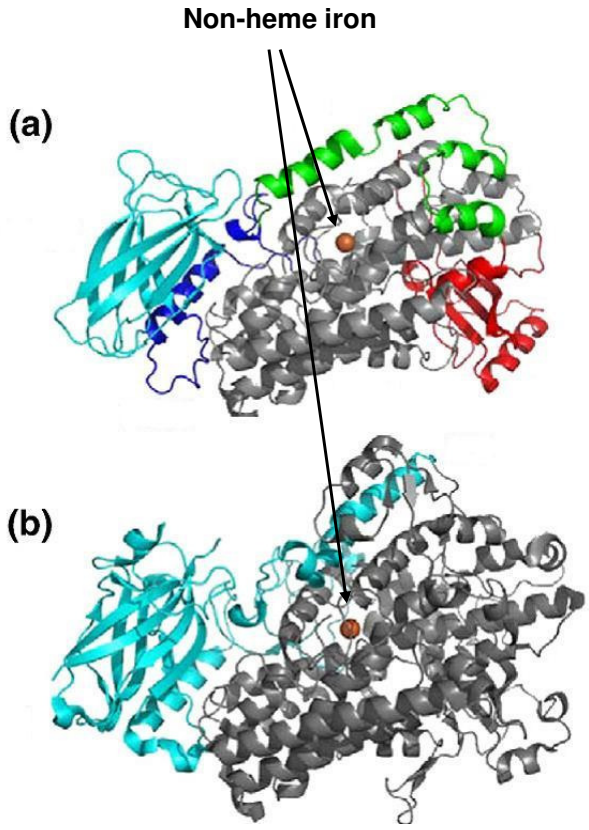

Fig. X *Proposed 3D-structure of (a) p12-LO based on (b) soybean 1-LO structure ([82] modified)*

How protein expression of p12-LO is regulated has scarcely been investigated. Nevertheless, regulation of expression by cytokines, interleukin 1 beta (IL-1β) and epidermal growth factor (EGF) has been proposed [83-85]. To keep in mind, different mechanisms of protein expression have to be assumed for p12-LO as platelets are anucleate and only able to perform limited protein biosynthesis. All p12-LO protein or the

respective mRNA must be generated before platelets are released into the blood stream.

p12-LO's main substrate arachidonic acid is metabolised to 12*(S)*-H(P)ETE and subsequently to 12*(S)*-HETE by a peroxidase activity [86]. p12-LO activity can also be induced by platelet stimulation with collagen or thrombin [87], although for collagen far higher concentrations are needed to induce p12-LO activity than for platelet activation during aggregation [69]. Thrombin concentrations used for aggregation studies are also effective to activate p12-LO [88, 89]. As collagen and thrombin are the main signalling agonists for platelets in physiological contexts [1], a connection of collagen and thrombin signalling pathways and p12-LO activating pathways can be assumed. There is evidence indicating a signalling cascade which originates at the Glycoprotein VI cluster receptor (GPVI) for collagen at the platelet membrane to activate p12-LO via PI3 kinase and $[Ca^{2+}]_i$ mobilisation [87, 90]. The proposed signalling cascade is more closely portrayed in Fig. XI [87, 90].

On the other hand, p12-LO is far more independent from $[Ca^{2+}]_i$ levels for activation as for example 5-LO in polymorphonuclear leukocytes (PMNL) [91]. In contrast, p12-LO can function without $[Ca^{2+}]_i$ if enough substrate is present [92]. A role for $[Ca^{2+}]_i$ elevation in activation of p12-LO is unclear, in particular as p12-LO displays a basal, constitutive activity. However it must be noted that most studies addressing p12-LO activation, measured as 12-H(P)ETE formation did not consider $cPLA_2$ as a critical component which releases AA as substrate for p12-LO. So to stimulate p12-LO activity also A23187 (Ca^{2+}-ionophore) and p12-LO substrate AA can be used [93].

p12-LO shows a very high throughput for its substrate arachidonic acid. ${}^{D}k_{cat}/K_m$ was found to increase temperature dependently to very large values showing a K_m of 8 µM for AA [74, 92, 94].

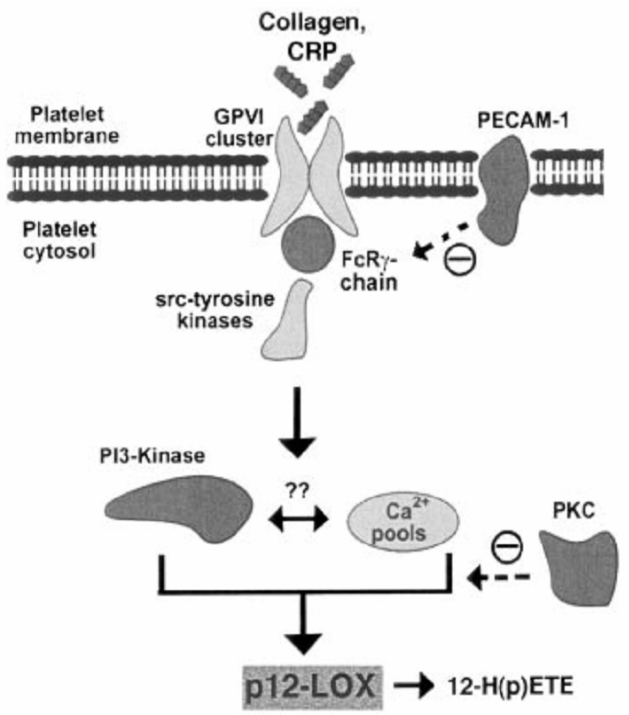

Fig. XI *Activation of p12-LO by collagen signalling ([87])*

The subcellular localisation of p12-LO in human platelets is a matter of debate. Subcellular localisation is proposed to be predominantly cytosolic by some authors [70, 74, 95, 96] or thought to be only at the platelet membranes by others [97]. In some studies p12-LO was found to be located mostly (65%) in the membrane or microsomal fractions of platelets [92]. The subcellular localisation might be connected to p12-LO activity demonstrated by translocation and activity studies of the enzyme [95, 98]. The enzyme liberating AA, $cPLA_{2,}$ translocates to the plasma membrane [15], so an accompanying translocation of p12-LO seems likely as p12-LO needs to access its substrate at the plasma membrane.
Neither regulation of p12-LO activation nor inhibition was so far correlated to a certain signalling pathway. p12-LO seems to be an enzyme that is permanently active, as a basal 12-HETE formation can be measured in non-stimulated platelets [75, 76]. Considering this circumstance an inhibitory regulation of p12-LO seems more likely than an activating regulation. PKA is a inhibitory kinase of many platelet functions and inhibitory regulation of p12-LO might be achieved via PKA signalling, even more probable since p12-LO actually contains a phosphorylation motif for PKA (Fig. XII) [99].

Fig. XII *Phosphorylation sites for PKA ([99] modified)*

To assign a pathophysiological role to p12-LO and its metabolite 12-HETE one has to discriminate between platelet and leukocyte 12-LO. Most (patho-) physiological roles of p12-LO and its metabolite 12*(S)*-HETE that have been identified so far could be attributed to both enzymes as both have the same main product and physiological functions of both enzymes may overlap. One has to take into account that some diseases like diabetes or atherosclerosis attributed to leukocyte 12-LO could well be purveyed also by p12-LO as the effective metabolite 12-HETE is produced by both enzymes. p12-LO was specifically identified to be involved in the mechanisms regulating platelet aggregation. Sekiya et al [100] were able to trace back regulation of platelet aggregation to 12-HETE, identifying a negative feedback mechanism that prevents excessive aggregation. When p12-LO was knocked out in mice using targeted gene disruption, an increased platelet sensitivity to ADP as agonist for platelet aggregation was observed [101]. In 2000, Akiba et al. found p12-LO product formation to be important for inhibition of platelet aggregation [102]. Together, p12-LO and its product 12*(S)*-HETE seem to be closely related to functioning platelet aggregation and its regulation. Also the timely progression of aggregation seems to depend on p12-LO activity and its product 12*(S)*-HETE as 12*(S)*-HETE supports the switch from primary agonist collagen to secondary agonist thrombin [103]. The most prominent connection of p12-LO product 12*(S)*-HETE to any pathophysiological events that has been observed so far is its role in cancer metastasis, invasiveness and motility of tumours as well

as angiogenesis connected to tumour growth [104-106]. p12-LO has been identified in human melanoma cell lines [107] and skin cancer cells [66] on mRNA and protein levels. Inhibition of 12-LO led to apoptosis of cancer cells in a gastric cancer cell model [108] although it was not clearly identified whether leukocyte or platelet 12-LO was investigated in this study. Nevertheless, apoptosis and cell survival of cancer cells seems to be regulated by 12-LO or its metabolite 12*(S)*-HETE [109]. Besides its involvement in skin carcinogenesis, p12-LO is closely connected to prostate cancer [110-113]. There is also evidence for a role of p12-LO in angiogenesis and tumour growth in these studies. Angiogenesis in particular seems to depend on p12-LO expression and activity [109, 113]. Also propulsion of endothelial sprout formation and modulation of angiogenesis has been proposed [114]. Invasive potential of different cancers was brought into connection with p12-LO activity as well [115]. Besides its apparently strong involvement in the different stages of cancer p12-LO is also involved in the genesis of psoriasis. 12*(S)*-HETE is a strong chemotactic stimulus for leukocytes and other epidermal cells [116] and p12-LO is expressed in epidermal cells in psoriasis [117] as is the human epidermis-type isoform12*(R)*-LO [67]. On the other hand, if one keeps in mind that p12-LO is producing its metabolite without stimulation over time periods of hours, one could possibly attribute p12-LO to “house-keeping” functions of a constitutively expressed protein with a basal continuously sustained activity.

Concerning p12-LO metabolites, not only 12-HETE should be mentioned but also lipoxins, the generation of which has been attributed to p12-LO metabolic functions. Lipoxins are tetraene containing eicosanoids with selective biological functions that are generated by single cell types or cell-cell interactions [118, 119]. In human tissues two major biosynthetic pathways for lipoxins are found, one metabolizing 15-H(P)ETE, the other being a transcellular route involving cell-cell interactions between platelets and PMNL in the conversion of leukocyte-derived LTB_4 [118, 120]. Lipoxins are involved in multifactorial events such as thrombosis and atherosclerosis whenever a cell-cell interaction of platelets and leukocytes is likely to occur *in vivo* [121].

Although p12-LO and its metabolites have been studied in many of their functions *in vivo*, still a lot of questions remain unanswered. However, a connection seems to exist between p12-LO activity and platelet functions. Moreover, as platelets are also involved in many central processes in cancer, inflammation and cardiovascular disease [4, 122, 123], a role for p12-LO and its metabolite 12-HETE seems likely.

1.6 Gender bias in inflammatory and cardiovascular disease

Modern medicine has increasingly focused on gender medicine in the last years. Sex hormones (progesterone, testosterone, estradiol) do not only affect sexual maturation and reproduction but also influence the immune system [124]. In the field of inflammatory diseases, a clear gender bias could be identified and the susceptibility for certain inflammatory diseases is far more prominent in the female sex. More women than men are affected by autoimmune disorders as rheumatoid arthritis and systemic lupus erythematosus. This observation has been connected to sex hormone levels and different production of cytokines [124, 125]. Other inflammatory diseases as asthma have been found to shift to a higher incidence in females after sexual maturity, an outcome related to a change in hormonal status after puberty [126]. In 2008, Pergola et al. proposed a pharmacological mechanism underlying gender disparities in inflammatory diseases linked to 5-LO activity and leukotriene formation [127]. Inhibitory modulation of 5-LO by sex hormones, in particular androgens, was uncovered explaining a lower leukotriene formation and therefore lower incidence for inflammatory diseases in the male sex.

But not only in inflammation is a gender bias a well known fact. Thus, an excess of cardiovascular complications in the male sex in comparison to the female sex has been observed **[128]**. As risks for cardiovascular disease rise after menopause, a regulation of platelet aggregation and thrombosis by female sex hormones, especially by progesterone, seems likely as progesterone production in the female ceases after menopause [129]. A high risk of cardiovascular events also accompanies the use of oral contraceptives that change the hormonal status in females. Oral hormone replacement therapy after menopause reduces cardiovascular risks [130]. Some studies

performed *in vivo* or *in vitro* established a differential regulation of platelet aggregation between male and female species. Platelet aggregation and secretion diminished in platelets of female pigs after sexual maturity in comparison to platelets isolated from male pigs [131], platelets isolated from male rats showed a greater aggregation than female platelets [132], and platelets from male and female human donors reacted differently to low-dose aspirin therapy [133]. However, the results are not as clear as for inflammatory disease and some groups found female platelets from different species to be more reactive than male, at least in untreated conditions [133, 134]. Nevertheless, a prominent role for sex hormones in regulation of different disorders seems obvious.

1.7 Progesterone and progesterone receptors

Progesterone is mainly involved in the regulation of normal female reproductive function controlling release of mature oocytes, facilitation of implantation and maintenance of early pregnancy, promoting uterine growth and suppressing myometrial contractility in uterus and ovaries, preparing breast tissue for milk secretion and milk protein production in the mammary gland, and mediating signals required for sexually responsive behaviour in the brain [135]. Progesterone (Fig. XIII) is synthesised mainly in the ovaries [135].

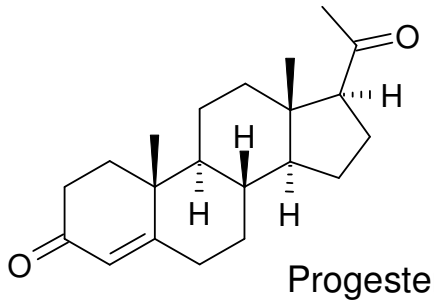

Fig. XIII *Chemical structure of progesterone and progesterone plasma levels*

Its secretion changes strongly during the menstrual cycle which can roughly be divided into two main phases, the follicular phase where progesterone plasma levels are low and the luteal phase with a high plasma level of progesterone (Fig. XIV). Before ovulation granulosa cells in the follicle are responsible for biosynthesis and secretion of estradiol (follicular phase). After follicle rupture (ovulation) the same granulosa cells form the corpus

luteum and as such start secreting progesterone (luteal phase). If no fertilisation occurs, the corpus luteum will degrade and menstruation proceeds. If fertilisation occurs, the corpus luteum continues to secrete progesterone during the first two to three months of early pregnancy until the placenta commences progesterone biosynthesis for pregnancy maintenance [135]. Progesterone plasma levels rise to very high concentrations of approximately 1000 nmol/l or even higher during pregnancy, the highest levels are reached at the end of the third trimester.

Phase/Sex	**follicular**	**luteal**	**menopause**	**pregnancy**	**male**
progesterone concentration (nmol/l)	< 5	3.5 - 67	< 4.4	< 1000	0.06 - 5.3

Fig. XIV *Progesterone plasma levels (http://www.biorama.ch/biblio/b90laka/lakap/pro010.htm)*

Once released from the ovaries, progesterone is transported in the blood by a progesterone carrying protein, transcortin, as opposed to binding of estradiol and testosterone to sex hormone binding globulin (SHBG) [136]. Finally in menopause, progesterone synthesis and secretion are reduced [129] as natural female fertility comes to an end. A short overview about the most important functions of progesterone in its target tissues during the normal reproductive phase of females is also given in Fig. XV.

Tissue	Function
Uterus/ovaries	Release of oocytes Facilitation of implantation Maintenance of pregnancy: *via myometrial quietening* Stimulation of stromal regeneration: *luteal phase of cycle*
Mammary gland	Lobular alveolar development Suppression of milk protein synthesis during pregnancy
Brain	Mediation of sexual responsiveness
Bone	Regulation of bone mass: *prevention of bone loss*

Fig. XV *progesterone functions in target tissues ([135])*

Effects of progesterone have classically been attributed to primarily genomic influences affecting protein expression and activation of nuclear receptors. Those “classical” modes of action of progesterone include regulation of gene expression in reproductional tissues as uterus and ovaries. Progesterone effects are mediated by its classical nuclear receptors that have initially been characterized in mammalian uterus in the early 70’s [137] and have been

found since then in many other human tissues. Two receptor subtypes have been identified and were characterized as progesterone receptors (PR) A and B, with the molecular masses of 81 and 115 kDa, respectively. The classical PRs fit into a large family of ligand-activated nuclear transcription regulators including receptors for other steroids as androgens or estradiol, retinoids, vitamin D and thyroid hormones [135]. In the absence of their ligand (i.e., progesterone) steroid receptors as PR exist in the cells as inactive complexes in the cytoplasm. Upon ligand binding they translocate to the nucleus and activate transcription of target genes [138], promoting protein biosynthesis and cell growth. Detailed molecular mechanisms of these regulatory functions are not known [135].
A second mode of action for steroid receptors besides the one described above mediates its actions via direct protein-protein interaction with transcriptional factors, repressing the activity of these factors. Those two modes of action both require translocation of the receptor-ligand complex to the nucleus and take at least 30 to 60 min [138].

In the last few years, other regulatory pathways of steroid hormones as progesterone have been proposed, mediating steroid effects in a fast, non-genomic way by activation of kinase signalling pathways or by direct influence on cellular structures [139]. So far, direct activation of MAP kinases, adenylylcyclase, PKC and GPCR has been described [138, 140]. Some of these actions are conveyed by the classical receptors but also new membrane-bound receptor subtypes have been identified proposing a completely new concept for sex hormone actions. A detailed classification of possible modes of non-genomic actions is given after the so-called Mannheim Classification in Fig. XVI.

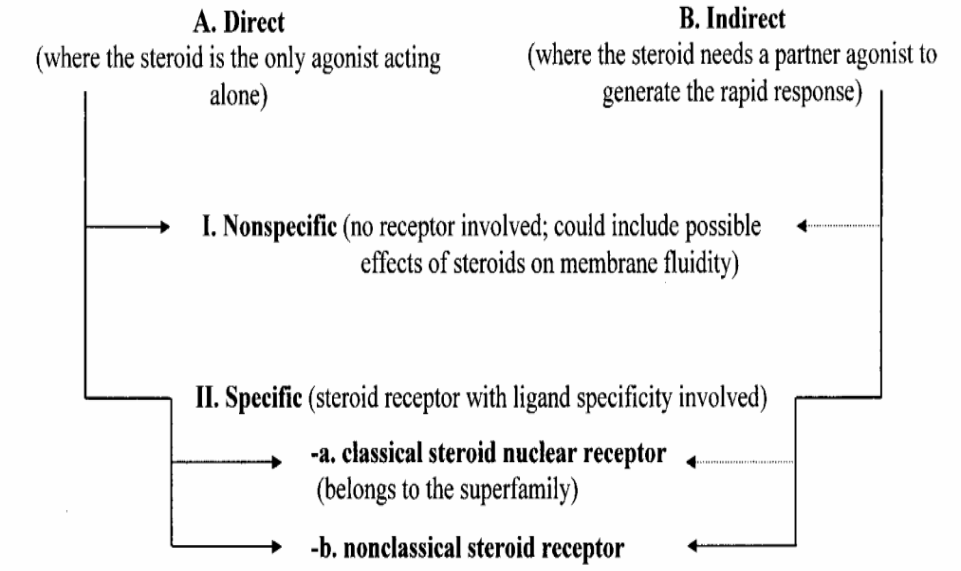

Fig. XVI *Mannheim classification of non-genomic steroid actions [141]*

Note that genomic actions are always connected to the existence of a nucleus in the cell and the possibility of *de novo* protein and mRNA synthesis. Genomic actions of progesterone depend on binding of progesterone plus its receptor to nuclear DNA and always affect their target tissue via regulation of DNA transcription and protein translation. This difference between genomic and non-genomic effects cannot be stressed enough (Fig. XVII).

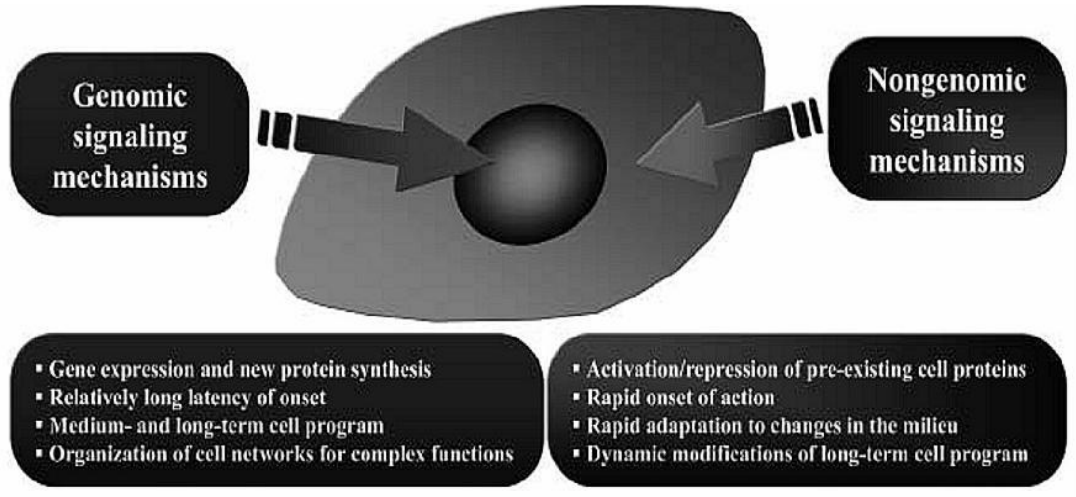

Fig. XVII *Differentiation between genomic and non-genomic actions ([139])*

Genomic regulatory effects in this definition cannot be observed in anucleate cells as platelets. Recently, also membrane-bound receptors unlike the classical steroid receptors could be identified by the use of BSA-coupled

and fluorescence marked steroids. Steroids could not enter the cells but still conveyed their actions although localised extracellularly at the cell membranes [141] where binding to non-nuclear receptors would occur. A good example for rapid actions of progesterone is the acrosome reaction observed in sperm cell maturation. Thus, progesterone induces sperm cell maturation in human sperm by stimulating a rapid $[Ca^{2+}]_i$ elevation and induction of acrosome reaction. In this process, a $[Ca^{2+}]_i$ dependent rise in intracellular cAMP leads to activation of PKA. Involvement of PKC has also been discussed [142, 143]. As classical progesterone receptor antagonist, RU 486 (mifepristone) that has a very high affinity to the progesterone receptor, may be even higher than progesterone itself [144] is only a weak antagonist of the progesterone induced acrosome reaction. Synthetic progestins normally potent activators of transcription via progesterone receptors are also only weak inducers of the acrosome reaction, the participation of a different kind of progesterone receptor was implied [141]. The presence of N-terminally truncated forms of the classical PR of approximately 50 kDa that could be detected by an antibody against the c-terminus of PR were reported and characterized by one group [145, 146]. This truncated form predominantly localizes to the plasma membrane and could be the mediator of progesterone effects on acrosome reaction via PKA and PKC. Non-genomic actions of steroid receptors that might be mediated by novel membrane receptors propose a new opportunity to explain rapid actions of the respective ligands that so far have only been connected to genomic regulation of protein expression and long-term effects. Progesterone aptitudes in induction of various reactions and activation of different signalling pathways are far more diverse than has been suggested to this point. Progesterone might be involved in rapid regulation of enzyme properties in nucleated as well as in non-nucleated cells.

1.8 Cinnamyl-3,4-hydroxy-α-cyanocinnamate (CDC)

In 1991, a series of new caffeic acid derivatives was synthesised that were identified to have a high potency to inhibit p12-LO [147].

One of these derivatives is now known as cinnamyl-3,4 hydroxy-α-cyanocinnamate or CDC. Its chemical structure is displayed in Fig. XVIII.

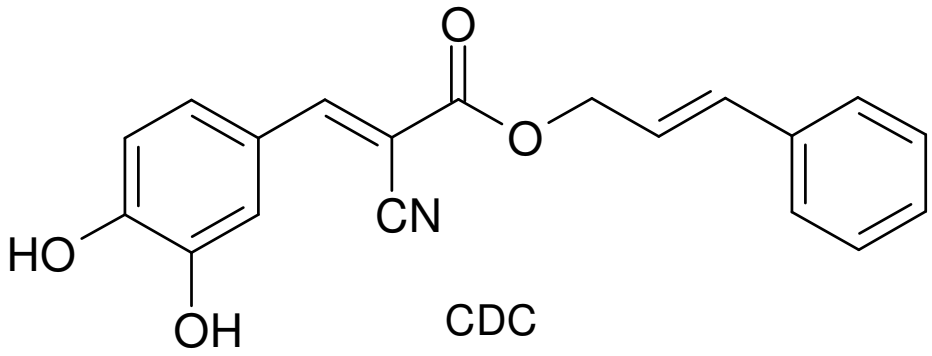

Fig. XVIII *Chemical structure of CDC*

The substance is coloured in an intensive yellow which can be explained by its extensive system of electron delocalisation. In standard HPLC analysis also a strong UV absorption of CDC at 280 nm can be observed at concentrations above 3 µM. Caffeic acid itself was known as a LO inhibitor with a high potency although a low selectivity for LO that blocks 5- as well as 15-LO activities [148, 149]. Introduced modifications were found to increase selectivity towards 12-LO. Inhibitor screening tests revealed an IC_{50} of 0.063 µM for isolated enzyme preparations of p12-LO [147]. The IC_{50} determined for 5- and 15-LO were much higher, 1.89 µM and 3.33 µM, respectively. Hence, CDC was designated a selective p12-LO inhibitor. However, CDC was only defined in cell-free enzyme preparations whereas cellular conditions have been neglected. Neither was defined which class of LO inhibitor it belonged to as there are many different possible mechanisms for LO inhibition such as iron ligand, redox active or non-redox competivtve inhibitors [91]. Still, CDC is often used to inhibit p12-LO or leukocyte 12-LO in cellular assays to determine the pathophysiological role of 12-LOs and their metabolites in cancer metastasis [113], monocyte adhesion [150], diabetic nephropathy [151], hypertension [152], vasodilatation [153], platelet activation [154] and many other research projects investigating disorders possibly connected to 12-LOs. In regard of the chemical structure of CDC, some other properties are brought to mind as CDC exhibits a potent redox system at its hydroxylated phenyl moiety and also possible iron- or Ca^{2+} -chelating properties could be proposed. In case CDC would change intracellular redox state of investigated cells or block $[Ca^{2+}]_i$ signalling by chelation, the use of CDC as “12-LO inhibitor” might lead to fatal misinterpretations of obtained results for involvement of

investigated cell functions in certain disorders. Many possibilities and molecular targets yet undiscovered necessitate a more thorough investigation of CDC functions in cellular systems.

1.9 MK-886

The indole derivative 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid (sodium salt) (Fig. XIX), also known as MK-886, was first identified as a potent inhibitor of leukotriene biosynthesis in intact leukocytes without directly inhibiting 5-LO or phospholipase A_2 [155]. MK-886 rather binds to the 5-LO activating protein (FLAP) that is needed for 5-LO activity [156] thus limiting 5-LO product formation by limiting 5-LOs access to its substrate AA with an IC_{50} of 2.5 nM [157]. In this way FLAP inhibition by MK-886 represents a new strategy of leukotriene biosynthesis inhibition apart from direct 5-LO suppression [158]. MK-886 shares structural similarities with other FLAP inhibitors as licofelone (Fig. XVIII) and MK-591 (not shown).

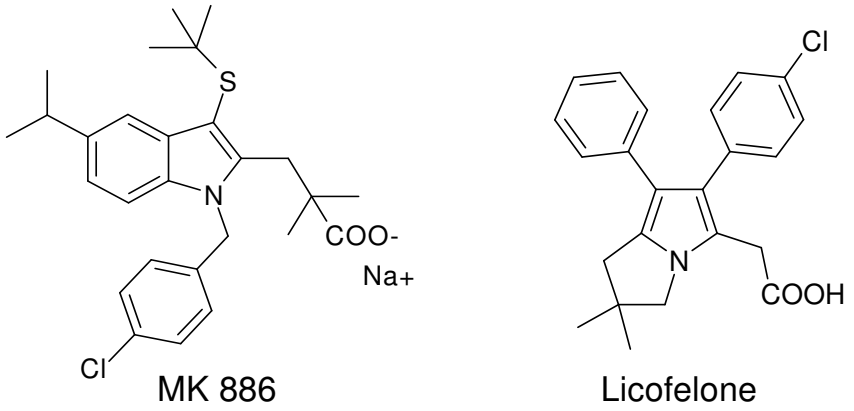

Fig. XIX *Chemical structures of MK-886 and licofelone*

Utilizing the high affinity and selectivity of MK-886 towards its target FLAP has helped as a pharmacological tool to assess the 5-LO pathway in many studies. MK-886 is used as a reference inhibitor for screenings of new 5-LO inhibitors or to discern the role of 5-LO in certain disorders as cancers or inflammatory diseases. However, some additional targets for MK-886 have been described. It not only affects 5-LO and FLAP but also was described to affect leukotriene biosynthesis by interference with leukotriene C4 synthase [159]. MK-886 also impairs prostaglandin E_2 formation by

interference with microsomal PGE_2 synthase [160]. Also COX-1 activity and platelet aggregation were recently found to be inhibited by MK-886 [157]. The peroxisome-proliferator-activated receptor α (PPARα) is also inhibited by MK-886 [161, 162]. Moreover, for MK-886 actions have been described that could not clearly be connected to inhibition of leukotriene synthesis. MK-886 also has antiproliferative effects and was found to induce apoptosis in various cancer cell lines [163-167], but only in concentrations far higher than needed for inhibition of leukotriene biosynthesis (1-50 µM). Some of these effects were more importantly found in cells that do not express 5-LO [163, 167] indicating a mechanism completely independent from 5-LO and pointing to other possible targets of MK-886. Interestingly, some other effects apart from enzyme inhibitions were recently identified for MK-886. Apparently it influences $[Ca^{2+}]_i$ levels in prostate carcinoma cells (PC-3) [168] and Madin Darby kidney cells [169]. Effective concentrations for $[Ca^{2+}]_i$ mobilisation also lay above those needed for inhibition of leukotriene biosynthesis with 10-50 µM as was the case for induction of apoptosis. Still, as $[Ca^{2+}]_i$ is a strong modulator of many intracellular functions and proteins, especially in polymorphonucelar leukocytes (PMNL) that also express 5-LO, a $[Ca^{2+}]_i$ elevating effect of MK-886 could have a tremendous impact on cell activity and enzyme regulation pathways. In this respect MK-886 should be reassessed in its function as a pharmacological tool.

1.10 Aim of the Study

p12-LO is the LO that was first discovered in mammals and still only little is known about the regulation of activity and about its pharmacological intervention. p12-LO is highly expressed and constitutively active in platelets. Although a possible function for regulation of platelet activation and aggregation was attributed to p12-LO activity and metabolite 12-HETE implicating a possibly prominent role for p12-LO in cardiovascular disease, a comprehensive pharmacological characterization of p12-LO activity and regulation is yet missing and urgently needed. When it became clear that a rapid regulation of human 5-LO in PMNL by androgens was responsible for a gender bias in inflammatory disease [127], a study of possible regulatory effects of sex hormones on p12-LO seemed the most obvious approach for a

characterization of p12-LO regulation. Both enzymes share a high homology in amino acid structure, both are expressed in cells of the blood lineage (leukocytes and platelets) and both are involved in the genesis of diseases with a strong gender bias, inflammatory disease for 5-LO and cardiovascular disease for p12-LO [126, 128]. In cardiovascular disease, female sex hormones are suspected to have a protective benefit. Regulatory influences and mechanisms of action of sex hormones have long since been studied; the main focus was directed on research of long-term genomic influences regarding transcriptional modifications. In the recent years, a different kind of influence was discovered: rapid non-genomic actions of sex hormones that directly influence intracellular signalling events. For example, on one hand androgenic regulation of 5-LO was too rapid to be mediated by classical genomic effects although the effect was observed in nucleate cells (PMNL) where genomic regulation would at least be possible [127]. Platelets on the other hand are anucleate cells, so classical genomic influences of sex hormones are not possible.

The aim of this study was to characterize p12-LO regulation of activity more clearly and to characterise its pharmacological inhibitors. First, a possible sex difference in regulation was investigated. Influence of human sex hormones estradiol, testosterone and progesterone on p12-LO activity in intact and cell free systems was determined and possible regulatory pathways were elucidated. Secondly, typical pharmacological tools to study p12-LO regulation were applied. Thus, the long recognized selective p12-LO inhibitor CDC and the FLAP inhibitor MK886 that should not influence p12-LO activity at all as platelets do not express FLAP were investigated. With these approaches a better characterization of p12-LO properties in intact cells was within reach and results of these studies are presented in this work.

2 Materials and Methods

2.1 Materials

[^{3}H]AA BioTrend Chemicals (Köln, Germany), 8-bromo-cAMP Biomol (Plymouth Meeting, PA, USA), AA Sigma-Aldrich (Deideshofen, Germany), β-mercaptoethanol Carl Roth (Karlsruhe, Germany), A23187 (Ca^{2+}-ionophore) Sigma-Aldrich (Deideshofen, Germany), CDC Biomol (Plymouth Meeting, PA, USA), COX-1 (ovine) Cayman Chemical (Ann Arbor, MI, USA), COX-2 (human recombinant) Cayman Chemical (Ann Arbor, MI, USA), $cPLA_2$ inhibitor Calbiochem (Bad Soden, Germany), Cyproterone acetate LKT Laboratories (St. Paul, MN, USA), 2,7-Dichlorofluorescin diacetate Sigma-Aldrich (Deideshofen, Germany), Dihydrotestosterone Sigma-Aldrich (Deideshofen, Germany), DMSO Carl Roth (Karlsruhe, Germany), Ethanol Merck (Darmstadt, Germany), Estradiol Sigma-Aldrich (Deideshofen, Germany), Fatty acid-free BSA Sigma-Aldrich (Deideshofen, Germany), Fura-2/AM Alexis Corp (Lausen, Switzerland), H89 Cayman Chemical (Ann Arbor, MI, USA), HEPES Applichem GmbH (Darmstadt, Germany), Hybond™ ECL Membrane GE Healthcare (Munich, Germany), Kollagenreagenz Horm® Nycomed Pharma GmbH (Wien, Austria), Methanol Merck (Darmstadt, Germany), Mifepristone Cayman Chemical (Ann Arbor, MI, USA), MK-886 BioTrend Chemicals (Köln, Germany), LSM 1077 Lymphocyte Separation Medium PAA (Coelbe, Germany), Plastic/PS-materials Greiner bio-one (Frickenhausen, Germany), PGE_2 Cayman Chemical (Ann Arbor, MI, USA), peqGold Protein Marker IV peqLab Biotechnology, (Erlangen, Germany), Progesterone Sigma-Aldrich (Deideshofen, Germany), PMA Alexis Corp (Lausen, Switzerland), SDS Carl Roth (Karlsruhe, Germany), Thrombin Sigma-Aldrich (Deideshofen, Germany), Tween 20 Carl Roth (Karlsruhe, Germany), U-46619 Calbiochem (Bad Soden, Germany), Ultima Gold™ XR Perkin Elmer (Boston, MA, USA)
Primary antibodies Cell Signalling (Boston, MA, USA), secondary antibodies Cy-5 labelled GE Healthcare/ Amersham (Munich, Germany), alkaline phosphatase labelled Sigma Aldrich (Deideshofen, Germany)

All other chemicals were purchased in analytical grade from Sigma-Aldrich (Deideshofen, Germany) or Applichem GmbH (Darmstadt, Germany) unless stated otherwise.
p12-LO primary antibody was a generous gift of Prof. C. Funk (Queen's University, Kingston Ontario, Canada).

2.2 Methods

2.2.1 Isolation of human platelets from venous blood

Venous blood from healthy donors was taken and leukocyte concentrates (buffy coats) were prepared by centrifugation at 4,000 × g, 20 min, room temperature (RT). Buffy coats were diluted 1:1 (v/v) with phosphate buffered saline pH 7.4 (PBS) and then with ice-cold 5% dextrane (w/v in PBS) in a ratio 1:4 (v/v), for sedimentation for 45 min. After dextrane sedimentation cells were immediately separated by centrifugation at 1,000 × g, 10 min, RT, w/o brake (Heraeus Sepatech, Varifuge 3.0, Hanau, Germany) on LSM 1077 Lymphocyte cushions. Platelets were isolated from supernatants after centrifugation of leukocyte concentrates to obtain platelet rich plasma (PRP). PRP was then mixed with ice-cold PBS pH 5.9 (3:2, v/v), centrifuged at 1,000 × g, 10 min, 4°C (Beckman GS-6R Centrifuge, Beckman Coulter, Fullerton, CA, USA) and the pelleted platelets were resuspended in ice-cold PBS pH 5.9/NaCl 0.9% (1:1, v/v) and washed by centrifugation (1,000 × g, 10 min, 4°C). Preparation of platelets at pH 5.9 is thought to minimize temperature activation**.** Finally, platelets were resuspended in ice-cold PBS pH 7.4 buffer containing 1 mg/ml glucose (PG buffer) as specified and kept on ice until further use. For assays determining $[Ca^{2+}]_i$ platelets were isolated from buffy coats as described above but kept at room temperature until Fura-2 loading.

In experiments investigating platelet aggregation, human platelets were freshly isolated from venous blood of healthy donors (Blood Centre, University Hospital Tübingen, Germany) who had not taken any medication for at least 10 days. Briefly, venous blood was collected in monovettes (Monovette®, Sarstedt, Nümbrecht, Germany) containing 0.106 mol/l trisodium citrate solution. PRP was obtained after centrifugation of whole blood at 200 × g, 15 min, RT w/o brake and placed into 15 ml conical tubes containing 10% (v/v) ACD-buffer (85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose) [170]. PRP was centrifuged at 800 × g, 10 min, RT, pelleted platelets were washed twice and finally resuspended in Tyrode´s buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.4, cells were kept at RT at all times.

2.2.2 Isolation of human polymorphonuclear leukocytes (PMNL) from venous blood

Human polymorphonuclear leukocytes (PMNL) were freshly isolated from buffy coats obtained from the Blood Centre, University Hospital Tübingen (Germany). In brief, leukocyte concentrates were prepared by centrifugation at 4,000 × g, 20 min, RT. Buffy coats were diluted 1:1 (v/v) with ice-cold PBS pH 7.4 and then with ice-

cold 5% dextrane (w/v in PBS) in a ratio 1:4 (v/v), for sedimentation for 45 min. After dextrane sedimentation neutrophils were immediately isolated by centrifugation at 1,000 × g, 10 min, RT, w/o brake (Heraeus sepatech, Varifuge 3.0, Hanau, Germany) on Lymphoprep cushions, and hypotonic lysis of erythrocytes as described [171]**.** PMNL (1×10^7 cells /ml; purity > 96-97%) were finally resuspended in PG buffer.

2.2.3 Preparation of platelet rich plasma (PRP) and plasma from venous blood

PRP was obtained from venous blood from healthy male and female donors (Blood Centre, University Hospital Tübingen, Germany) who had not taken any medication for at least 10 days. Briefly, venous blood was collected in monovettes (Monovette®, Sarstedt, Nümbrecht, Germany) containing 0.106 mol/l trisodium citrate solution. Monovettes were centrifuged at 200 × g, 15 min, RT w/o brake. The resulting supernatant (PRP) was used directly for incubations and determination of p12-LO and COX-1 activity in PRP. For preparation of plasma venous blood from healthy male and female donors (Blood Centre, University Hospital Tübingen, Germany) who had not taken any medication for at least 10 days was collected in monovettes (Monovette®, Sarstedt, Nümbrecht, Germany) containing 0.106 mol/l trisodium citrate solution. Monovettes were directly centrifuged at 600 × g, 12 min, RT and plasma as resulting supernatant was collected. The supernatant was analyzed to confirm the absence of cellular contaminations and stored at -20°C until further use [127]. If necessary, hormone plasma levels were determined at the Central Lab of Tübingen University (Germany).

2.2.4 Determination of p12-LO and COX-1 product formation in intact human platelets

As p12-LO and COX-1 are the major enzymes in platelets converting AA into oxidized metabolites 12-HETE and 12-HHT [86, 172], those AA metabolites were used as parameters to determine p12-LO and COX-1 activity. Freshly isolated platelets (10^8/ml in PG buffer) were supplemented with 1 mM $CaCl_2$. Washed platelets were pre-incubated with the indicated agents at 37°C for the indicated times. Reactions were either terminated directly after the indicated times without platelet stimulation to determine spontaneous basal 12-HETE and 12-HHT formation or p12-LO and COX-1 product formation was activated by cell stimulation with thrombin (1 U/ml), A23187 (1/2.5/3 µM), collagen (8/12 µg/ml) or AA (1/3/10 µM) in the indicted concentrations. In this case, the reactions were stopped 10 min after addition of stimulus with 1 ml of ice-cold methanol and 30 µl

HCl, 200 ng prostaglandin B_1 (PGB_1, internal standard) and 500 µl PBS were added. The p12-LO (12-HETE) and COX-1 (12-HHT) products were extracted and then analyzed by HPLC as described [75, 127, 173]. In detail, after centrifugation (800 × g, 10 min, RT) samples were applied to C-18 solid-phase extraction columns (100 mg; IST, Mid Glamorgan, UK), which had been preconditioned with 100 % methanol, 25% methanol and water (1ml, each). The columns were washed with 1 ml water, 1 ml methanol 25% (v/v) and p12-LO and COX-1 products were eluted with 300 µl methanol and then diluted with 120 µl water. 100 µl diluted extract was analyzed by HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters (Eschborn, Germany)) using 76% methanol aq. + 0.007% TFA (v/v) as mobile phase at a flow rate of 1.2 ml/min and UV detection at 235/280 nm. The amount of 12-HETE and 12-HHT was determined by peak area integration. p12-LO and COX-1 product formation is expressed as ng of 12-HETE or 12-HHT per 10^8 cells.

2.2.5 Determination of p12-LO and COX-1 activity in human whole blood and PRP

For assays in whole blood, freshly drawn blood from healthy male and female adult donors was obtained by venipuncture and collected in monovettes containing 0.106 mol/l trisodium citrate solution (Sarstedt, Nümbrecht, Germany). Aliquots of 2 ml were pre-incubated with the test compounds or with vehicle for 20 min at 37°C, and formation of p12-LO and COX-1 products was started by addition of A23187 (30 µM). The reaction was stopped on ice and the samples were centrifuged at 600 × g, 10 min, 4°C (Thermofisher MicromaxRX, Fisher Scientific, Schwerte, Germany). Aliquots of the resulting plasma (500 µl) were then mixed with 2 ml of methanol and 200 ng PGB_1 were added as internal standard. The samples were placed at -20°C for 2 h and centrifuged again (600 × g, 15 min, 4°C). The supernatants were collected and diluted with 2.5 ml PBS and 75 µl HCl (1M). Formed 12-HETE and 12-HHT was extracted and analyzed by HPLC as described for intact human platelets in 2.2.4. For analysis of p12-LO and COX-1 activity in platelet rich plasma (PRP), PRP was prepared from freshly drawn blood obtained from healthy male and female adult donors as described above. PRP was prepared by centrifugation of the monovettes as stated in 2.2.3 and the resulting supernatant was directly used for incubations. Reactions were stopped by addition of 2 ml methanol to 500 µl PRP and further sample preparation was performed as stated above for whole blood samples.

2.2.6 Determination of p12-LO and COX-1 product formation in platelet homogenates

Freshly isolated washed platelets in PBS buffer containing 1 mM EDTA were homogenized by sonification (3 × 20 s, 4°C**,** Branson Sonifier Cell Disruptor B15, Branson , Danbury, CT, USA) in a small volume (1 ml platelet suspension max.)**.** Cell rupture was checked under the microscope. Cell homogenates were diluted to the desired volume (1 ml corresponding to 10^8 cells) and were pre-incubated with the indicated substances for 10 or 20 min on ice respectively with 2 mM $CaCl_2$ present. Homogenates were then incubated at 37°C with or without addition of AA (0.2/10 µM) as substrate for further 10 min. After 10 min at 37°C the reactions were stopped with 1 ml of ice-cold methanol and 30 µl HCl, 200 ng prostaglandin B_1 ((PGB_1), internal standard) and 500 µl PBS were added. The p12-LO (12-HETE) and COX-1 (12-HHT) products were extracted and then analyzed by HPLC as described in 2.2.4**.**

2.2.7 Determination of cell-free COX-1/-2 activity

Inhibition of the activities of isolated ovine COX-1 and human COX-2 was performed as described [174, 175]**.** Though the purified COX-1 is not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effectiveness of compounds on the activity of isolated COX-1 enzyme [175]**.** Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris buffer pH 8.5 mM GSH, 5 µM haemoglobin, 100 µM EDTA at 4°C and pre-incubated with the test compounds for 5 min. Samples were pre-warmed for 60 s at 37°C and AA (5 µM for COX-1 and 2 µM for COX-2) was added to start the reaction for additional 5 min at 37 °C. Reactions were stopped with 1 ml of ice-cold methanol and 30 µl HCl, 200 ng prostaglandin B1 ((PGB1), internal standard) and 500 µl PBS were added and COX product 12-HHT was extracted and then analyzed by HPLC as described in 2.2.4 [75, 173]. The amount of 12-HHT was determined by peak area integration. COX-1/2 product formation is expressed as % rest activity of solvent control.

2.2.8 Determination of TXB_2-levels in human platelets

To assess stimulus-induced TXB_2 formation, freshly isolated platelets (10^8/ml in PG buffer) were pre-incubated with the indicated compounds for 20 min at 37°C before stimulation with thrombin (1 U/ml), A23718 (3 µM), collagen (8 µg/ml) or AA (3 µM) and further incubation for 10 min at 37°C. After 10 min, reactions were stopped on ice, cells were centrifuged at 1,000 × g, 10 min, 4°C (Beckman GS-6R, Beckman Coulter, Fullerton, CA, USA) and TXB_2 was quantified in the resulting

supernatants using a TXB_2 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.2.9 Determination of AA release in human platelets and cell-free $cPLA_2$ activity

Human PRP was labelled with 19.2 nM [^{3}H] AA (1 µCi/ml, specific activity 200 Ci/mmol) for 2 hours at 37°C. Then, cells were washed twice with PBS pH 5.9 plus 1 mM $MgCl_2$, 11.5 mM $NaHCO_3$, 1 mg/ml glucose, and 1 mg/ml fatty acid-free BSA. Platelets were finally resuspended in PGC buffer (10^8/ml) and subsequently stimulated with thrombin (1 U/ml) or A23187 (2.5 µM) for 5 min at 37°C and then put on ice for 10 min, followed by centrifugation (5,000 × g, 15 min). Aliquots (300 µl) of the supernatants were mixed with 2 mL of Ultima Gold™ XR and measured (Micro Beta Trilux, Perkin Elmer) to detect the amounts of [^{3}H]-labelled AA released into the medium. [176]**.** To measure AA release over long incubation times, a method for AA derivatization was applied. In short, freshly isolated human platelets (10^8/ml cells in PG buffer) were supplemented with the p12-LO inhibitor CDC (15 µM) to avoid the conversion of released AA to LO and COX-1 metabolites. The reaction was started by addition of the indicated test compounds for 30 to 120 min. Reactions were either terminated after the indicated incubation times or cells were stimulated with A23187 (2.5 µM). After 5 min at 37°C, the reaction was stopped by addition of 2 ml methanol. After adjusting pH 3 and addition of magarinic acid (11.1 nmol) as internal standard, released AA was separated by solid phase extraction (RP-18 material and elution with methanol) and coupled to 2,4-dimethoxyaniline hydrochloride (0.14 mg, 0.75 µmol) using N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide (1.46 mg, 9.4 µmol) as coupling reagent for 1 h at 37°C. Derivatized AA was analyzed by RP-HPLC (gradient: 71% methanol / 29% water to 100% methanol in 20 min, detection at 272 nm). Amounts of released AA were determined by comparison of peak areas to standard (margarinic acid) and were expressed in % release of standard [127]. To determine the activity of cell-free $cPLA_2$ recombinant $cPLA_2$ was expressed in SF-9 cells and purified by affinity chromatography. Vesicle solutions were prepared freshly for each assay: In detail, 50 nmol lipids (i.e. a mixture of PAPC (= 1-Palmitoyl-2-Arachidonyl-sn-Glycero-3-Phosphocholine) and POG (= 1-Palmitoyl-2-Oleoyl-sn-Glycerol) 2:1) were dried completely under nitrogen at continuous rotation. 190 µl of vesicle buffer (20 mM Tris pH 7.4, 134 mM NaCl, 1 mg/ml fatty acid free-BSA) were added. To disperse the lipids homogenously, the mixture was put through 2-3 freeze-thaw cycles (N_2 liq./ 37°C) and the solution was extruded 11 times through a polycarbonate membrane (pore size 100 nm) to form LUV (large unilamellar

vesicles). For each sample 190 µl of vesicle solution was used. The enzyme solution contained 500 ng/µl purified $cPLA_2$ in TNG buffer (10 mM Tris pH 8.0, 300 mM NaCl, 20 % Glycerol) diluted 1:10 (v/v). For the assay, to 190 µl vesicle solution, 1mM EDTA or 1 mM $CaCl_2$, the respective substances and 10 µl of enzyme solution were added. The mixture was incubated for 60 min at 37°C. Reaction was stopped with 1.6 ml of methanol, and 1 nmol γ-linolenic acid (internal standard), 40 µl HCl (1N) and 1.6 ml PBS were added. Released AA was separated by solid phase extraction (RP-18 material and elution with methanol), derivatized with p-anisidinium chloride (Merck) and analyzed by HPLC as described above with detection at 250 nm.

2.2.10 Determination of reversibility of inhibition

Washed human platelets (10^8/ml in PG buffer) were incubated with the indicated compounds for 30 min. Reactions were stopped on ice and cells were centrifuged at 1,000 × g, 10 min, 4°C (Beckman GS-6R, Beckman Coulter, Fullerton, CA, USA). Buffer was removed and replaced by PG buffer containing BSA fatty acid free 1 mg/ml (PG-BSA buffer) in half the samples to bind the examined substances and remove them from the cell sample. The other half was used as control samples where no buffer exchange was performed, although they were centrifuged in the same way as the washed samples. Cells were resuspended in the respective buffers, centrifuged again at the same conditions, and PG-BSA buffer was removed and replaced with fresh PG buffer. Cells were incubated for another 30 min and reactions were terminated by addition of 1 ml methanol. p12-LO and COX-1 products were extracted and analyzed by RP-HPLC as described in 2.2.4.

2.2.11 Determination of p12-LO translocation

Freshly isolated human platelets (1×10^9/ml in PG buffer) were supplemented with $CaCl_2$ 1 mM and aliquoted (1 ml) to 1.5 ml Eppendorf cups. Samples were pre-warmed at 37°C for 5 min on the heating block. To determine the influence of the indicated substances on p12-LO translocation and induction of p12-LO translocation, the indicated substances were added after pre-warming and cells were incubated for further 30 min. Then reactions were either stopped or stimulated with thrombin 1 U/ml to induce translocation. To determine subcellular localisation after stimulation with p12-LO activators, platelets were stimulated for 10 min at 37°C with thrombin 1 U/ml or collagen 12 µg/ml after pre-warming. Reactions in both cases were stopped by putting Eppendorf cups into liquid nitrogen. Cells were lysed by repeated freeze-thaw cycles (3 ×), cell rupture was checked under the microscope. Aliquots of 300 µl of the cell homogenates were centrifuged at 100.000 × g, 1h, 4°C, (Beckman L7-65 Ultracentrifuge, Beckman Coulter,

Fullerton, CA, USA) to prepare soluble (S100) and pelletable (P100) fractions [95, 98]. After centrifugation 200 µl supernatant (= S100) were mixed with 150 µl 2 × SDS loading buffer (SDS-LB) and boiled for 5 min at 96°C. The remaining supernatant was discarded, the pellet (= P100) was rinsed once with PG buffer and resuspended in 300 µl PG buffer. The pellet suspension was sonified (1 × 10 sec on ice) to fully disperse membrane aggregates. 200 µl of the pellet solution was mixed with 150 µl SDS-LB and boiled for 5 min at 96°C. Samples were stored at -20°C until separation by SDS-PAGE followed by WB using specific antibodies (2.2.18 and 2.2.19).

2.2.12 Platelet aggregation (turbidimetric)

Aggregation of washed human platelets was determined using a turbidimetric light-transmittance device (two channel Chrono-Log aggregometer, Haverton, PA, USA) as described [157]. In short, washed human platelets were prepared as described in 2.2.1 and adjusted to 2×10^8/ml. The instrument was calibrated with a platelet suspension for basal (0%) light transmission and with Tyrode`s buffer alone for 100% light transmission. Aliquots of 0.5 ml platelets were incubated with the indicated substances for 0-120 min at RT. For aggregation, the response to 1 U/ml thrombin, 1 µg/ml collagen, 1 µM U-46619 or 200 µM AA is given as % of the maximal light transmission A_{max}. $CaCl_2$ (1 mM) was added right before the start of the measurement. Aggregation was recorded under continuous stirring (1000 rpm) at 37°C for 5 min.

2.2.13 Measurement of intracellular $[Ca^{2+}]_i$ levels in platelets and PMNL

Washed human platelets (6×10^8/ml PG buffer) were incubated with 2 µM Fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy) ethane-N,N,N',N'-tetraacetic acid) for 40 min at 37°C. After washing platelets (10^8/ml in PG buffer) were directly transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY) with continuous stirring. One min prior addition of the indicated substances 1 mM $CaCl_2$ was added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively, and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [177]**.** F_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA. For samples where a long-time influence of substances on $[Ca^{2+}]_i$ levels was determined platelets were pre-incubated with the indicated agents for 20, 30, 60 or 120 min at 37°C after Fura-2 loading and washing. Cells were transferred to a thermally controlled (37 °C) fluorimeter cuvette in a spectrofluorometer as described and were stimulated with thrombin 1

U/ml or collagen 12 µg/ml 30 s after start of measurement. $[Ca^{2+}]_i$ was determined as described above. Determination of $[Ca^{2+}]_i$ levels in PMNL was performed as described previously [171]. In short, for measurement of $[Ca^{2+}]_i$ mobilization in PMNL washed human PMNL (6×10^8/ml PG buffer) were incubated with 2 µM Fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy) ethane-N,N,N',N'-tetraacetic acid) for 40 min at 37°C. After washing PMNL (10^6/ml in PG buffer) were directly transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY) with continuous stirring. One min prior addition of the indicated substances 1 mM $CaCl_2$ was added and $[Ca^{2+}]_i$ mobilization was measured as stated above.

2.2.14 Measurement of ROS formation in human platelets

Measurement of peroxides was conducted using the peroxide-sensitive fluorescence dye DCF-DA. Freshly isolated platelets (10^8/ml in PG buffer) were pre-incubated with the indicated substances for 20 min at 37°C. After 20 min cells were pre-incubated with DCF-DA (1 μg/ml) for 2 min at 37°C in a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, USA) with continuous stirring. The fluorescence emission at 530 nm was measured after excitation at 485 nm. The mean fluorescence data measured 5 min after stimulus addition are given as arbitrary units [178].

2.2.15 Determination of kinase phosphorylation status

Human platelets were obtained as described (2.2.1) and adjusted to 1×10^9/ml in PG buffer. Cells were supplemented with $CaCl_2$ 1 mM and incubated with progesterone or vehicle control for 0-120 min at 37°C. After the indicated times the reaction was terminated by adding a half-volume of 2 × SDS-LB and boiled at 95°C for 5 min. Samples were stored at -20°C until separation by SDS-PAGE followed by WB using specific antibodies (2.2.18 and 2.2.19). Control samples were supplemented with $CaCl_2$ 1 mM and stimulated with thrombin (1 U/ml), collagen (12 µg/ml) or PMA (100 nM) for 7 min at 37°C (positive controls) or left resting on ice without $CaCl_2$ addition (negative controls). Reactions were terminated as described above and samples were stored at -20°C until separation by SDS-PAGE followed by WB using specific antibodies (2.2.18 and 2.2.19).

2.2.16 Sample preparation for p12-LO protein amount determination and progesterone receptor blots

Suspensions of freshly isolated washed platelets (5×10^8/ml in PG buffer) or PC-3 cells (1×10^6/ml, see 2.2.17) were lysed by addition of ice-cold 2 × SDS-LB 1:1 (v/v). Samples were heated at 96°C for 5 min and stored at -20°C until SDS-PAGE and Western Blot analysis (2.2.18 and 2.2.19).

2.2.17 Cell culture of PC-3 cells

Human prostate cancer cell line PC-3 was used as a control for the progesterone receptor in Western blot analysis. In short, PC-3 cells were grown in PC-3 medium (RPMI + 5% Penicillin/ Streptomycin + 2 mM Glutamate + 10% FCS) to 70% confluency. The cells were harvested and diluted in SDS loading buffer to a density of. Protein samples were prepared for Western Blot as described in 2.2.16 and stored at -20°C until SDS-PAGE and Western Blot analysis (2.2.18 and 2.2.19).

2.2.18 SDS-PAGE

Cell suspensions or given sample preparations were incubated as specified and the reaction was stopped by the addition of ice-cold 2 × SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-LB; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% (v/v) β-mercaptoethanol), vortexed, sonificated (3 × 15 s) if required and heated for 5 min at 95 °C. The protein samples (18 µl) were mixed with 4 µl of glycerol/0.1% bromophenolblue (1:1, v/v) and analyzed by SDS-PAGE using a Mini Protean system (Bio-Rad, Hercules, CA, USA) according to Laemmli [179]. Depending on the molecular weight of the proteins to be analyzed, the polyacrylamid (PAA) concentration was adjusted to 8% (progesterone receptor) or 10% (p12-LO translocation, p12-LO protein expression, kinase phosphorylation assay). Molecular weight of the proteins was estimated by comparison with prestained broad range molecular weight marker peqGOLD IV (peqLab Biotchenology, Erlangen Germany).

2.2.19 Western Blot

After electroblot (tank blotting method) of gels from SDS-PAGE to nitrocellulose membrane (Amersham Pharmacia, Little Chalfont, UK), membranes were blocked with 5% (w/v) BSA blocking buffer (50 mM Tris/HCl, pH 7.4, and 100 mM NaCl (Tris-buffered saline (TBS)) plus 0.1% Tween 20 (TBS-Tween)) for 1 h at RT. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau S staining. Membranes were washed (TBS-Tween) and then incubated with the respective primary antibody (AB) overnight at 4°C (Table I). Antibodies were diluted in TBS Tween, 0.05% NaN_3 (v/v), 5% milk or BSA as indicated. The membranes were washed and incubated with 1:1000 dilution of

secondary AB (Cy-5 conjugated IgGs or alkaline peroxidase-conjugated IgGs) for 1 or 3 h at RT. After washing (4 × with TBS-Tween and TBS), proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in detection buffer (100 mM Tris/HCl, pH 9.5; 100 mM NaCl, 5 mM $MgCl_2$) or analyzed by fluorescence detection (Ettan DIGE). All steps regarding the incubation of the membrane were performed under gentle agitation.

Primary antibody	Isotype	Dilution	Company
p12-LO	Rabbit IgG	1:500	Gift of C.Funk
pERK	Mouse IgG	1:1000	Cell Signalling
pp38	Rabbit IgG	1:1000	Cell Signalling
pAkt	Rabbit IgG	1:1000	Cell Signalling
pMARCKS	Rabbit IgG	1:1000	Cell Signalling
Progesterone receptor	Mouse IgG	1:1000	Santa Cruz

Table I. Primary antibodies

2.2.20 Statistics

Data are expressed as mean ± S.E.; IC_{50} and EC_{50} values are approximations determined by graphical analysis (linear interpolation between the points neighbouring 50% activity). Statistical evaluation of the data was performed by one-way ANOVA tests for independent or correlated samples followed by Bonferroni post-hoc tests (GraphPad Software Inc.). Where appropriate, Student´s t test for paired and correlated samples was applied. A p value of < 0.05 (*) was considered significant.

3 Results

3.1 p12-LO and gender influence on p12-LO activity

As leukotriene biosynthesis was found to be differentially regulated in male and female neutrophils [127], a possible gender influence on the regulation of p12-LO product formation was investigated in human platelets. To this aim, the p12-LO pathway was analysed at different levels, as protein expression, cell-free and cellular activity, subcellular localisation and substrate availability.

3.1.1 Determination of p12-LO activity in washed platelets

To evaluate the influence of gender on spontaneous cellular p12-LO product formation freshly isolated washed platelets from male and female donors were incubated at 37°C in PG buffer for different time periods, without cell stimulation. No difference in 12-HETE formation was found between cells isolated from male and female donors (Fig. 1.1).

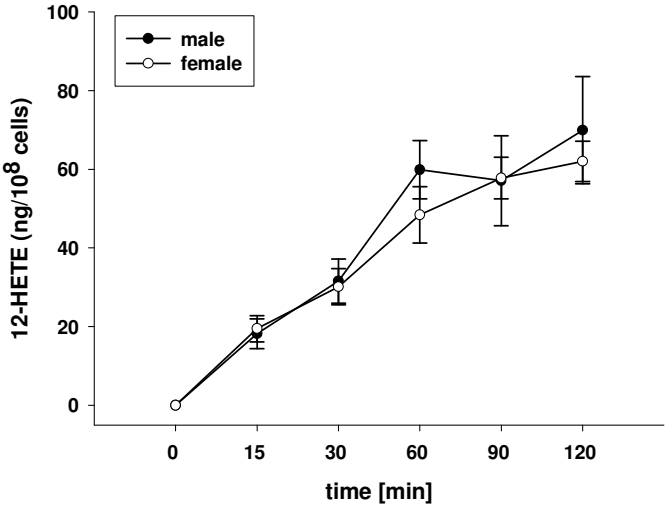

Fig. 1.1 Spontaneous p12-LO product formation in human platelets *Washed human platelets (10^8 /ml in PG buffer) isolated from male and female donors were incubated at 37°C for the indicated times. $CaCl_2$ (1 mM) was added 50 s before start of the incubation. The reaction was terminated with 1 ml methanol and samples were extracted and analysed by RP-HPLC. Data are given as mean ng/10^8 cells ± S.E. (n = 6).*

Next, cells were stimulated with different cell activating agents, as platelet specific and physiopathologically relevant activators (thrombin and collagen), with the p12-LO substrate arachidonic acid (AA) or other well-recognized stimulatory agents (i.e., A23187). No significant differences

were observed between p12-LO activities in male and female platelets under these conditions (Fig. 1.2).

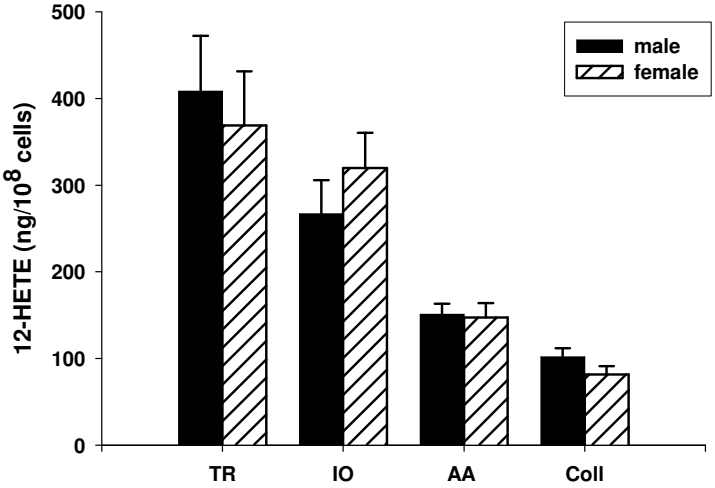

Fig. 1.2 p12-LO product formation in stimulated human platelets *Washed human platelets (10^8 / ml PG buffer) isolated from male and female donors were stimulated with thrombin (TR, 1 U/ml), A23187 (IO, 1 µM), AA (1 µM) or collagen (Coll, 12 µg/ml), after pre-warming for 1 min. $CaCl_2$ (1 mM) was added 50 s prior stimulation. Reactions were terminated after 10 min and samples were extracted and analyzed via RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3)*

3.1.2 Determination of p12-LO activity in platelet rich plasma (PRP) from male and female donors

Washed platelets are no longer in contact with any plasma components (e.g., sex hormones) that could be necessary for differential regulation of p12-LO between male and female donors. Thus platelet rich plasma (PRP) was used to investigate 12-HETE formation under more physiological conditions including plasma components. However, 12-HETE formation in PRP did not significantly differ between male and female donors, whether basal p12-LO activity was measured for 15 or 120 min at 37°C (Fig. 2.1 A) or whether cells were stimulated for 10 min with thrombin, collagen, A23187 or AA after a preincubation period of 15 minutes (Fig. 2.1 B).

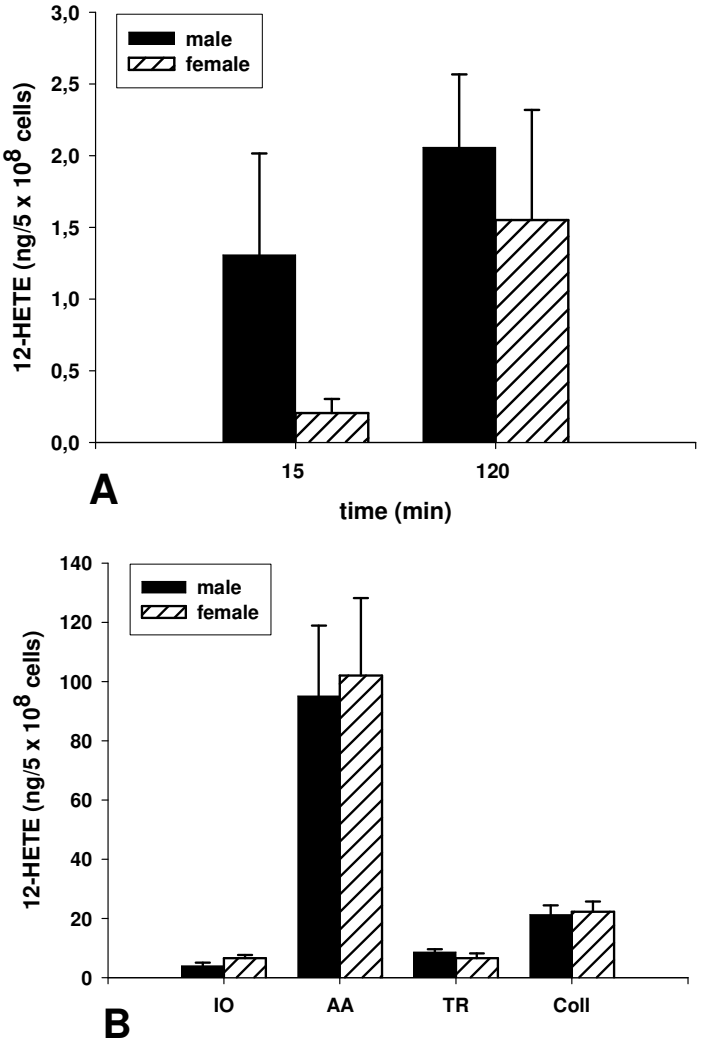

Fig. 2.1 Spontaneous and stimulated p12-LO product formation in human PRP from male and female donors *PRP prepared from freshly drawn venous citrate blood from male and female donors was incubated at 37 °C* ***(A)*** *for 15 and 120 min without stimulation or* ***(B)*** *stimulated for 10 min at 37 °C with A23187 (IO 30 µM), AA (100 µM), thrombin (TR 1 U/0.5 ml) or collagen (Coll 8 µg/ 0.5 ml), after a short preincubation time of 15 min. $CaCl_2$ (2 mM) was added to provide adequate concentrations when preincubation was started. Reactions were stopped by addition of 2 ml methanol and samples were analyzed via RP-HPLC. Data are given as mean ng/ 5 × 10^8 platelets + S.E. (n = 5) and have been normalized to represent 5 × 10^8 cells, as cell numbers varied strongly between donors.*

When PRP was stimulated with a combination of A23187 and AA, female cells produced more 12-HETE than male cells (Fig. 2.2). However, the differences were considered not significant.

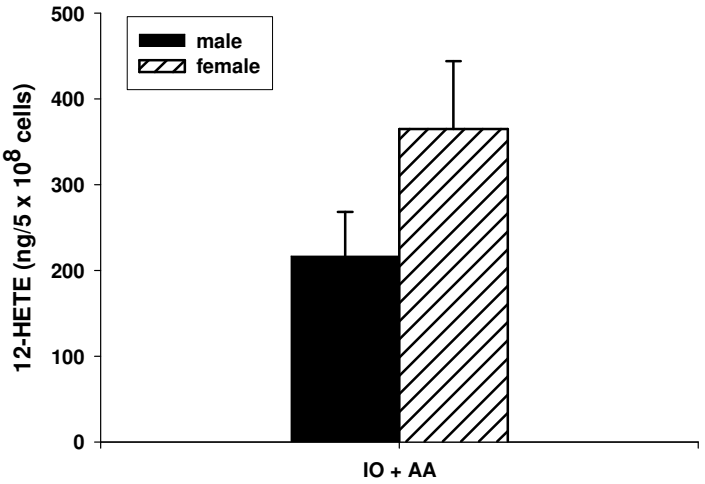

Fig. 2.2 p12-LO product formation in PRP from male and female donors stimulated with A23187 and AA *PRP isolated from freshly drawn venous blood was stimulated with a combination of A23187 (IO 30 µM) and AA (100 µM) after a short preincubation time of 15 min. $CaCl_2$ (1 mM) was added at the start of preincubation. Reactions were stopped after 10 min by addition of 2 ml methanol and samples were analyzed via RP-HPLC. Data are given an mean ng/ 5×10^8 platelets + S.E. (n = 5) and have been normalized to represent 5×10^8 cells, as cell numbers strongly varied between donors.*

3.1.3 Determination of p12-LO product formation in platelet homogenates

Platelet homogenates prepared from male and female cells were kept non-stimulated on ice or incubated for 10 min at 37°C in presence of 0.2 µM exogenous AA. No difference in p12-LO product formation between male and female cell homogenates could be observed (Fig. 3). Interestingly, small amounts of 12-HETE were measured also when no AA was added and homogenates were kept on ice.

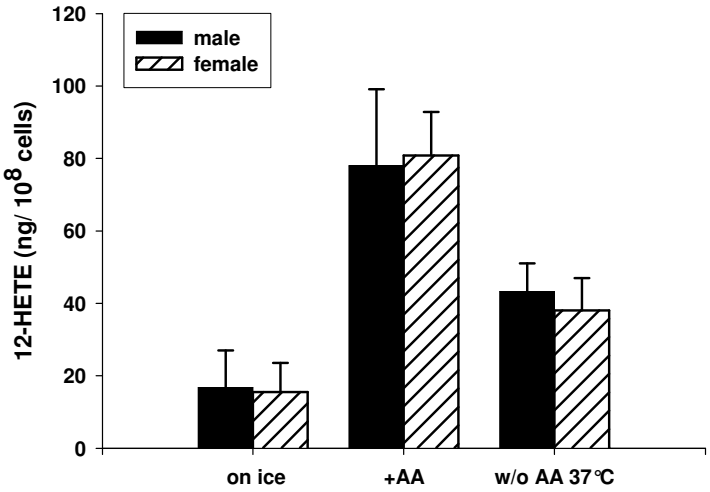

Fig. 3 12-HETE production in homogenates from male and female cells *Cell homogenates from washed human platelets (corresponding to 10^8/ml in PBS-EDTA) were prepared by sonification. Cell rupture was checked under the microscope. Homogenates were kept on ice or incubated at 37°C with exogenous AA (AA 0.2 µM) for 10 min. $CaCl_2$ (2 mM) was added 50 s before AA addition. Reactions were stopped after 10 min with 1 ml methanol and samples were extracted and analyzed via RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3).*

3.1.4 p12-LO protein expression in male and female platelets

To determine the expression of p12-LO in male and female platelets, cells from both genders were diluted to the same cell numbers, lysed and analyzed via Western Blot. p12-LO was detected by a specific p12-LO antibody. The amounts of p12-LO protein were equal in male and female cells (Fig. 4).

Fig. 4 p12-LO protein amounts in platelets from male and female donors *Washed human platelets (5×10^8/ ml in PG buffer) were lysed by addition of SDS-loading buffer. Proteins were separated by SDS-PAGE and Western blot analysis was performed. Data shown represents at least n = 3 different experiments. Ponceau staining of the blotted membranes was used as a loading control (not shown).*

3.1.5 Subcellular localisation of p12-LO in male and female platelets

Subcellular localisation of p12-LO was assessed in male and female platelets as translocation is considered a necessary step for p12-LO to access its substrate AA [98]. Surprisingly, p12-LO was predominately found to be localized in the pelletable fraction even without platelet stimulation (Fig. 5 A).

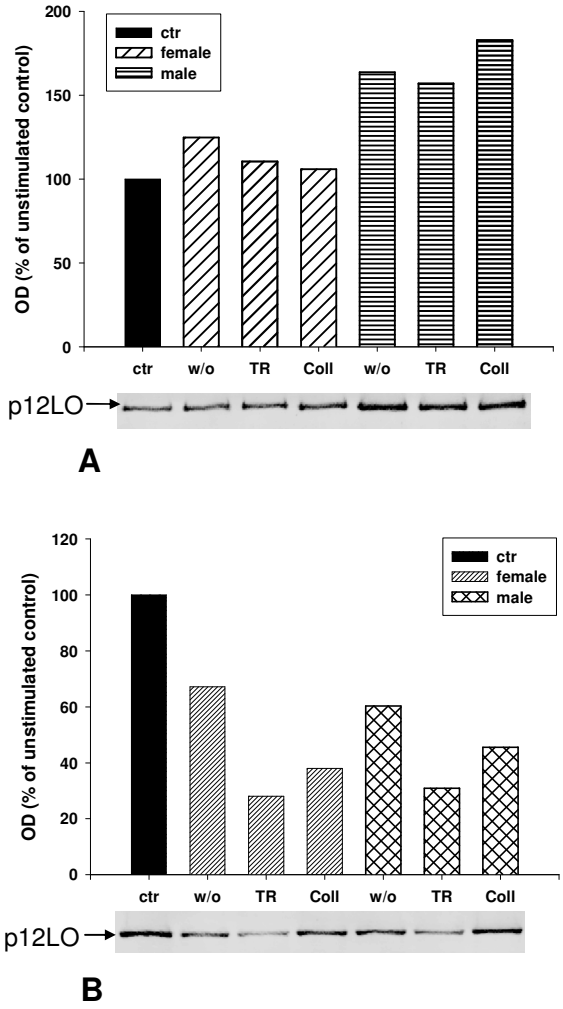

Fig. 5 Subcellular localisation of p12-LO in resting and stimulated cells
Washed human platelets (1×10^9/ml in PG buffer) were pre-warmed for 5 min at 37°C with $CaCl_2$ (1 mM) added, stimulated with thrombin (TR 1 U/ml) or collagen (Coll 12 µg/ml) for 10 min or left non-stimulated. p12-LO localisation is shown for ***(A)*** *membrane fraction (P100) and* ***(B)*** *cytosolic fraction (S100). As control (ctr) cells resting on ice with no $CaCl_2$ supplementation were used. Reactions were stopped by freezing samples in N_2 liq., cells were lysed by repeated freeze-thawing cycles. S100 and P100 fractions were prepared by centrifugation at 100,000 × g, 1 h, 4°C. Samples were analyzed via SDS-PAGE and Western Blot (Ettan Dige). Data shown are representative for n =3 individual experiments.*

A constant localisation of p12-LO at the plasma membrane was found consistent with a basal p12-LO activity that was observed in non-stimulated platelets (Fig. 1.1). However, when the cytosolic fraction was analysed, p12-LO was found to translocate from the cytosol in the same manner in male and female cells (Fig. 5 B). Translocation from the cytosolic fraction could be induced in both genders upon stimulation with thrombin (1 U/ml) and collagen (12 µg/ml). A slight translocation from the cytosol in comparison to the control sample could be detected even in non-stimulated cells that

were incubated for 10 min at 37°C with $CaCl_2$ (1 mM) present (Fig. 5 B). No increase of p12-LO in the pelletable fraction after stimulation could be detected although p12-LO obviously moved from the cytosol, neither via calculation of optical densities of protein bands in comparison to the control sample nor by visual inspection of Western Blot membranes.

3.1.6 AA release in male and female platelets

The release of AA did not significantly differ between male and female cells, regardless cell-stimulation (spontaneous release or A23187) or incubation time (30, 60 and 120 min) (Fig. 6 A, Fig. 6 B).

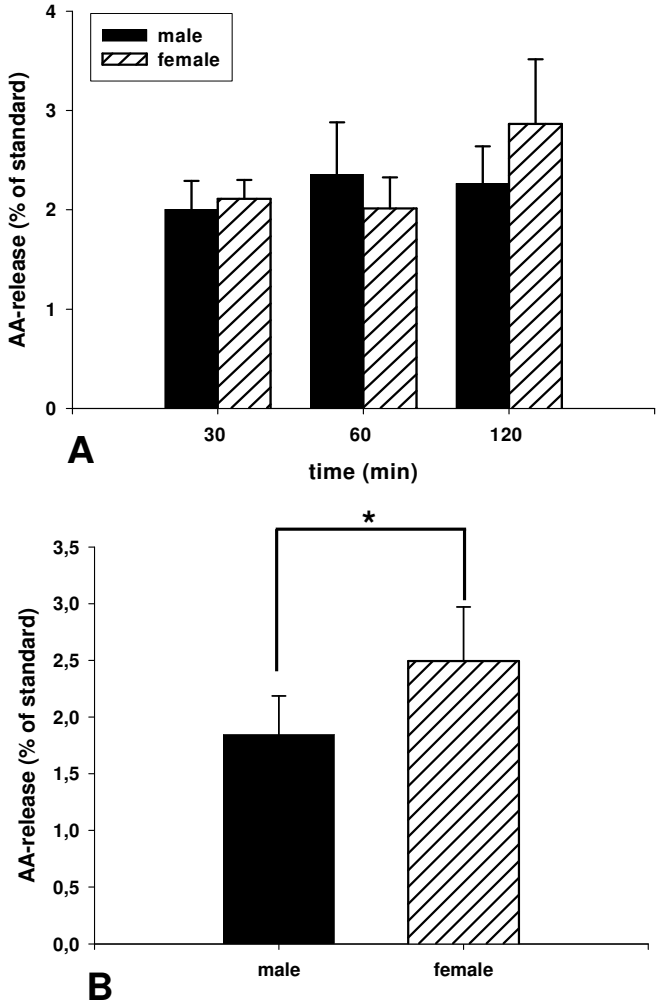

Fig. 6 AA release in intact human platelets from male and female donors
*Washed human platelets (10^8/ml in PG buffer) from male and female donors were incubated for **(A)** the indicated times and reactions stopped directly or were **(B)** incubated for 30 min and stimulated for 5 min with A23187 (2.5 µM) for 5 more min. $CaCl_2$ (1 mM) and CDC (15 µM, a 12-LO inhibitor) were added to the samples shortly before the incubation. Reactions were stopped with 2 ml methanol. Released AA was derivatized with a mixture of EDC-dimethoxy aniline for 1 h and samples were directly analysed by RP-HPLC analysis. Data are given as mean % AA of standard (30 µg/ml margarinic acid) + S.E. (n = 3) *p < 0.05 paired t-test.*

3.1.7 Determination of COX-1 product formation in washed platelets and PRP

Besides p12-LO, a major AA-metabolizing pathway in human platelets is represented by COX-1. Spontaneous formation of 12-HHT at different times (Fig. 7.1 A) as well as agonist stimulated 12-HHT formation (Fig. 7.1 B), as measure of COX-1 activity, was found to be the same in male and female washed platelets (Fig. 7.1).

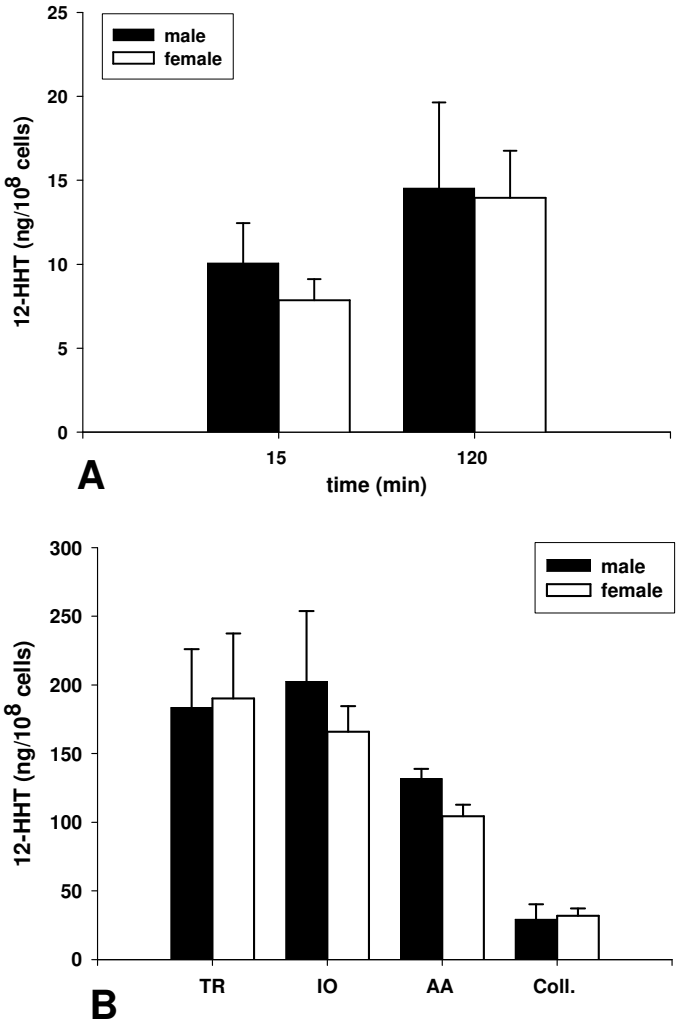

Fig. 7.1 COX-1 product formation in isolated human platelets from male and female donors *Washed platelets (10^8/ml in PG buffer) from male and female donors were* ***(A)*** *incubated for 15 and 120 min without stimulus or* ***(B)*** *stimulated with thrombin (TR 1U/ml), A23187 (IO 1 µM) AA (1 µM) or collagen (Coll. 12 µg/ml) for 10 min. $CaCl_2$ 1 mM was added 50 s prior to start of the incubation. Reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC. COX-1 product formation is given as amount of 12-HHT as stable COX-1 metabolite in mean ng/10^8 cells + S.E. (n = 3).*

PRP prepared from freshly drawn venous blood was used to determine basal and stimulated COX-1 activity in more physiological conditions. No difference between male and female cells was found for both basal or

A23187-, AA-, thrombin- or collagen-stimulated 12-HHT formation (Fig. 7.2).

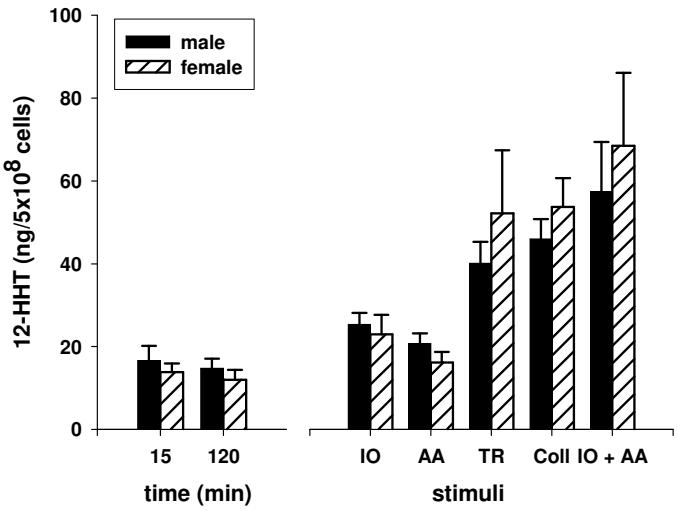

Fig. 7.2 COX-1 product formation in human PRP from male and female donors *PRP freshly isolated from male and female donors was incubated at 37°C for 15 or 120 min. After 15 min PRP was either stimulated with A23187 (IO 30 µM), AA (AA 100 µM), thrombin (TR 1 U/0.5 ml), collagen (Coll 8 µg/0.5 ml) or a combination of A23187 and AA, respectively, for 10 min or reactions were stopped directly after 15 min without stimulation. Also basal 12-HHT formation after 120 min was determined. Reactions were terminated by addition of 2 ml methanol and samples were analyzed by RP-HPLC (n = 5). COX-1 product formation is given as amount 12-HHT as stable COX-1 metabolite in mean ng/ 5 × 10^8 cells +S.E. Values have been calculated to represent 5 × 10^8 cells as cell numbers varied strongly between donors.*

3.2 Effects of progesterone on p12-LO activity

So far, no difference in p12-LO activity between male and female cells could be observed. Nevertheless, most investigations were performed in absence of naturally occurring sex hormones and other plasma components, so replacement of sex hormones as well as studies of p12-LO function in plasma and PRP samples were the next steps.

3.2.1 Spontaneous 12-HETE formation in washed platelets treated with sex hormones

The effect of sex hormones on spontaneous 12-HETE formation in washed platelets was analysed. Under these conditions, 1 µM progesterone completely suppressed spontaneous 12-HETE production, whereas no effects were observed for 0.1 µM estradiol or 0.01 µM dihydrotestosterone (DHT). The effect of progesterone was observed in male as well as in female cells (Fig. 8) over long time periods, being most prominent after 120 minutes preincubation in comparison to vehicle controls.

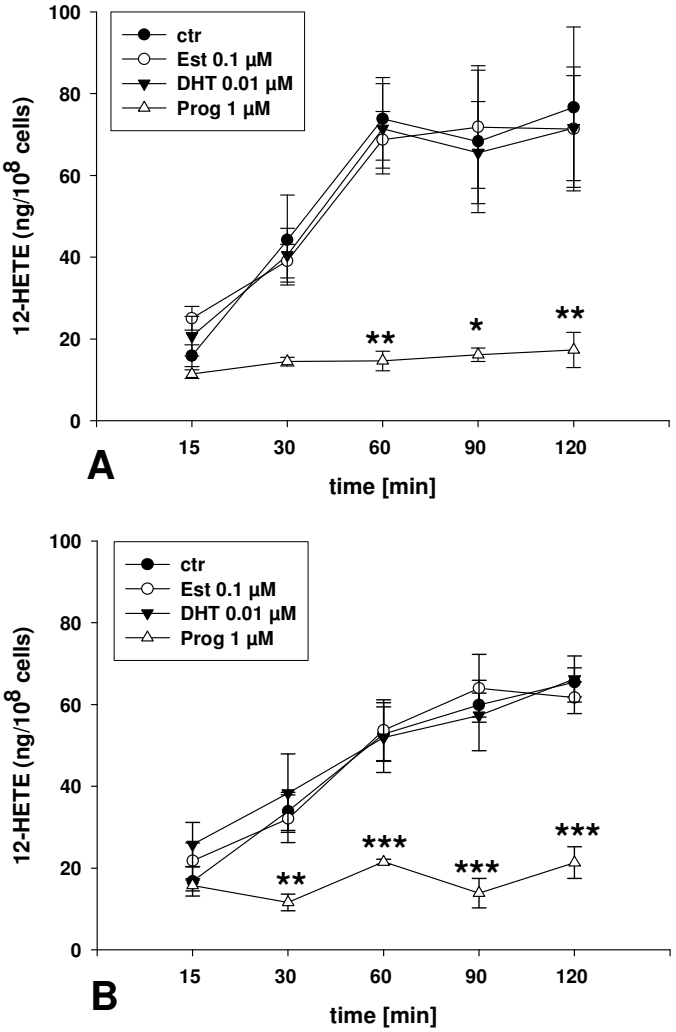

Fig. 8 Effects of sex hormones on p12-LO product formation in isolated human platelets from male and female donors *Washed human platelets (10^8/ml in PG buffer) from **(A)** male and **(B)** female donors were incubated for the indicated times at 37°C with ethanol 0.1% as solvent control (ctr), estradiol (0.1 µM), dihydrotestosterone (DHT 0.01 µM) or progesterone (1 µM) respectively. $CaCl_2$ (1 mM) was added directly before sex hormone addition. Reactions were terminated by addition of 1 ml methanol and samples were extracted and analyzed by RP-HPLC. Data are given as mean ng/10^8 cells +/- S.E. (n = 3 - 6).*p< 0.05, **p < 0.01, ***p < 0.001 vs. solvent control, ANOVA + Bonferroni post hoc tests.*

3.2.2 12-HETE formation in stimulated washed platelets treated with sex hormones

Next, the effect of sex hormones on stimulated 12-HETE formation was analysed. Progesterone, but not estradiol or DHT, significantly impaired collagen-induced p12-LO product formation. Progesterone prevented the increase of product formation by collagen stimulation by about 50% at a concentration of 1 µM (Fig. 9.1). This influence of progesterone on collagen-induced 12-HETE production was already observed after 15 min pre-incubation (data not shown). Collagen stimulation of p12-LO activity was also influenced by very low concentrations of progesterone (0.1 and 0.3

µM), when a pre-incubation of 60 min with the hormone prior collagen stimulation was performed (Fig. 9.1).

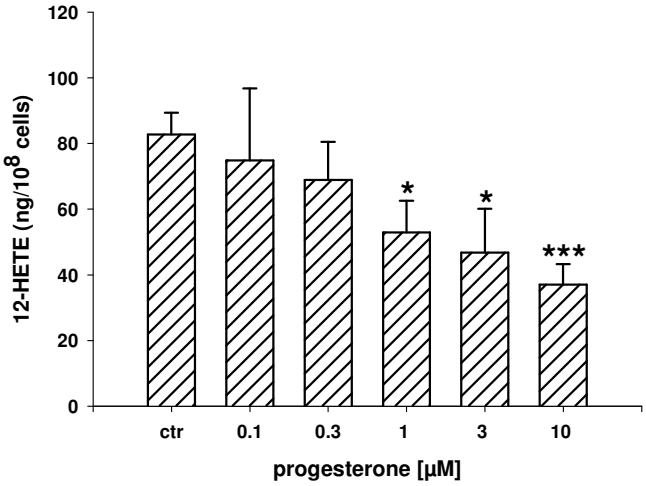

Fig. 9.1 p12-LO product formation in collagen stimulated platelets supplemented with progesterone *Washed human platelets (10^8/ml in PG buffer) from female donors were for 60 min at 37°C with the indicated concentrations of progesterone. After pre-incubation platelets were stimulated with collagen 12 µg/ml for 10 min. $CaCl_2$ (1 mM) was added before hormone supplementation. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped with 1 ml methanol and samples were analyzed via RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3).***p< 0.0007 vs. solvent control, unpaired t-test.*

On the contrary, progesterone did not significantly influence thrombin, A23187 or AA stimulated 12-HETE formation at concentrations of 1 or 3 µM (Fig. 9.2).

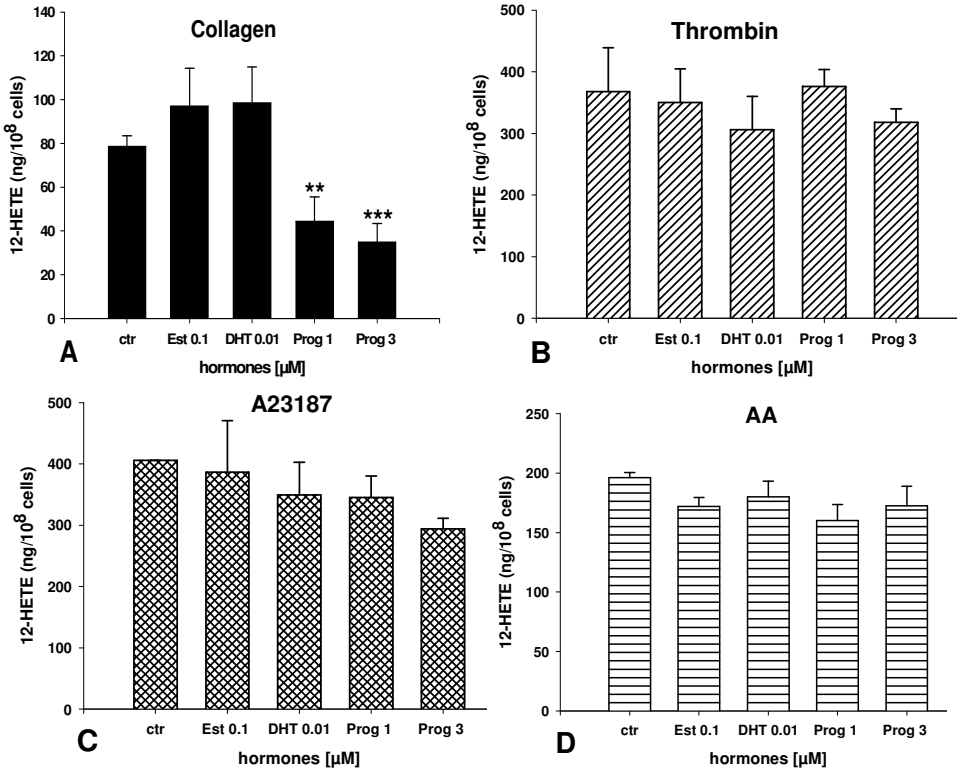

Fig. 9.2 p12-LO product formation in stimulated human platelets supplemented with sex hormones *Washed human platelets (10^8/ml in PG buffer) from female donors were incubated for 120 minutes at 37°C with estradiol (Est 0.1 µM), DHT (0.01 µM) and progesterone (Prog 1 and 3 µM) respectively. $CaCl_2$ (1 mM) was added shortly before hormone supplementation. After 120 min platelets were stimulated with **(A)** collagen (12 µg/ml), **(B)** thrombin (1 U/ml), **(C)** A23187 (1 µM) or **(D)** AA (1 µM) for 10 min. As solvent control 0.1% ethanol was used (ctr). Reactions were stopped with 1 ml methanol and samples were analyzed via RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3) **p < 0.045 ***p < 0.0003 unpaired t-test vs. solvent control*

However, when platelets were incubated with supra-physiological concentrations of progesterone (10 µM), a reduction of A23187- and AA-stimulated cellular p12-LO activity was observed (Fig. 9.3 A), although being not significant. A strong and very significant inhibition of p12-LO product formation by 10 µM progesterone was instead observed when the thrombin or collagen were used as stimuli. This effect was observed in female (Fig. 9.3 B) as well as in male cells (data not shown).

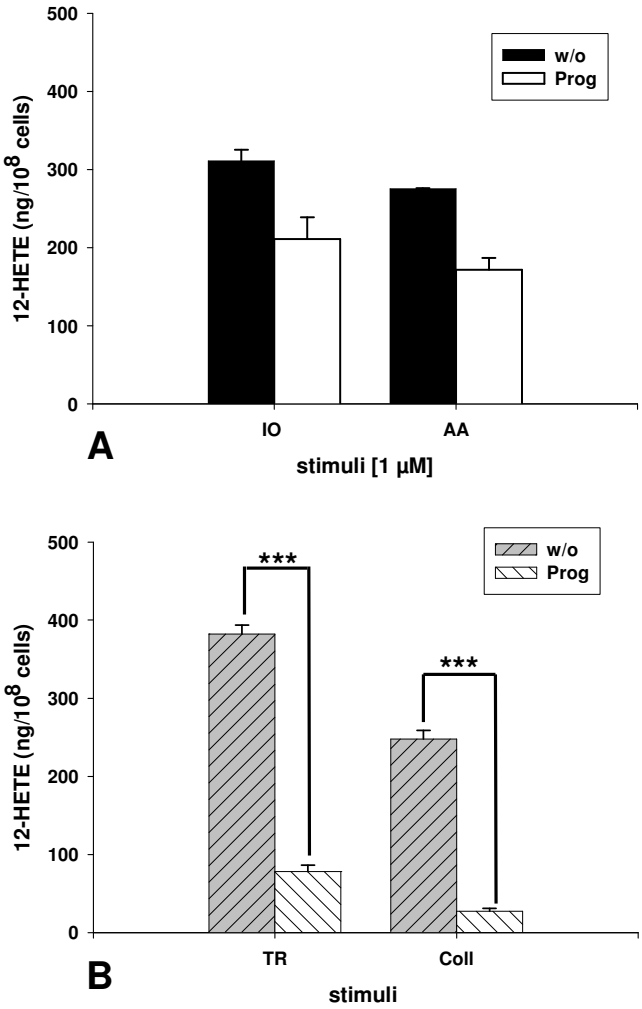

Fig. 9.3 p12-LO product formation in stimulated human platelets incubated with high progesterone concentrations *Washed human platelets (10^8/ml in PG buffer)from female donors were incubated at 37°C for 120 minutes with progesterone 10 µM. After 120 min, platelets were stimulated with **(A)** A23187 (IO 1 µM) or AA (AA 1 µM) or **(B)** thrombin (TR 1 U/ml) or collagen (Coll 12 µg/ml) for 10 min. Reactions were stopped with 1 ml methanol and samples analyzed via RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3) Data are given as mean ng/10^8 cells + S.E. (n = 3). ***p < 0.0001 vs. solvent control, unpaired t-tests vs. olvent control*

Importantly, the effect of progesterone was selective for p12-LO, since COX-1 product formation was not influenced by progesterone at any concentration in non-stimulated (Fig. 9.4) as well as in stimulated cells (data not shown)

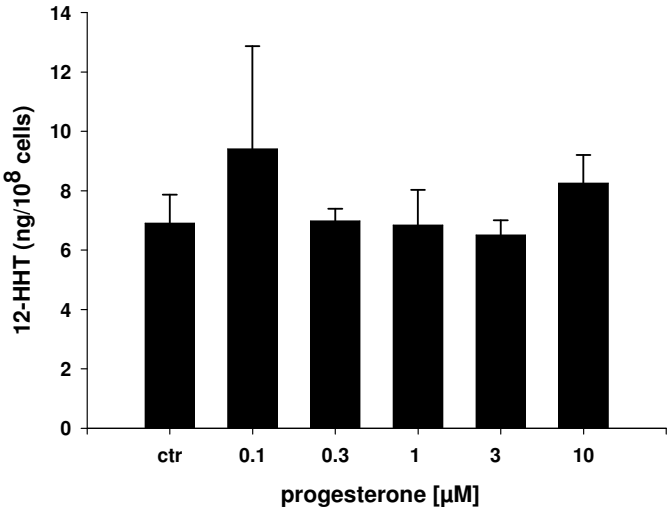

Fig. 9.4 COX-1 activity in resting platelets treated with different progesterone concentrations *Washed platelets (10^8/ml in PG buffer) from male and female donors were incubated for 60 min without stimulus. $CaCl_2$ 1 mM was added 50 s. prior to start of the incubation. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC. COX-1 product formation is given as amount 12-HHT as stable COX-1 metabolite in mean ng /ml + S.E. (n = 3).*

3.2.3 p12-LO activity in female platelets resuspended in plasma from male and female donors

To verify the effects of progesterone on p12-LO product formation under more physiologically relevant conditions, platelets were resuspended in plasma from male or female donors. Plasma levels of sex hormones (testosterone, progesterone and estradiol) in the donors' blood had been measured immediately after the blood was drawn. Mean values for the investigated sex hormones determined of all study donors are shown in Table1.

Gender	Progesterone	Estradiol	DHT
male	2.89 (+/- 0.23)	0.16 (+/- 18,63)	21.09 (+/-1.85)
female	13.47 (+/-6,78)	0.37 (+/- 80.48	2,06 (+/- 0.20)

Table 1 *Mean hormone plasma levels (nmol/l) +/- S:E:*

Cells resuspended in a selected male plasma sample (progesterone concentration 4.2 nM) produced more 12-HETE than cells resuspended in selected female plasma (progesterone 68 nM) (Fig. 10.1 A). Analysis of 12-HHT levels in the same samples showed some gender differences, but a clear pattern was not evident. These preliminary evidences suggested that unrecognized additional factors present in the plasma might have multiple effects on different levels of the AA pathways in platelets (Fig. 10.1 B).

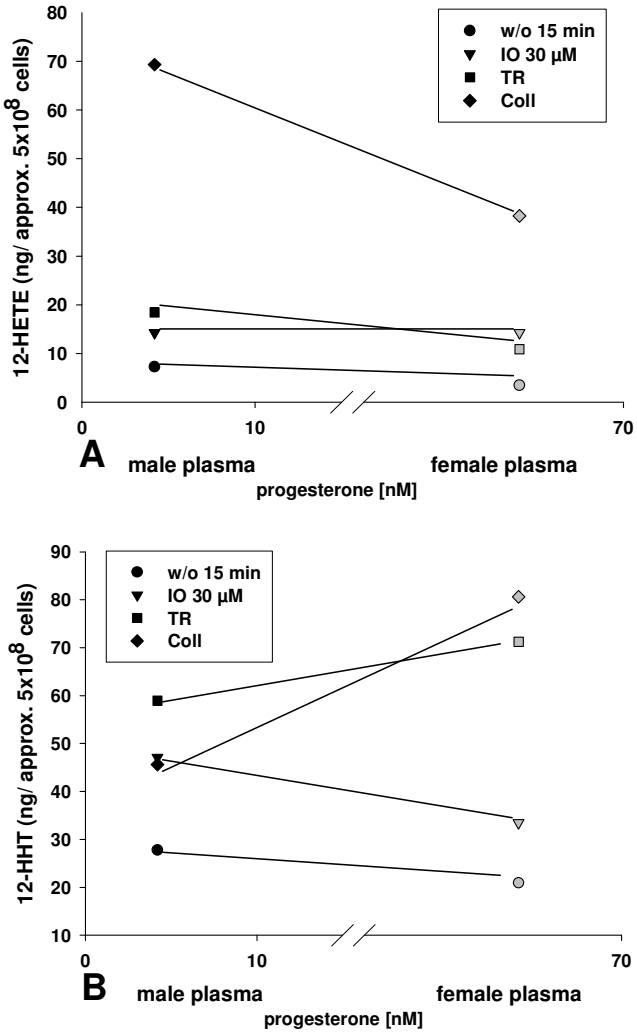

Fig. 10.1 p12-LO and COX-1 activity in human platelets resuspended in male and female plasma with different progesterone levels *Washed human platelets (approx. 5 × 10^8/0.5 ml) from female donors were resuspended in plasma from male and female donors. After a preincubation time of 15 min at 37°C* ***(A)*** *12-HETE and* ***(B)*** *12-HHT formation was measured. Either basal activities were measured or cells were stimulated with A23187 (IO 30 µM), thrombin (TR 1 U/0.5 ml) or collagen (Coll 8 µg/0.5 ml) respectively. Reactions were stopped by addition of 2 ml methanol and samples were analyzed by RP-HPLC. 12-HETE and 12-HHT formation in relation to progesterone plasma levels was compared. Data are given as ng/5 × 10^8 cells.*

This was confirmed by the analysis of platelets resuspended in male and female plasma with approximately the same progesterone levels. In fact, also under these conditions p12-LO activity resulted lower in female plasma (Fig. 10.2 A) and a strong variability for 12-HHT was observed (Fig. 10.2 B).

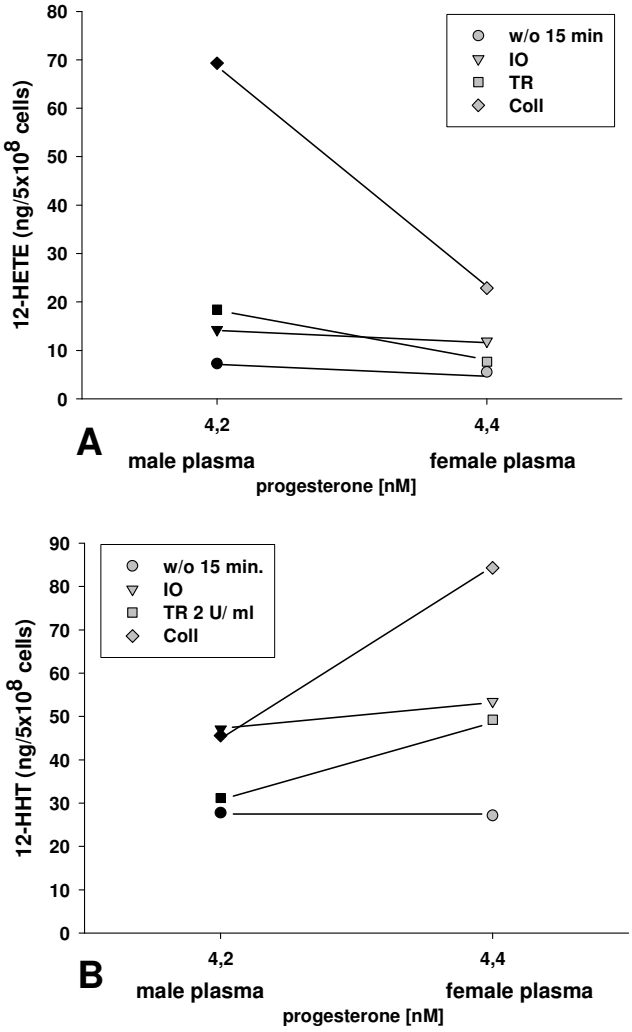

Fig. 10.2 p12-LO and COX-1 activity in human platelets resuspended plasma from male and female donors with similar progesterone plasma levels
Washed human platelets were resuspended in plasma from male and female donors (approx. 5 × 10^8 cells/0.5 ml). After a preincubation time of 15 min at 37°C ***(A)*** *12-HETE and* ***(B)*** *12-HHT formation was measured. Either basal activities were measured or cells were stimulated with A23187 (IO 30 µM), thrombin (TR 1 U/0.5 ml) or collagen (Coll 8 µg/0.5 ml) respectively. Reactions were stopped by addition of 2 ml methanol and samples were analyzed by RP-HPLC.* ***(A)*** *12-HETE and* ***(B)*** *12-HHT formation in relation to progesterone plasma levels was compared (male 4.2 nM, female 4.4 nM). Data are given as ng/5 × 10^8 cells*

The effective concentration of progesterone to influence p12-LO activity in isolated platelets was found to be between 0.3 and 1 µM (as previously shown, Fig. 8). Progesterone plasma levels in the female donors tested were below these concentrations (table 1).

Nevertheless, when female platelets were incubated in male plasma supplemented with different concentrations of progesterone and were stimulated with thrombin 1 U/0.5 ml or collagen 8 µg/0.5 ml respectively, a slight inhibition of p12-LO activity could be observed (Fig. 10.4).

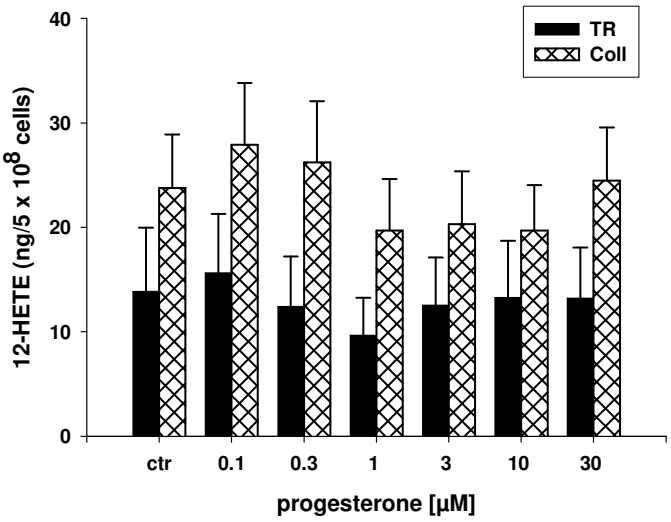

Fig. 10.4 12-HETE formation in platelets resuspended in male plasma supplemented with different progesterone concentrations *Washed human platelets from female donors were resuspended in pooled male plasma (approx. 5 × 10^8 /0.5 ml). After a preincubation time of 120 min at 37°C cells were stimulated with thrombin (1 U/0.5 ml) and collagen (8 µg/0.5 ml) respectively. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped by addition of 2 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean ng/5 × 10^8 cells + S.E. (n = 3-4)*

Taken together, progesterone had inhibitory effects on platelet 12-HETE formation, also when added to plasma. However, additional gender-related plasma factors might influence the behaviour of platelets, in particular in relation to the AA pathway, and therefore do not allow to establish a clear correlation between plasma (and the sex hormones therein) and 12-HETE production.

3.2.4 p12-LO activity in plasma and PRP from pregnant donors

In contrast to females and males with regular sex hormonal states, pregnant females show strongly elevated estrogen and progesterone plasma levels. To evaluate whether female hormones might influence p12-LO activity during pregnancy, the effect of plasma of pregnant donors on p12-LO activity in female platelets was analysed. However, no close correlation of p12-LO activity with progesterone plasma levels could be established (Fig. 10.3 A) when p12-LO activity of platelets resuspended in plasma from 5 different pregnant donors was compared. Plasma progesterone levels of individual donors are given in Table 2. 12-HETE formation was analysed in PRP from pregnant donors (Fig. 10.3 A) side by side with non-pregnant females and males (data not shown).

Pregnant donor	Progesterone (nmol/l)
Pregnant 1	275.3
Pregnant 2	361.4
Pregnant 3	437.1
Pregnant 4	445.7
Pregnant 5	1353.8

Table 2 *Progesterone levels of pregnant donors*

All data collected were pooled and mean p12-LO activities in male, female and pregnant plasma were determined (Fig. 10.3 B). No correlation between progesterone levels and p12-LO activity was found. Nevertheless, after 120 min incubation a trend emerged in spontaneous 12-HETE formation (Fig. 10.3 B) with lowest 12-HETE formation in pregnant and highest in male plasma. Thus, a connection to different progesterone plasma levels seems possible.

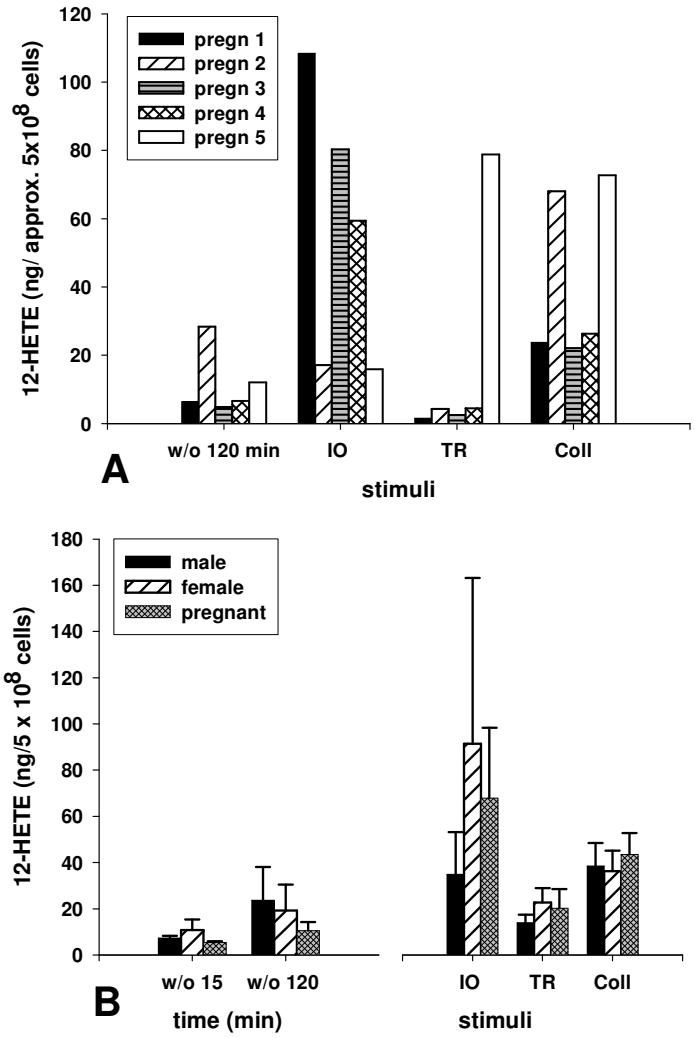

Fig. 10.3 p12-LO activity in human platelets resuspended in plasma from pregnant donors with different progesterone plasma levels *Washed human platelets (5 × 10^8/ml) from female donors were resuspended in human citrate plasma from **(A)** pregnant and **(B)** male, female or pregnant donors. Cells were either incubated without stimulus for 15 or 120 min or were stimulated after a pre-incubation time of 15 min with 30 µM A23187 (IO), 1 U/0.5 ml thrombin (TR) or 8 µg/0.5 ml collagen (Coll) for 10 min. Reactions were terminated with 2 ml of methanol and analyzed by RP-HPLC. Pregnant plasma samples are n = 1 each donor, pooled data n = 6-9. Data in (A) are given as ng/5 × 10^8 cells, in (B) are given as mean ng/5 × 10^8 cells*

To collect more data on p12-LO activity in pregnant donors, also PRP isolated from male, female and pregnant donors was analyzed in regard to 12-HETE and 12-HHT formation. Two independent experiments were performed. The respective plasmatic concentrations of sex hormones of all donors are reported in Table 3.

Gender	Date	Progesterone	Estradiol	DHT
Male	090910	3.2 nM	0.143 nM	13.1 nM
Female	090910	4.1 nM	0.384 nM	1.9 nM
Pregnant	090910	309.3 nM	2406.6 nM	2.2 nM
Male	090708	2.3 nM	0.146 nM	20.8 nM
Female	090708	1 nM	0.401 nM	1.8 nM
Pregnant	090708	946.7 nM	>367.0 nM	5.9 nM

Table 3 *Plasmatic concentrations of sex hormones in studied samples*

When the PRP from a pregnant donor with approx. 1 µM plasma progesterone was analysed, stimulated 12-HETE formation resulted lower than in PRP prepared from male or female (which did not significantly differ in 12-HETE formation, Fig. 11.1 A). However, when the experiment was performed with PRP from a pregnant donor with approx 0.3 µM progesterone levels, the effect observed was not as prominent, however, a lower p12-LO activity could still be found (Fig. 11.1 B). Interestingly, analysis of COX-1 activity in the PRP from both pregnant donors showed also reduced levels of 12-HHT (Fig. 11.1 C, Fig. 11.1 D).

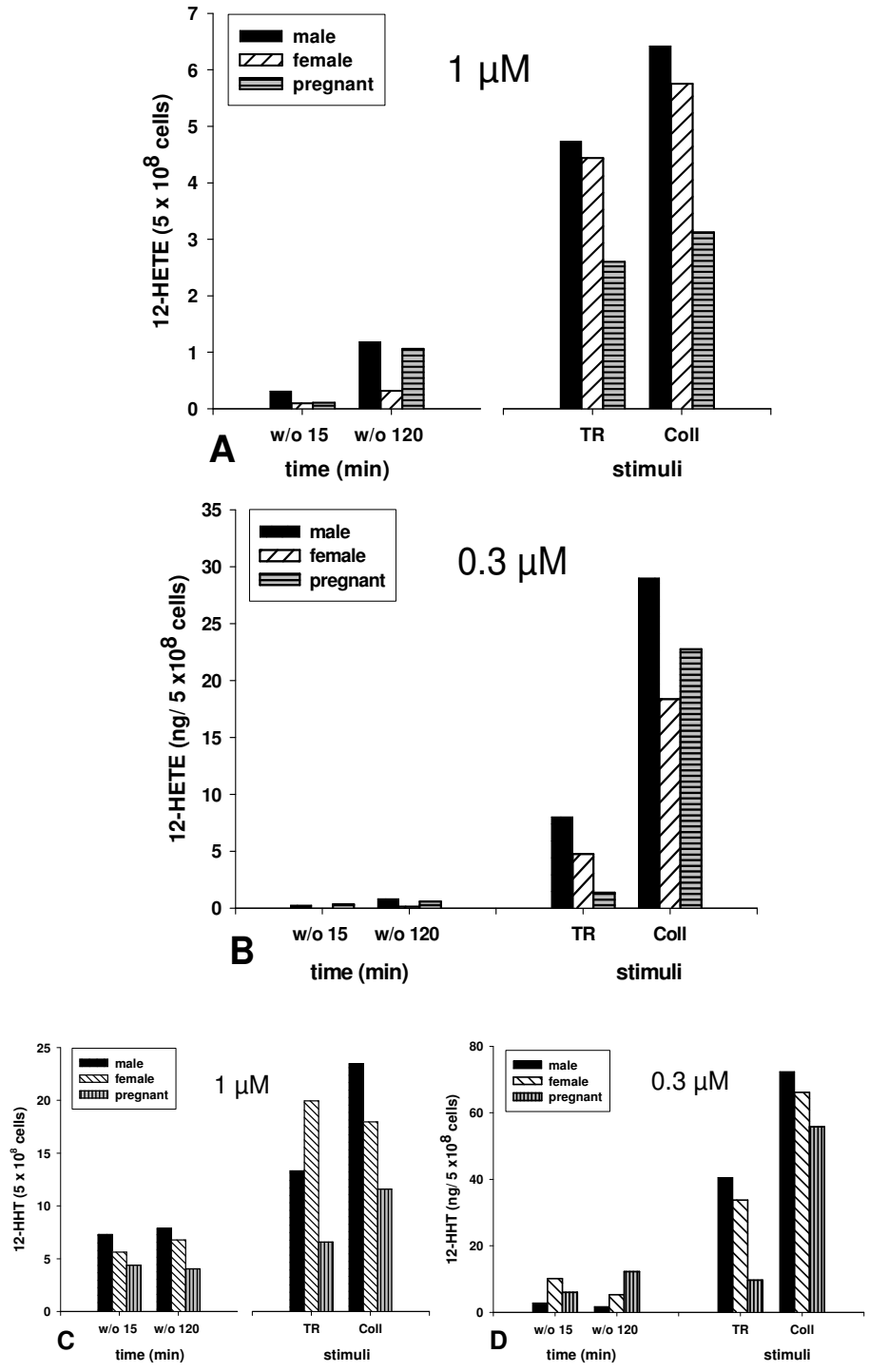

Fig. 11.1 p12-LO and COX-1 activity in PRP isolated from male, female and pregnant donors *PRP isolated from freshly drawn venous blood was incubated for either 15 or 120 minutes without stimulation at 37°C or stimulated for 10 min at 37 °C with thrombin (TR 1 U/0.5 ml) and collagen (Coll 8 µg/0.5 ml), after a preincubation time of 15 minutes, $CaCl_2$ (1 mM) was added at the beginning of the preincubation time. Progesterone levels of pregnant donors were determined at 1 µM for (A) and (C), 0.3 µM for (B) and (D). 12-LO activity measured is displayed in **(A)** and **(B)**, values for COX-1 activity are given in **(C)** and **(D)**. Reactions were stopped by addition of 2 ml methanol. Samples were extracted and analyzed via RP-HPLC analysis. Data are given as ng/ 5 × 10^8 platelets (n = 2). Values have been normalized to represent 5 × 10^8 cells, as cell numbers strongly varied between donors.*

To recreate progesterone concentrations in plasma as they are found during pregnancy, PRP from male donors (low natural progesterone levels, < 5 nM) were supplemented with different concentrations of progesterone. p12-LO product formation was lower in progesterone treated PRP than in the vehicle control samples (Fig. 11.2). The effect was clearly evident in collagen stimulated samples. Basal 12-HETE formation without stimulation was not determined in this set-up.

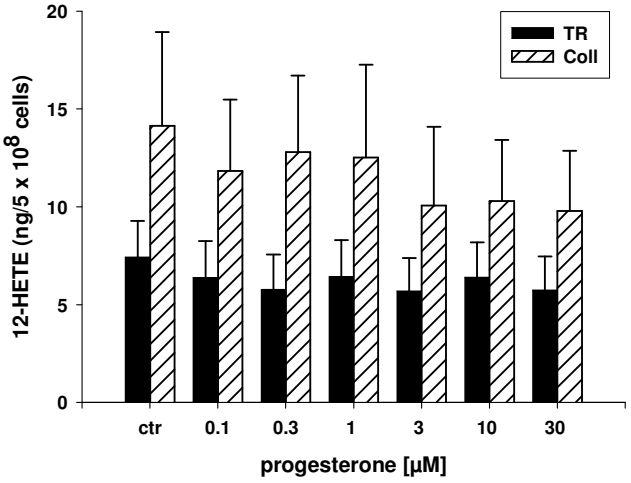

Fig.11.2 p12-LO activity in PRP isolated from male donors and supplemented with different progesterone concentrations *PRP isolated from freshly drawn venous blood was supplemented with different concentrations of progesterone (0.1 µM to 30 µM). PRP was incubated for 120 min. Then cells were stimulated with thrombin (TR 1 U/0.5 ml) or collagen (Coll 8 µg/0.5 ml). As control (ctr) 0.1% ethanol was used. Reactions were stopped by addition of 2 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean ng/5 × 10^8 platelets + S.E. (n = 4). Values have been calculated to represent 5 × 10^8 cells, as cell numbers varied strongly between donors.*

3.2.5 AA release in intact platelets from male and female donors treated with low hormone concentrations

As previously shown, basal p12-LO activity was impaired by addition of low progesterone concentrations (1 µM, Fig. 8). Nevertheless, AA release in intact female platelets treated with the same concentrations of progesterone was not different compared to vehicle treated cells (Fig. 12), both in cells stimulated after a preincubation time of 30 min or left non-stimulated.

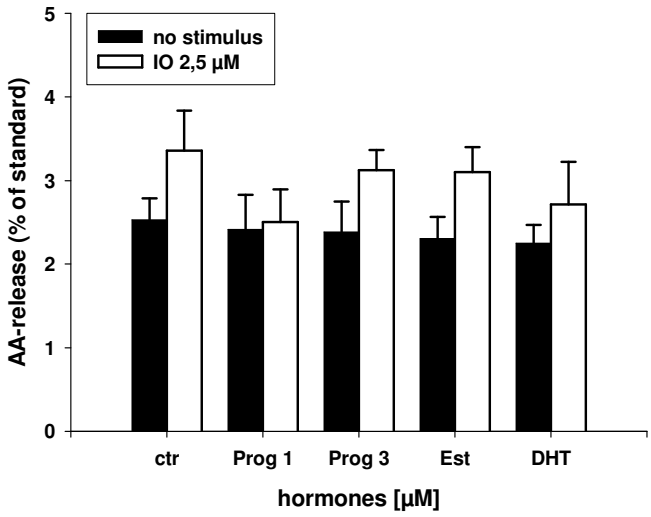

Fig. 12 Influence of low hormone concentrations on AA release in intact platelets *Washed human platelets (10^8/ml in PG buffer) from female donors were incubated with the indicated hormones (progesterone Prog 1 and 3 µM, estradiol Est 0.1 µM, DHT 0.01 µM) for 30 min at 37°C. $CaCl_2$ 1 mM was added shortly before hormone supplementation. Samples were either stimulated with A23187 (IO 2.5 µM) for 5 min or left untreated. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped with 2 ml methanol, released AA was derivatized and samples were analyzed by RP-HPLC. Data are given as mean % AA-release of standard (30 µg/ml margarinic acid) + S.E. (n = 3)*

3.2.6 AA release in intact platelets from female donors treated with high hormone concentrations

High concentrations of progesterone, estradiol or DHT were tested for their ability to impair spontaneous AA release in intact platelets. Even at concentrations of 10 µM and long preincubation times of 30, 60 or 120 minutes, no significant influence of either hormone could be detected on AA release in intact cells (Fig. 13.1).

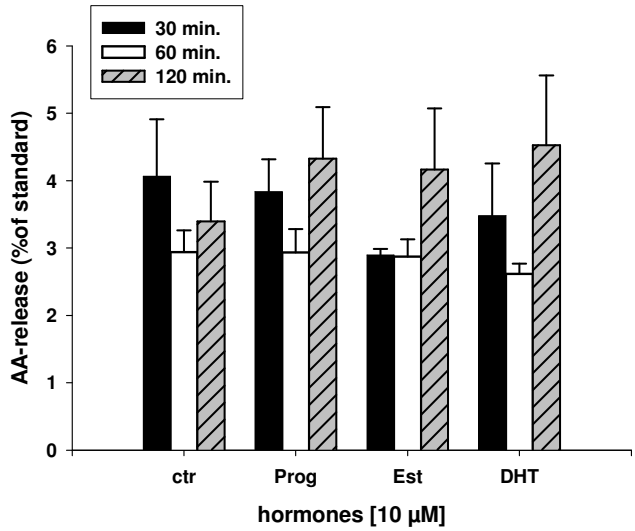

Fig. 13.1 Influence of high hormone concentrations on AA release in intact platelets at different time points *Washed human platelets (10^8/ml in PG buffer) from female donors were incubated with the indicated hormones (progesterone Prog, estradiol Est, DHT) in concentrations of 10 µM for 30, 60 or 120 min at 37°C. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped with 2 ml methanol, released AA was derivatized and samples were analyzed by RP-HPLC. Data are given as mean % AA-release of standard (30 µg/ml margarinic acid) + S.E. (n = 3)*

Also, no significant influence of sex hormones was detected when cells were stimulated with A23187 to release AA after a preincubation time of 30 minutes with the different hormones (10 µM, Fig. 13.2).

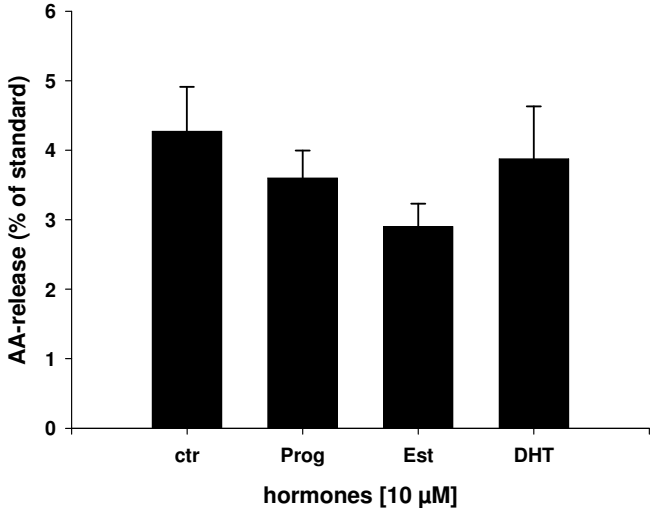

Fig. 13.2 Influence of high hormone concentrations on AA release in intact platelets after A23187 stimulation *Washed human platelets (10^8/ml in PG buffer) were incubated with the indicated hormones (progesterone Prog, estradiol Est, Dihydrotestosterone DHT) at a concentration of 10 µM for 30 min at 37°C. Cells were stimulated after 30 min with A23187 (2.5 µM for 5 min). As control (ctr) 0.1% ethanol was used. Reactions were stopped with 2 ml methanol, released AA was derivatized and samples were analyzed by RP-HPLC. Data are given as mean % AA release of standard (30 µg/ml margarinic acid) +S.E. (n = 3)*

3.2.7 Influence of progesterone on cPLA$_2$ activity in a cell free system

To further exclude a connection of AA release to the effect of progesterone on p12-LO product formation, recombinant and partly purified cPLA$_2$ was incubated with 10 µM progesterone. cPLA$_2$ activity was measured by the ability to release AA (% rest activity) and was not inhibited by progesterone in a cell free assay (Fig. 14).

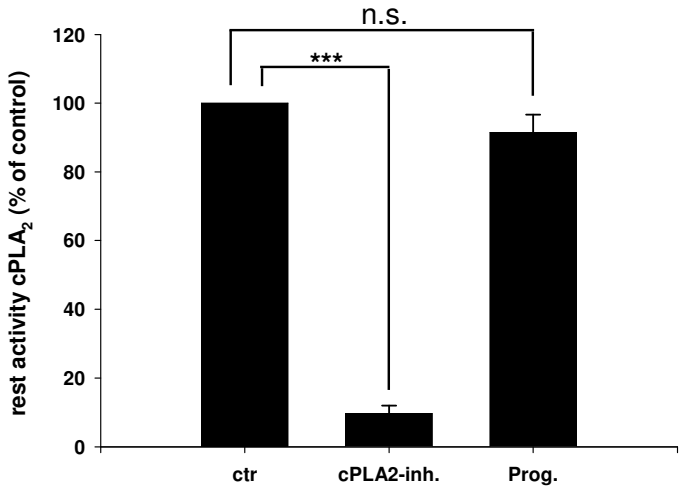

Fig. 14 Influence of progesterone on cPLA$_2$ activity in a cell-free system
*Purified cPLA$_2$ was incubated as described in the methods section. Progesterone (Prog, 10 µM), a defined cPLA$_2$ inhibitor (pyrrolidine-1, 10 µM) or DMSO as solvent control (ctr) were added. Reactions were stopped by addition of 2 ml methanol, released AA was derivatized and samples were analyzed by RP-HPLC. Data is given as mean % rest activity + S.E. (n = 3). ***p< 0.001 vs. solvent control, ANOVA + Bonferroni post hoc tests. Measurements were performed together with Moritz Verhoff (University of Tübingen, Germany)*

3.2.8 Determination of p12-LO activity in platelet homogenates treated with sex hormones

To evaluate whether the effect of progesterone observed in intact cells could be related to a direct inhibition of p12-LO activity, a cell free assay was performed. To this aim, platelets from female donors were first homogenized and then incubated (after homogenization) with progesterone (10 µM), estradiol (0.1 µM) or DHT (0.01 µM) (Fig. 15.1). AA (0.2 µM) was added as substrate. No direct inhibition of p12-LO in the homogenates was observed by any of the sex hormones, product amounts in hormone treated samples were the same as in vehicle treated cells.

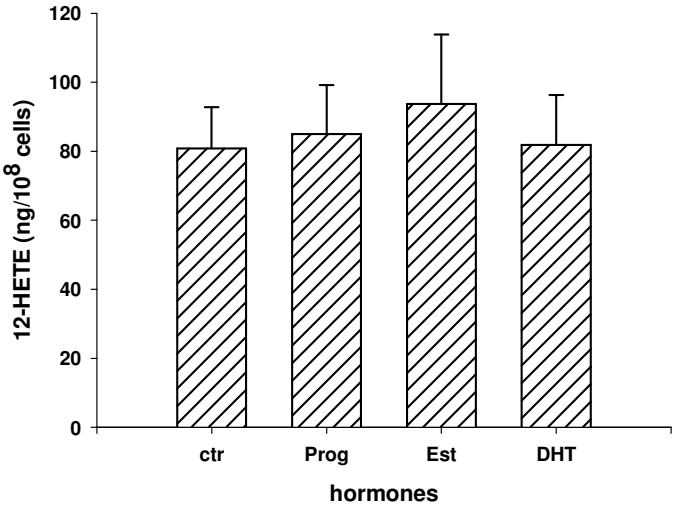

Fig. 15.1 Effects of sex hormones in platelet homogenates incubated with sex hormones *Washed human platelets were homogenized by sonification, cell rupture was checked under the microscope. Cell homogenates (1 ml corresponding to 10^8 cells in PBS-EDTA) were pre-incubated on ice with hormones (Progesterone Prog 10 µM, estradiol Est 0.1 µM, DHT 0.01 µM,) for 10 min, then incubated at 37°C for 10 min with 0.2 µM AA as substrate. $CaCl_2$ (1 mM) was added 50 s. before hormone addition. As solvent control (ctr), 0.1% ethanol was used. Reactions were stopped with 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3)*

These results indicate that progesterone (as well the other hormones tested) is not directly targeting p12-LO enzymatic activity. Interestingly, no significant effects of progesterone (and DHT or estradiol) were observed, also when cells were first incubated with hormones and then homogenates were prepared (Fig. 15.2). This suggests that an intact cellular environment is needed for the action of progesterone on p12-LO product formation, and then only the cellular p12-LO activity results influenced, as this effect is not persistent after homogenization.

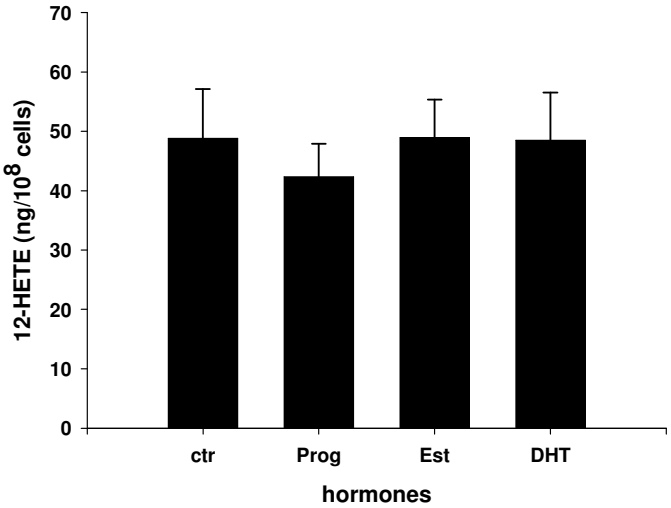

Fig. 15.2 Effects of sex hormones on 12-LO activity in homogenates after preincubation of intact platelets *Washed human platelets (10^8/ml in PG buffer) were incubated with the respective sex hormones (progesterone Prog 10 µM estradiol Est 0.1 µM, DHT D 0.01 µM,) for 30 min, resuspended in new buffer, and homogenized by sonification. Cell rupture was checked under the microscope. Homogenates were then incubated at 37°C for another 10 min with addition of 0.2 µM AA as substrate. $CaCl_2$ 1 mM was added 50 s before hormone addition. As control (ctr) 0.1% ethanol was used. Reactions were stopped with 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3)*

3.2.9 Reversibility of the progesterone effect

Progesterone was observed to have significant effects on cellular p12-LO activity. However, no difference between male and female platelets was observed (Fig. 1.1). To evaluate possible reasons for this discrepancy, the reversibility of the progesterone effect was evaluated. Platelets from female donors were incubated with progesterone (3 µM for 30 min). Progesterone was removed by a washing step with BSA 1 mg/ml in PG buffer and cells were then resuspended in PG buffer and incubated for another 30 min. Interestingly, platelets regained almost their full ability to form 12-HETE after progesterone had been removed from the sample (Fig. 16). Removal of progesterone was confirmed by evaluation of the HPLC-chromatogram (absorption at 280 nm) (data not shown).

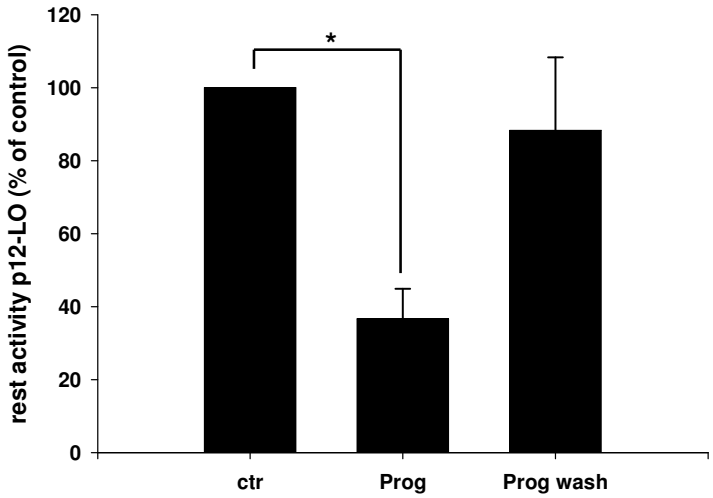

Fig. 16 Reversibility of progesterone effect on p12-LO in intact human platelets *Washed human platelets from female donors (10^8/ml in PG buffer) were incubated at 37°C for 30 min with ethanol as solvent control (ctr) or progesterone 3 µM (Prog) and a washing step was performed as described in the methods section. Reactions were finally stopped with 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean % rest activity of solvent control + S.E. (n = 3) * p 0.0245 vs. solvent control, ANOVA + Bonferroni post hoc tests*

3.2.10 Influence of progesterone on p12-LO subcellular localisation

p12-LO activity is assumed to be connected to p12-LO translocation to the platelet membrane [95]. Inhibition of this translocation step could be responsible for a reduced 12-HETE formation in progesterone treated cells. However, progesterone (10 µM) was neither able to change subcellular localisation in non-stimulated cells nor to inhibit thrombin-induced translocation of p12-LO (30 min preincubation with progesterone, Fig. 17).

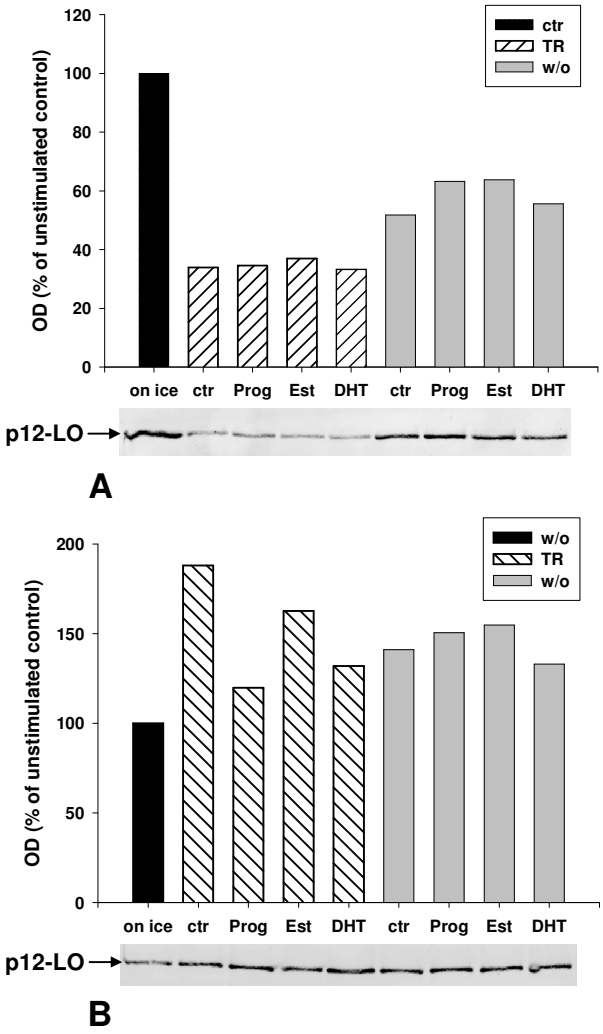

Fig. 17 Effect of sex hormones on translocation of p12-LO in intact cells (A as S100 and B as P 100) *Female washed platelets (1 × 10^9/ml in PG buffer) were incubated for 30 min at 37°C with the indicated hormones (10 µM). $CaCl_2$ (1 mM) was added 5 min before hormone addition. After 30 min samples were either stimulated with thrombin (TR 1 U/ml) or left non-stimulated. Reactions were stopped in N_2 liq., cells were lysed by repeated freeze-thawing cycles (3×) and an ultracentrifugation step (1h, 4°C, 100,000 × g) was performed to separate **(A)** cytosolic (S 100) and **(B)** pelletable (P 100) fractions. S 100 and P 100 were mixed 1:1 with SDS-loading buffer and separated via SDS-PAGE. p12-LO was determined by Western blot and Ettan Dige analysis. Data shown is representative for 3 individual experiments.*

Longer preincubation times with progesterone did not result in an inhibition of translocation (data not shown), despite very prominent effects on 12-HETE formation.

3.2.11 Platelet aggregation after progesterone treatment

To evaluate whether the effect of progesterone might be related to a general change of platelet physiology, the aggregation of platelets was evaluated. Platelets were freshly isolated from citrate blood and incubated for different

times with progesterone (10 µM) or DMSO as solvent control. When cells were stimulated to aggregate with thrombin or collagen, no difference in aggregation was seen between platelets treated with progesterone and platelets treated with solvent, even when long preincubation times of 120 minutes were applied (Fig. 18)

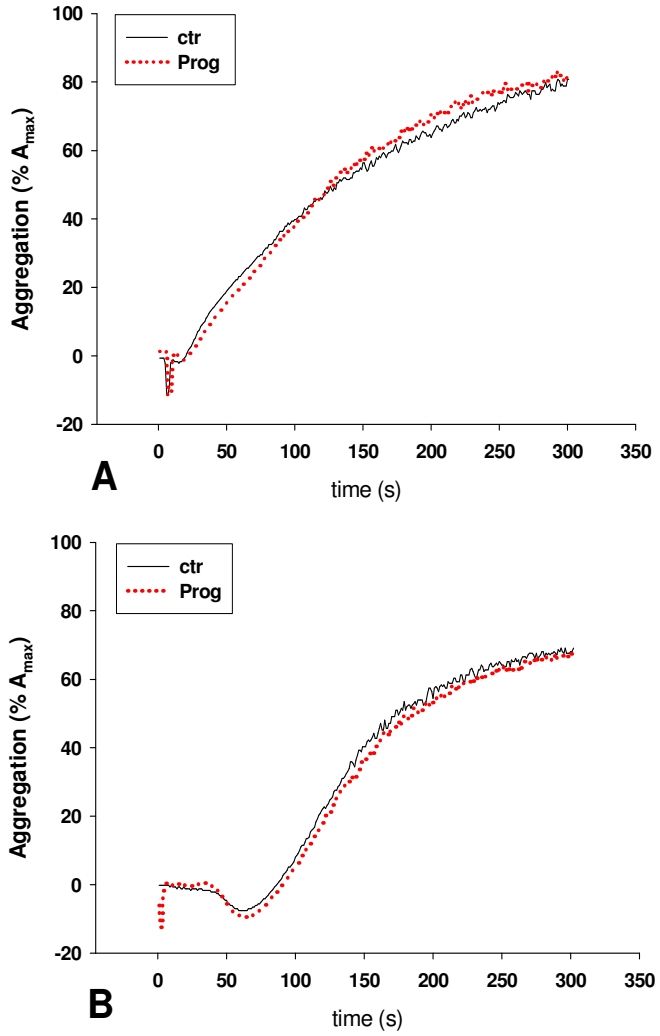

Fig. 18 Agonist induced platelet aggregation after treatment of platelets with progesterone *Washed human platelets were resuspended in Tyrode´s buffer (2 × 10^8/ml) and preincubated with progesterone (Prog 10 µM) or DMSO (ctr 1 µl) for 120 min at RT. Platelet aggregation was recorded for 5 min using a turbidimetric light-transmittance device. $CaCl_2$ (1 mM) was added right before the start of the measurement. Cells were stimulated with* ***(A)*** *1 U/ml thrombin or* ***(B)*** *1 µg/ml collagen, the aggregation response is given as percentage of the maximal light transmission A_{max}. Curves are representative for 3 independent determinations.*

3.2.12 Influence of progesterone on kinase activation status

Sex hormones have been reported to influence signalling kinases via non-genomic mechanisms, leading to rapid cellular effects. Since platelets are devoid of a nucleus and therefore lack genomic regulation, it was evaluated

whether the effect of progesterone on p12-LO might be mediated by activation or inhibition of kinase-mediated phosphorylation events. However, progesterone did not influence the basal phosphorylation state of the signalling kinases ERK, Akt, p38 or PKC or their main phosphorylation target MARCKS for PKC (Fig. 19). The result was the same whether male (data not shown) or female cells were used. An influence of agonist induced kinase activation was not examined since a progesterone effect on p12-LO activity was clearly observed in non-simulated and in stimulated platelets as well.

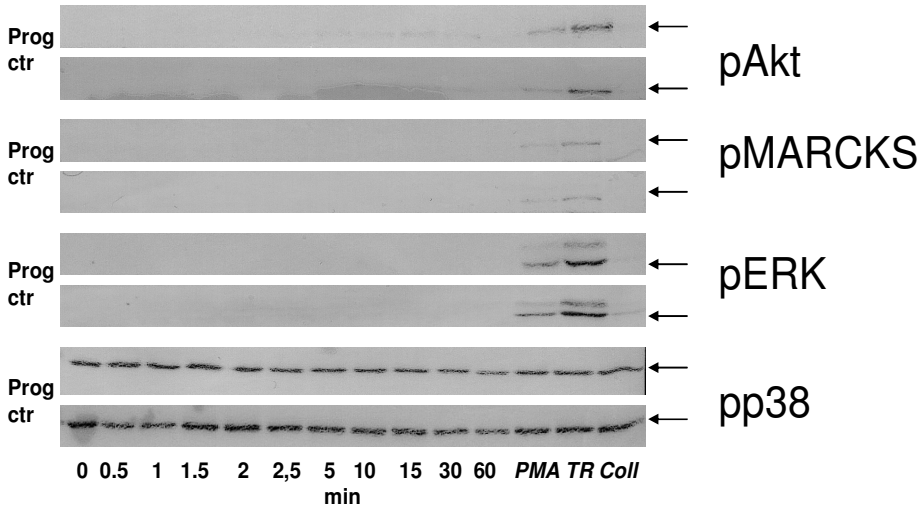

Fig. 19 Influence of progesterone on phosphorylation status of signalling kinases in human platelets *Washed human platelets (10^8/0.1 ml PG buffer) were incubated for the indicated times with progesterone (10 µM) or ethanol (0.1%) as solvent control, respectively. $CaCl_2$ (1 mM) was added on ice before aliquoting the cells. As positive control cells were stimulated with thrombin (1 U/ml), collagen (12 µg/ml) or PMA (100 nM) for 7 min at 37°C. Samples were stopped after the indicated times by addition of SDS-loading buffer 1:1 and were separated by SDS-PAGE. Activation of Akt, PKC (via MARCKS), ERK and p38 was determined by Western blot using specific antibodies. Data shown are representative for 3 individual experiments.*

3.2.13 Influence of hormone addition on $[Ca^{2+}]_i$ in intact platelets

It has previously been reported that progesterone metabolites are able to influence $[Ca^{2+}]_i$ in platelets [180]. Thus, the effect of sex hormones on $[Ca^{2+}]_i$ levels was evaluated. However, neither progesterone nor estradiol or DHT induced $[Ca^{2+}]_i$ influx in platelets, when directly added to the cells (Fig. 20.1).

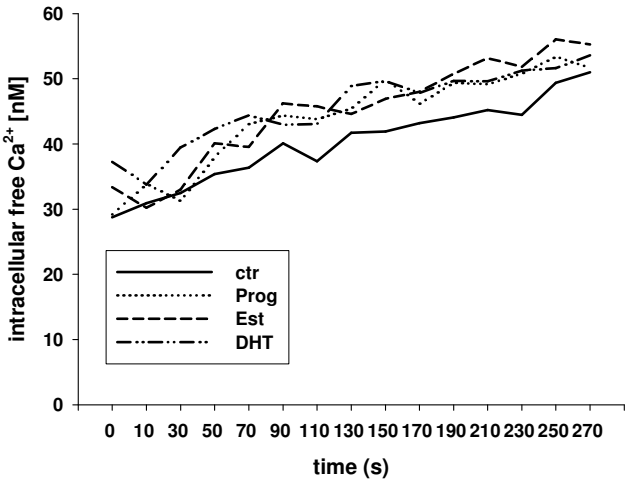

Fig. 20.1 Effect of direct sex hormone addition on $[Ca^{2+}]_i$ influx in intact platelets *To Fura-2 loaded female cells (10^8/ml PG buffer) $CaCl_2$ (1 mM) was added 60 s prior start of measurement of $[Ca^{2+}]_i$. Progesterone (Prog), estradiol (Est) and DHT were added at a concentration of 10 µM after 30 s. Cells were left untreated and $[Ca^{2+}]_i$ was measured for 280 s. As solvent control (ctr) 0.1% DMSO was used. Data shown is representative of 3 individual experiments.*

Also, hormone addition immediately followed by stimulation with thrombin or collagen did not result in a significant change of agonist-mediated $[Ca^{2+}]_i$ mobilisation (Fig. 20.2).

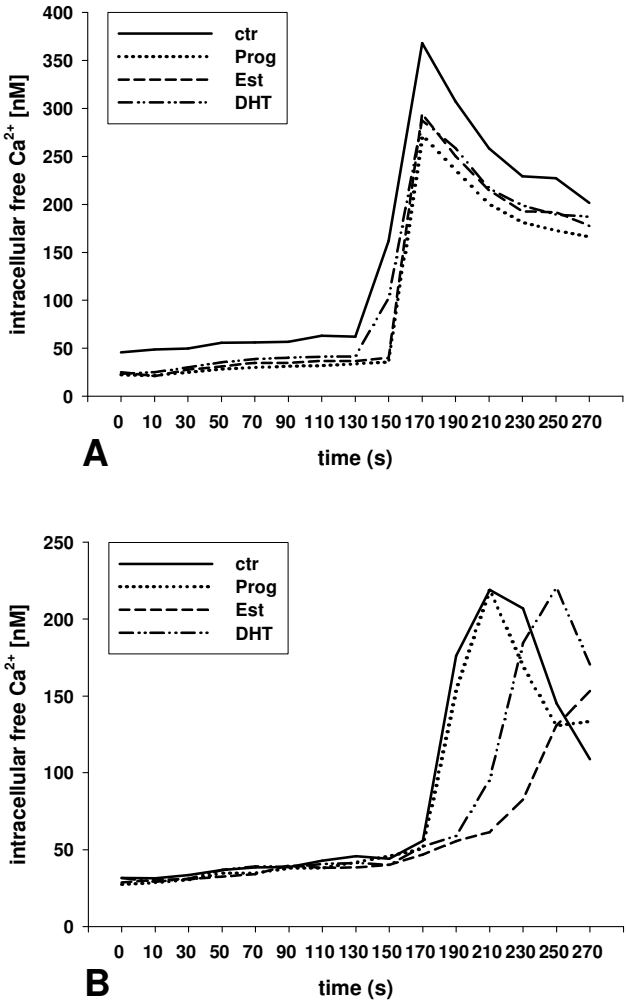

Fig. 20.2 Effect of direct sex hormone addition on agonist* induced *$[Ca^{2+}]_i$ influx in intact platelets *To Fura-2 loaded female cells (10^8/ml in PG buffer) $CaCl_2$ (1 mM) was added 60 s prior start of measurement of $[Ca^{2+}]_i$, Hormones progesterone (Prog), estradiol (Est) and DHT were added at a concentration of 10 µM 30 s. Cells were stimulated with **(A)** thrombin 1 U/ml or **(B)** collagen 12 µg/ml after 110 s and $[Ca^{2+}]_i$ was measured for 280 s. As solvent control (ctr) 0.1% DMSO was used. Data shown is representative of 3 individual experiments.*

Pre-incubation of platelets with hormones (10 µM) for 60 minutes did not change $[Ca^{2+}]_i$ levels after stimulation of cells with thrombin or collagen (Fig. 20.3). Longer pre-incubation times did not give any effect either (data not shown).

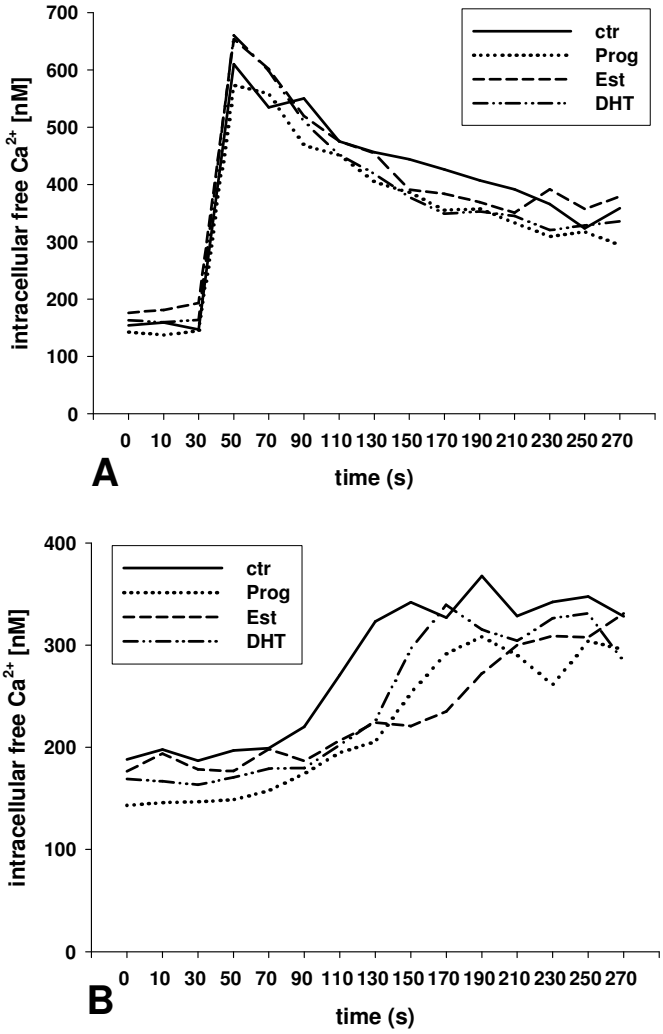

Fig. 20.3 Effect of sex hormones on agonist induced $[Ca^{2+}]_i$ elevation in intact platelets after incubation for 60 minutes *To Fura-2 loaded female cells (10^8/ml PG buffer) progesterone (Prog), estradiol (Est) and DHT were added at a concentration of 10 µM and samples were incubated for 60 min at 37°C under light protection. $CaCl_2$ 1 mM was added 60 s prior to start of measurement. Cells were stimulated with **(A)** thrombin (1 U/ml) or **(B)** collagen (12 µg/ml) 30 s after start of measurement of $[Ca^{2+}]_i$. As solvent control (ctr) 0.1% DMSO was used. Data shown is representative of 3 individual experiments.*

3.2.14 Influence of protein kinase A (PKA) activators and inhibitors on p12-LO activity and on the effect of progesterone

The most prominent and well-recognized inhibitory pathway for platelet functions is represented by protein kinase A [181]. To investigate whether the progesterone effect involves PKA activation, female platelets were treated with the PKA inhibitor H89 (10 µM) [182] and progesterone (3 µM) simultaneously. H89 treatment reversed progesterone-induced inhibition of p12-LO slightly although no full recovery of p12-LO activity was achieved (Fig. 21). Also, the PKA activator PGE_2 inhibited 12-HETE formation in

platelets at a concentration of 5 µM but its effect could not be reversed by co-incubation with H89. The PKA inhibitor itself did not show any effects on p12-LO.

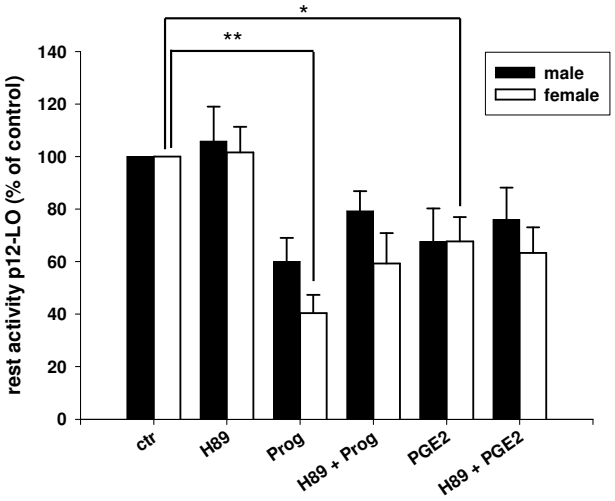

Fig. 21 Influence of PKA inhibitors and activators on p12-LO activity *Washed human platelets (10^8/ml PG buffer) from male and female donors were incubated for 60 min at 37°C with vehicle controls, progesterone (Prog 3 µM), H 89 (10 µM), PGE_2 (5 µM) and combinations, as indicated. $CaCl_2$ (1 mM) was added 50 s before addition of the substances. As control (ctr) ethanol and DMSO combined in a concentration of 0.1% each were used. After 60 min reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as % rest activity of solvent control + S.E. (n = 3)*

3.2.15 Influence of the cAMP analogue 8-bromo-cAMP on p12-LO activity

To confirm a possible role of PKA as mediator of the progesterone effect on p12-LO the capacity of cAMP analogue 8-bromo-cAMP on p12-LO activity was tested in intact cells. When 8-bromo-cAMP (1 mM) was added to intact cells and incubated for 60 min, a very slight inhibition of p12-LO activity could be measured in comparison to the control sample.

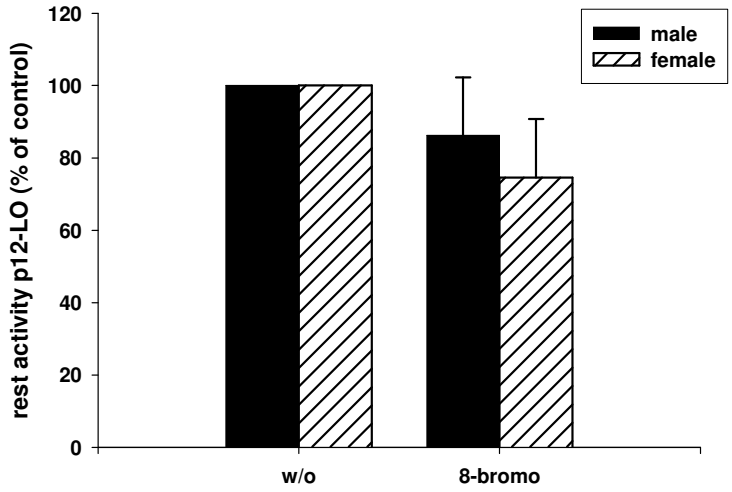

Fig. 22 Influence of cAMP analogue 8-bromo-cAMP on p12-LO product formation *Washed human platelets (10^8/ml in PG buffer) were incubated for 60 min. at 37°C with 8-bromo-cAMP (1 mM). $CaCl_2$ (1 mM) was added 50 s prior 8-bromo-cAMP. Reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as % rest activity of control (w/o) + S.E. (n = 3)*

3.2.16 Occurrence of classical progesterone receptors in platelets

Next, the occurrence of the classical progesterone receptor was evaluated in platelets. Platelets of both genders do not express the classical hormone receptor for progesterone. Western Blot signals for the corresponding proteins were not found (Fig. 23.1). As positive control PC-3 cell lysates were used (according to the antibody manual supplied by the manufacturer). However, also signals of the control cells were very weak.

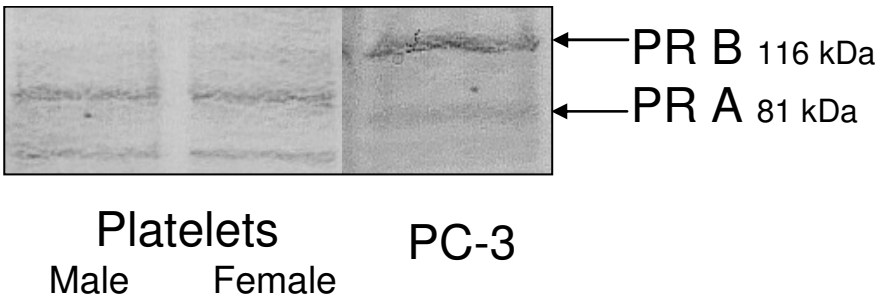

Fig. 23.1 Occurrence of classical progesterone receptors PR-A and PR-B in human platelets *Washed platelets from male and female donors (1×10^9/ml in PG buffer) were lysed by addition of SDS-loading buffer 1:1 to the cells. Proteins were separated by SDS-PAGE and analyzed by Western blot. As positive controls PC-3 cells were used in a concentration of 1×10^7/ml. Data shown is representative for n = 3 experiments performed separately.*

Next, it was evaluated whether mifepristone, a progesterone receptor antagonist with a 5-fold higher affinity to the classical progesterone receptor than progesterone itself, could block the effect of progesterone.

Interestingly, when female platelets were co-incubated with mifepristone (3 µM) and progesterone (3 µM), mifepristone was not able to abolish the progesterone effect on p12-LO product formation (Fig. 23.2). The results were the same if male cells were used (data not shown).

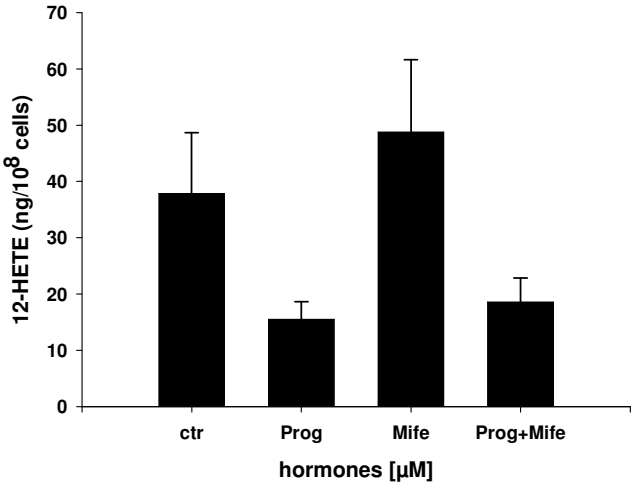

Fig. 23.2 Effect of progesterone receptor antagonist mifepristone on progesterone inhibition of p12-LO product formation in intact human platelets *Washed female platelets (10^8/ml PG buffer) were incubated with progesterone (Prog 3 µM), mifepristone (Mife 3 µM) and a combination of both for 120 min. $CaCl_2$ (1 mM) was added 50 s before hormone addition. As solvent control (ctr) ethanol and DMSO in a concentration of each 0.1% were used in combination. Reactions were stopped by addition of 1 ml methanol, samples were analyzed by RP-HPLC: Data are given as mean ng/10^8 cells + S.E. (n = 3).*

A progesterone derivative [183], cyproterone with a slight progestinic activity also inhibited basal 12-HETE formation, although in a slightly weaker manner (Fig. 23.3). The results were the same if male cells were used (data not shown).

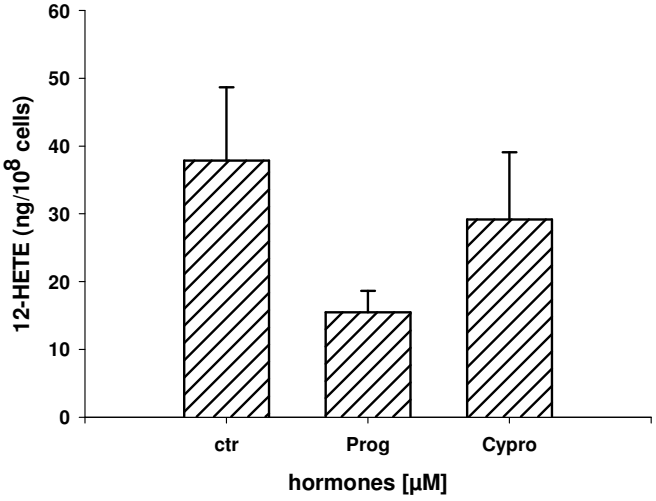

Fig. 23.3 Effect of cyproterone on p12-LO product formation in intact human platelets *Washed female platelets (10^8/ ml in PG buffer) were incubated with progesterone (3 µM) and cyproterone (3 µM) for 120 min, respectively. $CaCl_2$ (1 mM) was added 50 s before hormone addition. As solvent control (ctr) 0.1% ethanol was used. Reactions were stopped by addition of 1 ml methanol, samples were analyzed by RP-HPLC: Data are given as mean ng/10^8 cells + S.E. (n = 3)*

3.3. p12-LO inhibitor cinnamyl-3,4 dihydroxy-α-cyanocinnamate (CDC) and its pharmacological profile in platelets

Cinnamyl-3,4 dihydroxy-α-cyanocinnamate (CDC) was characterized as selective p12-LO inhibitor [147]. CDC was therefore used in this work as a control inhibitor in p12-LO activity assays and has also frequently been used by different work groups to define p12-LO involvement in various pathophysiological conditions [112, 154]. However, CDC not only inhibited p12-LO activity in the control samples but surprisingly also inhibited COX-1 with comparable potency. To further characterize its pharmacological profile, CDC was more closely investigated.

3.3.1. Influence of CDC on p12-LO and COX-1 in intact platelets

CDC was added to freshly isolated washed platelets. CDC inhibited p12-LO activity as well as COX-1 activity in intact cells (Fig. 24). Though CDC had been termed a selective p12-LO inhibitor [147], its capacity to inhibit COX-1 activity was even more prominent in some cases than its inhibition of 12-HETE formation in intact cells. In particular when cells were stimulated with AA (3 µM), p12-LO activity was not inhibited, while COX-1 product formation was blocked with an IC_{50} of 3 µM CDC (Fig. 24 D).

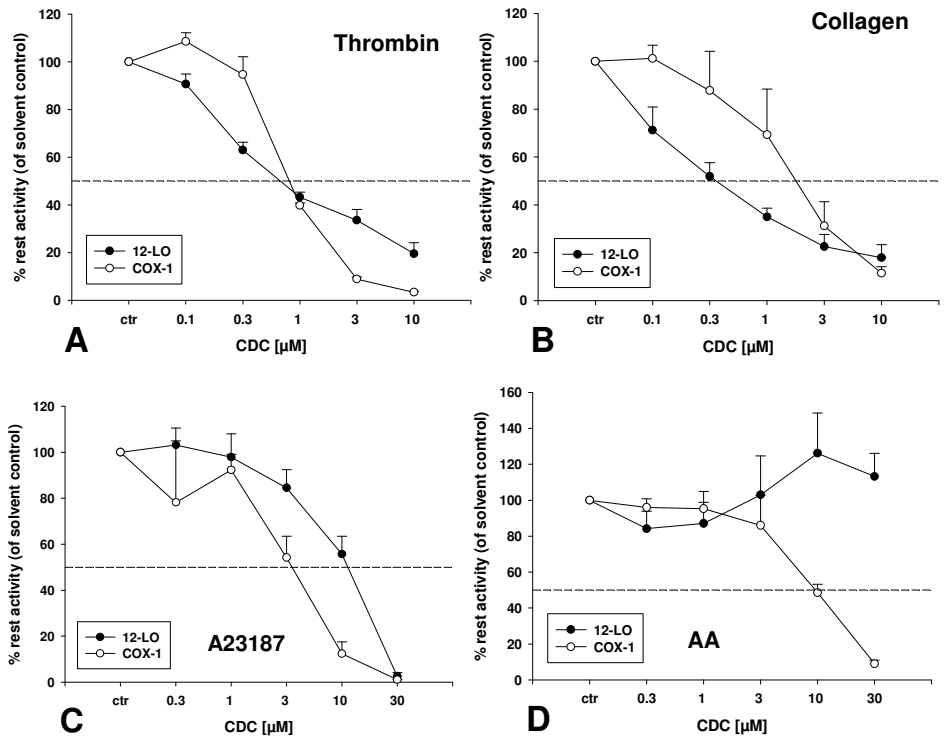

Fig. 24 Effect of CDC on p12-LO and COX-1 activity in washed human platelets stimulated with different platelet agonists *Washed human platelets (10^8/ml PG buffer) were pre-incubated with CDC in the indicated concentrations for 20 min at 37°C. Platelets were then stimulated with* ***(A)*** *1 U/ml thrombin,* ***(B)*** *8 µg/ml collagen,* ***(C)*** *2.5 µM A23187,* ***(D)*** *3 µM AA for 10 min. $CaCl_2$ (1 mM) was added 60 s before CDC. Reactions were terminated by addition of 1 ml methanol and samples were analyzed by RP-HPLC. As solvent control (ctr) 0.2% DMSO was used. Data are given as % remaining activity of solvent control for p12-LO and COX-1 respectively, + S.E. (n = 3). IC_{50} values for CDC were determined to be between 0.3 and 10 µM for both enzymes depending on assay design except for AA stimulation, where the IC_{50} for p12-LO was > 30 µM.*

3.3.2. Inhibition of p12-LO and COX-1 by CDC in platelet homogenates

To distinguish between a direct inhibition of COX-1 and p12-LO and effects only mediated in the intact cell, platelet homogenates were treated with different concentrations of CDC. p12-LO was potently inhibited in platelet homogenates, CDC could be confirmed as a direct p12-LO inhibitor. But also COX-1 was potently inhibited as observed in intact platelets, indicating that CDC might also be a direct inhibitor of COX-1 (Fig. 24).

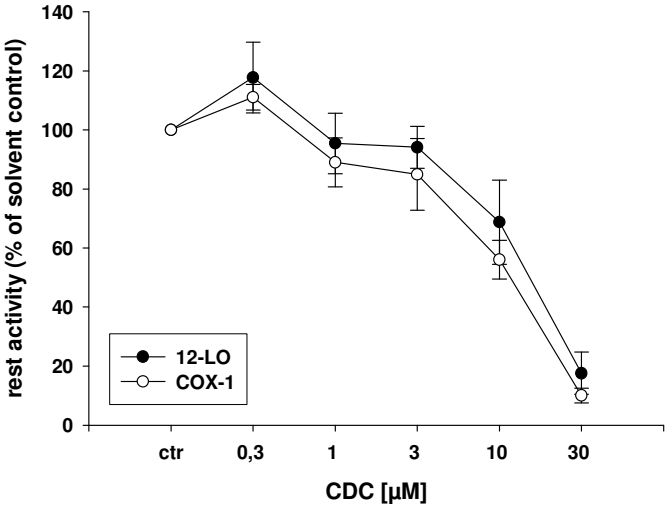

Fig. 25 Effect of CDC on p12-LO and COX-1 activity in platelet homogenates *Washed platelets were homogenized by sonification, cell rupture was checked under the microscope. Cell homogenates (1 ml corresponding to 10^8 cells in PBS-EDTA) were pre-incubated with CDC at the indicated concentrations for 20 min on ice, then for another 10 min at 37°C with AA (3 µM) as substrate. $CaCl_2$ was added 60 s before CDC on ice. As solvent control (ctr) 0.2% DMSO was used. Reactions were terminated with 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean % remaining activity of solvent control + S.E. (n = 3) IC_{50} for COX-1 and p12-LO inhibition was determined as 10 µM.*

3.3.3. Influence of CDC on COX-1/2 and $cPLA_2$ in a cell free system

More detailed studies of CDC effects on COX-1/2 in a cell free system revealed that CDC did in fact not inhibit purified COX-1/2 enzymes at all and thus a direct inhibitory mechanism of COX enzymes can be excluded (Fig. 26.1).

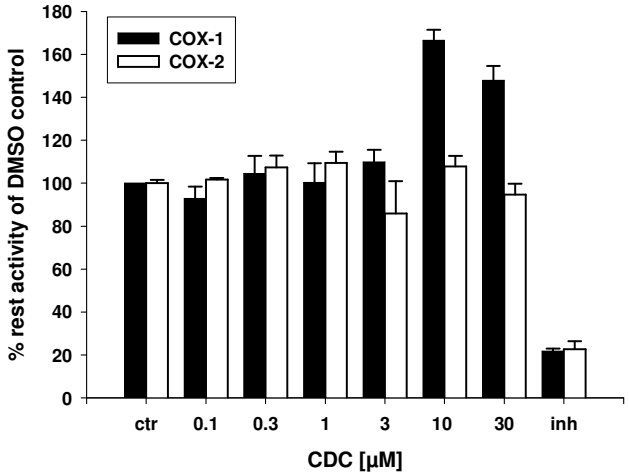

Fig. 26.1 Effect of CDC on COX-1/2 activity in a cell free assay *Purified COX-1 and COX-2 enzymes were pre-incubated with CDC in the indicated concentrations at 4°C for 5 min. Samples were then pre-warmed for 60 s at 37°C, AA (5 µM) was added and samples were incubated for 5 more min. Reactions were stopped by addition of 1 ml methanol, COX-1/2 product 12-HHT was extracted and analyzed by RP-HPLC. As control inhibitors (inh) indomethacin (10 µM) for COX-1 and celecoxib (10 nM) for COX-2 were used, solvent control (ctr) was 0.1% DMSO. Data are given as mean % rest activity of solvent control + S.E. (n = 3)*

CDC was not able to impair $cPLA_2$ activity in a cell-free assay (Fig. 26.2), a direct inhibition of $cPLA_2$ by CDC could be excluded. The strong inhibition of COX-1 activity in intact platelets and platelet homogenates could thus not completely be attributed to a direct inhibition and lowered AA-release but seemed to target more complex regulatory mechanisms.

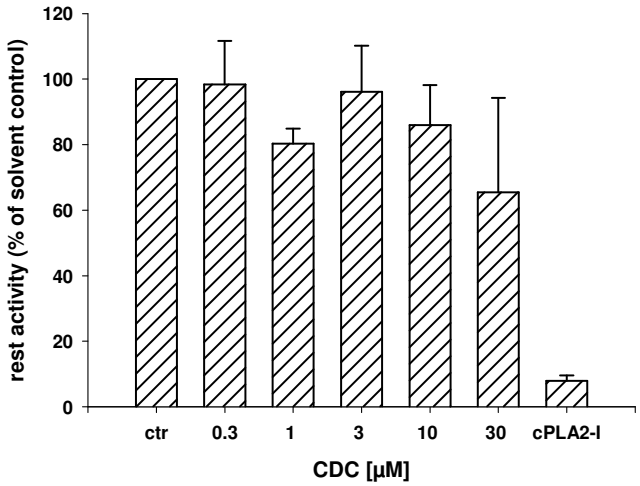

Fig. 26.2 Effect of CDC on $cPLA_2$ activity in a cell free system *Purified $cPLA_2$ was incubated for 60 min with CDC in the indicated concentrations as described in the methods section. Reactions were stopped with 1 ml methanol, released AA was extracted and derivatized with p-anisidinium chloride for 60 min. Samples were then analyzed by RP-HPLC. As controls EDTA and a $cPLA_2$ inhibitor (*pyrrolidine-1 *10 µM) were used, solvent control (ctr) was 0.1% DMSO. Data are given as % rest activity of DMSO control*

3.3.4. Influence of CDC on AA release in intact platelets

AA release was also measured in intact cells using tritium-labelled AA to determine the effect of CDC to decrease AA release in a cellular environment (Fig. 27). Thrombin- as well as A23187 -induced AA release were suppressed by CDC with IC_{50} determined at 3 µM for A23187 and at 0.3 µM for thrombin.

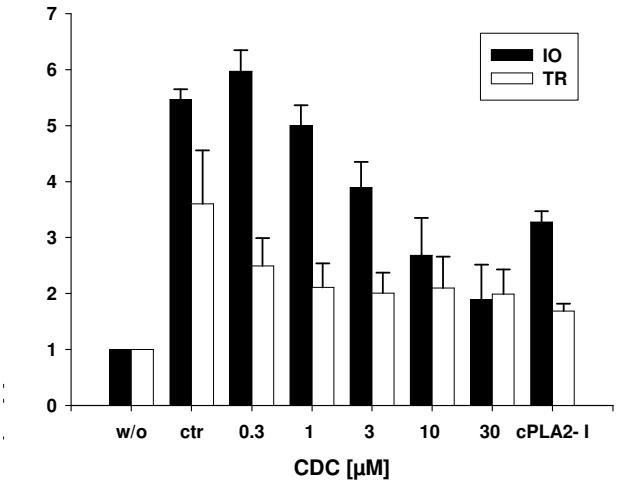

Fig. 27 Effect of CDC on AA release in intact platelets stimulated with thrombin and A23187 *Freshly isolated PRP was labelled with [^{3}H] AA (1 µCi/ml) for 120 min at 37°C. Cells were washed to remove non-incorporated AA and were treated with CDC (10^8/ml) at the indicated concentrations. To stimulate AA-release thrombin (TR 1 U/ml) or A23187 (IO 2.5 µM) were added, as solvent control (ctr) 0.1% DMSO was used. Total amounts of radioactive AA and AA-metabolites were measured via scintillation counter. Data are given as % rest activity of DMSO control + S.E. (n = 3).*

3.3.5. Influence of CDC on $[Ca^{2+}]_i$ mobilisation in platelets

Enzyme activities in platelets are closely regulated by the amounts of $[Ca^{2+}]_i$ [11]. Impairing $[Ca^{2+}]_i$ mobilisation could add to the inhibitory effect of CDC on COX-1 and p12-LO activity in platelets. Thus the influence of CDC on agonist-induced change in $[Ca^{2+}]_i$ levels was determined. CDC was able to suppress $[Ca^{2+}]_i$ mobilisation in comparison to solvent control when cells were stimulated by thrombin (Fig. 28.1). Thrombin-induced $[Ca^{2+}]_i$ mobilisation was reduced markedly by 10 µM CDC and completely abolished by 30 µM CDC (Fig. 28.1).

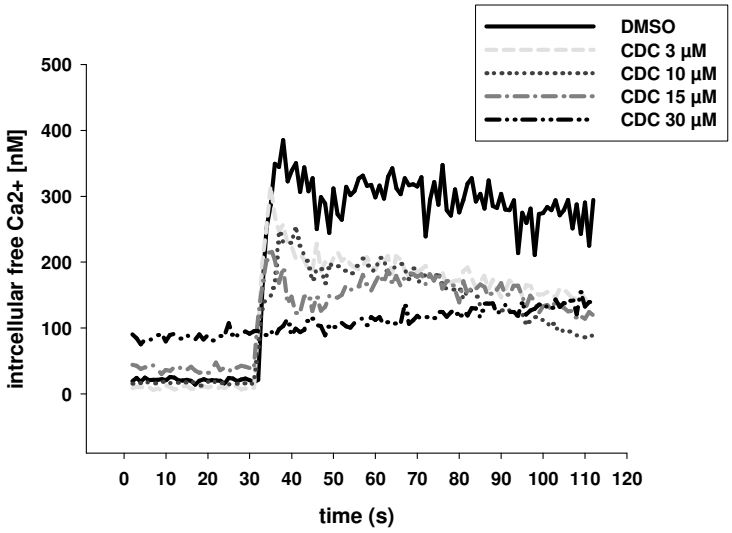

Fig. 28.1 Effect of CDC on thrombin-induced $[Ca^{2+}]_i$ mobilisation in intact human platelets *To Fura-2 loaded female cells (10^8/ml in PG buffer) CDC in the indicated concentrations or DMSO as vehicle control were added and samples were incubated for 20 incubation at 37°C under light protection. $CaCl_2$ (1 mM) was added 60 s prior to start of measurement. Cells were stimulated with thrombin 1 U/ml 30 s after start of measurement of $[Ca2+]_i$. As solvent control (ctr) 0.1% DMSO was used. Data shown is representative of 3 individual experiments.*

When collagen was used as agonist to induce $[Ca^{2+}]_i$ mobilisation a strong inhibitory effect by CDC could already be observed at 0.3 µM and $[Ca^{2+}]_i$ elevation was completely blocked at a concentration of 10 µM CDC (Fig. 28.2).

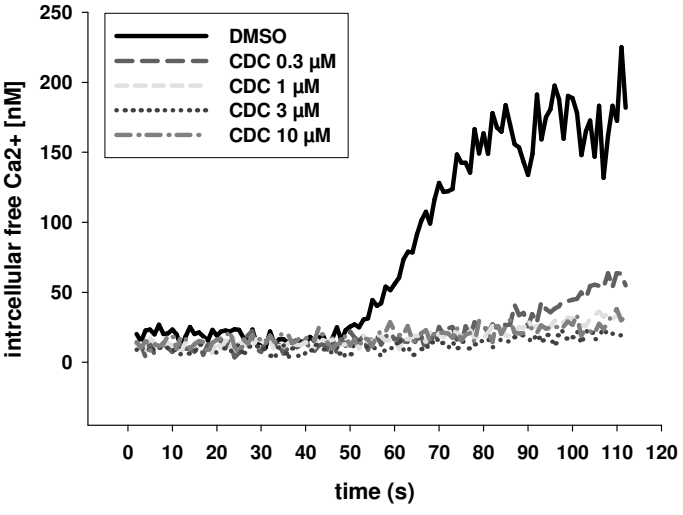

Fig. 28.2 Effect of CDC on collagen-induced $[Ca^{2+}]_i$ mobilisation in intact human platelets *To Fura-2 loaded female cells (10^8/ml in PG buffer) CDC at the indicated concentrations or DMSO as vehicle control were added and samples were incubated for 20 incubation at 37°C under light protection. $CaCl_2$ (1 mM) was added 60 s prior to start of measurement. Cells were stimulated with collagen (12 µg/ml) 30 s after start of measurement of $[Ca^{2+}]_i$. As solvent control (ctr) 0.1% DMSO was used. Data shown are representative of 3 individual experiments.*

3.3.6 Influence of CDC on ROS formation in platelets

CDC as structural derivative of caffeic acid [147] could possibly possess anti-oxidative characteristics. Its ability to prevent formation of reactive oxygen species (ROS) was determined. When thrombin (0.5 U/ml) or collagen (8 µg/ml) were used to stimulate cells, almost no ROS formation could be measured in platelets treated with 10 or 30 µM CDC in comparison to solvent control, a marked decrease of ROS formation was already observed at 0.3 µM CDC (Fig. 29).

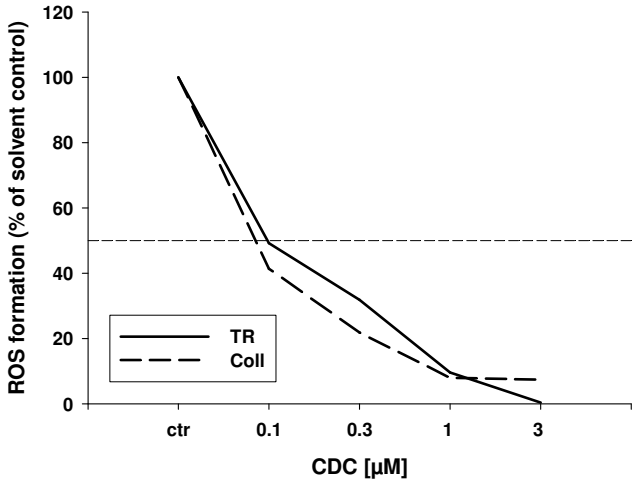

Fig. 29 Effect of CDC on agonist induced ROS formation in intact human platelets *Washed human platelets were incubated with CDC at the indicated concentrations for 20 min at 37°C. Measurement was started 5 min after addition of fluorescent dye. ROS formation was induced by stimulation of cells with thrombin (TR 1 U/ml) and collagen (Coll 12 µg/ml). 0.2% DMSO was used as solvent control (ctr). Data are given as % ROS formation of solvent control and are representative for 3 experiments performed separately. EC_{50} = 0.1 µM.*

3.3.7 Inhibition of p12-LO and COX-1 by CDC in whole blood and PRP

To characterize the efficiency of CDC in a more physiological system, whole blood assays were performed. IC_{50} values were determined at about 30 µM for p12-LO and > 30 µM for COX-1 (Fig. 30.1).

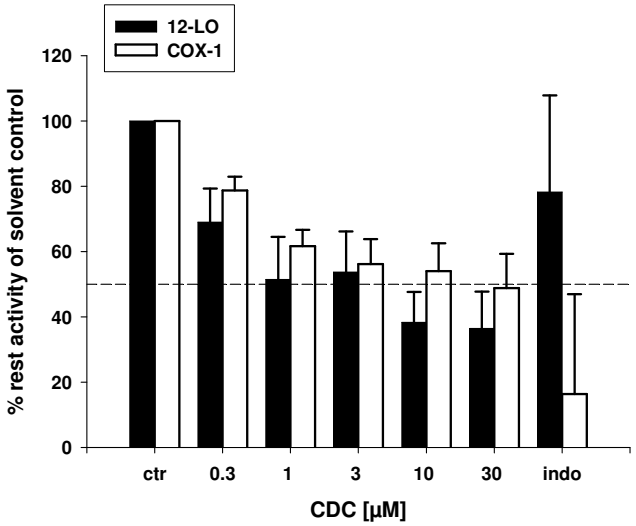

Fig. 30.1 Effect of CDC on p12-LO and COX-1 product formation in whole blood *Freshly drawn venous citrate blood was incubated with CDC at the indicated concentrations for 20 min at 37°C. After 20 min p12-LO and COX-1 activity was induced by addition of 30 µM A23187. Reactions were stopped after 10 min on ice, samples were centrifuged (600 × g, 10 min, 4°C) and plasma supernatants were collected. Samples were prepared for HPLC as described and p12-LO and COX-1 product formation was analyzed. As solvent control (ctr) 0.1% DMSO was used. Data are given as mean % rest activity of solvent control + S.E. (n = 3-4). IC_{50} were determined at 3 µM for p12-LO and 30 µM for COX-1.*

However, when CDC was added to PRP prepared from freshly drawn citrate blood, the inhibitory potential of CDC was less prominent, although a similar result as in whole blood could be seen. CDC inhibited both COX-1 and p12-LO with an IC_{50} > 30 µM (Fig. 30.2).

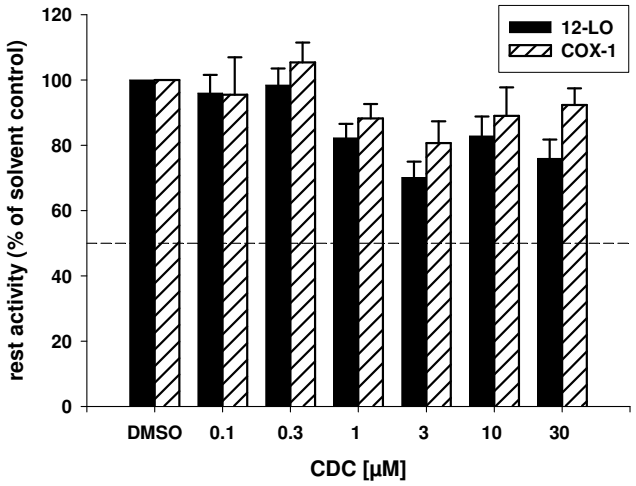

Fig. 30.2 Effect of CDC on p12-LO and COX-1 product formation in PRP *PRP freshly prepared from human venous citrated blood was incubated with CDC at the indicated concentrations for 20 min at 37°C. After 20 min. p12-LO and COX-1 activity was induced by addition of 100 µM AA. $CaCl_2$ (2 mM) was added 50 s prior CDC. Reactions were stopped after 10 min by addition of 2 ml methanol and samples were analyzed by RP- HPLC. Data are given as mean % rest activity of solvent control + S.E. (n = 3). IC_{50} for p12-LO and COX-1 were determined at > 30 µM.*

3.3.8 Influence of CDC on platelet aggregation induced with different stimuli

CDC was able to block COX-1 activity as well as $[Ca^{2+}]_i$ elevation in thrombin and collagen stimulated cells. As both events are closely connected to platelet aggregation the effect of CDC on platelet aggregation was investigated. Freshly prepared washed human platelets were treated with different concentrations of CDC. At concentrations of 10 and 30 µM an inhibition of collagen-induced aggregation was observed (Fig. 31.1).

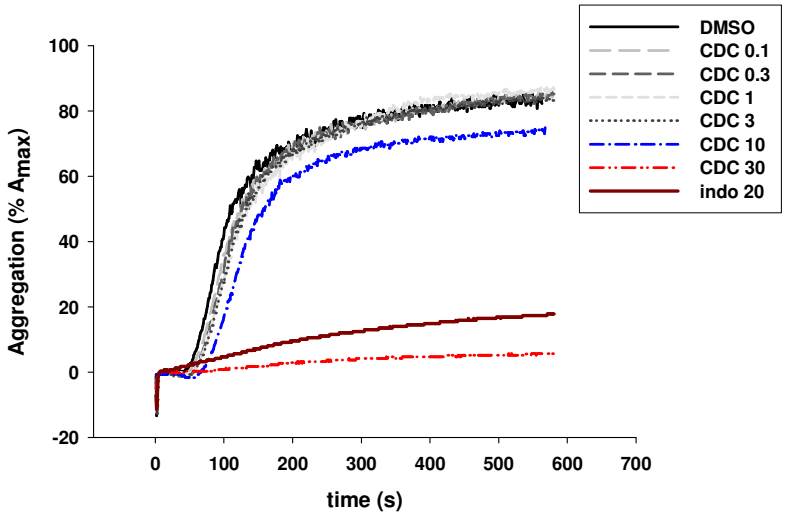

Fig. 31.1 Effect of CDC on collagen induced platelet aggregation of platelets
Washed human platelets were resuspended in Tyrode´s buffer (2×10^8/ml). Platelet aggregation was recorded for 5 min using a turbidimetric light-transmittance device. $CaCl_2$ 1 mM was added right before the start of the measurement. Cells were pre-incubated for 20 min with CDC in the indicated concentrations. As control indomethacin (indo 20 µM) was used as inhibitor of platelet aggregation. Cells were stimulated with 0.5 µg/ml collagen, the aggregation response is given as percentage of the maximal light transmission A_{max}. Curves are representative for 3 independent determinations.

When cells were stimulated with AA (200 µM), thromboxane analogue U 46619 (1 µM) or thrombin (1U/ml) to induce aggregation CDC did not prevent aggregation even at concentrations of 30 µM (Fig. 31.2 A-C).

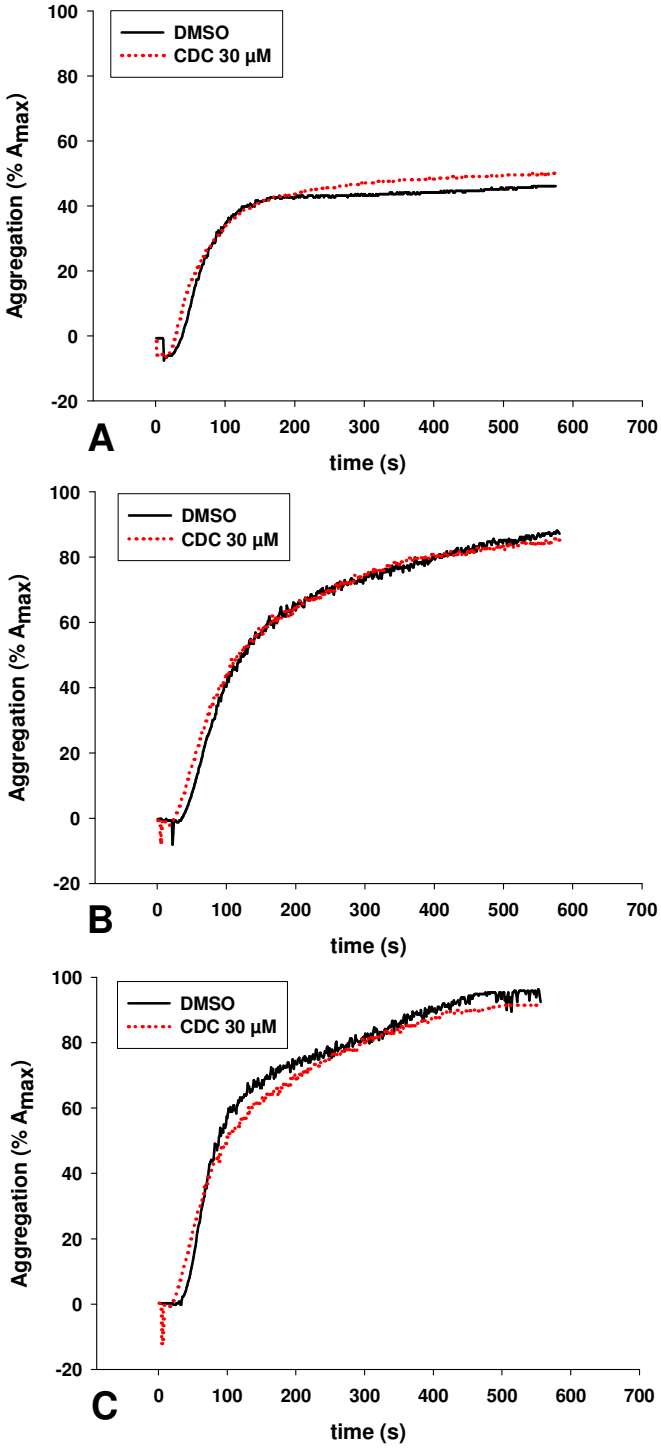

Fig. 31.2 Effect of CDC on AA-, U46619- and thrombin-induced platelet aggregation *Washed human platelets were resuspended in Tyrode´s buffer (2 × 10^8/ml). Platelet aggregation was recorded for 5 min using a turbidimetric light-transmittance device. $CaCl_2$ (1 mM) was added right before the start of the measurement. Platelets were preincubated for 20 min with CDC (10 and 30 µM). As controls indomethacin 10 µM was used as inhibitor of platelet aggregation and 0.1% DMSO as solvent control. Platelets were stimulated with* ***(A)*** *AA (200 µM),* ***(B)*** *U46619 (1 µM) or* ***(C)*** *thrombin (1 U/ml). The aggregation response is given as percentage of the maximal light transmission A_{max}. Curves are representative for 3 independent determinations.*

Also, TXB_2-levels after stimulation of intact cells and in homogenates were determined. TXB_2 is the stable metabolite of TXA_2 which is closely connected to platelet aggregation [20]. TXB_2 formation was significantly reduced at CDC concentrations of 3 to 10 µM as well in intact cells stimulated with 1 U/ml thrombin or 8 µg/ml collagen (Fig. 31.3).

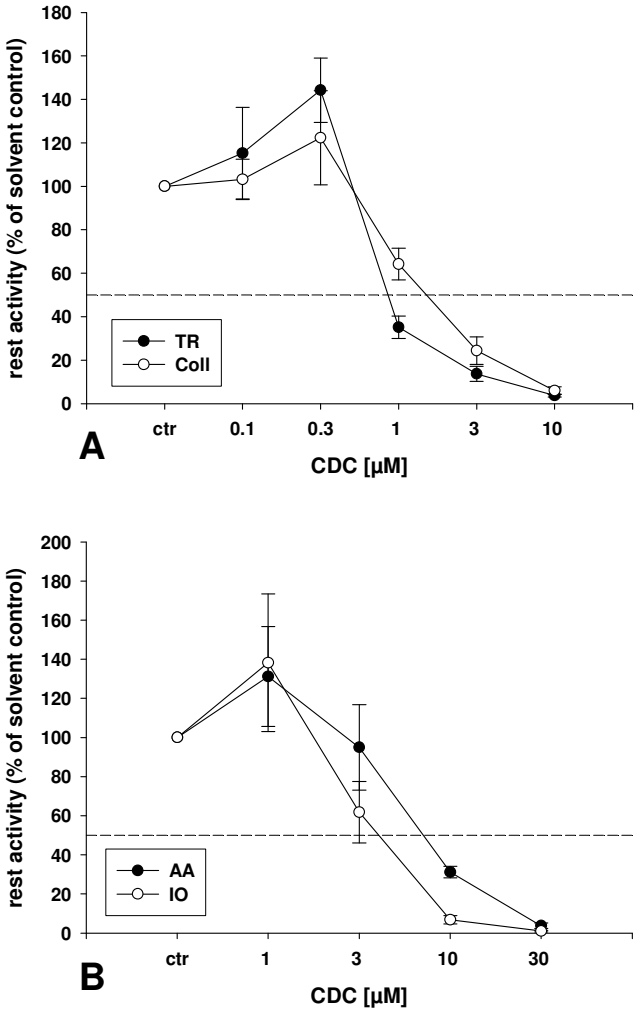

Fig. 31.3 CDC Inhibition of TXB_2 formation in washed human platelets *Washed human platelets (10^8/ml) were pre-incubated with CDC in the indicated concentrations for 20 min at 37°C. After 20 min cells were stimulated with* ***(A)*** *thrombin (1 U/ml) and collagen (8 µg/ml) and* ***(B)*** *AA (3 µM) and A23187 (2.5 µM) respectively. $CaCl_2$ (1 mM) was added 60 s before CDC. As solvent control 0.2% DMSO was used. Reactions were stopped on ice and TXB_2 formation was determined following the manufacturer's manual of the used ELISA kit. Data (mean + S.E.; n = 3) are given as percentage of solvent control (100%).*

Altogether, CDC proved to be much more than just a p12-LO inhibitor that can easily be used as a pharmacological tool for analysis of the p12-LO pathway. In fact, when using CDC many regulatory pathways could possibly be affected and contribute to the observed effect independent of p12-LO.

3.4 Effects of MK-886 on p12-LO in platelets

MK-886 is a well known and frequently used FLAP and mPGES-1 inhibitor and was also described as a COX-1 inhibitor [157]. As the p12-LO and the 5-LO pathway are structurally related [184] it was reasonable to investigate whether MK-886 may affect p12-LO in intact platelets. COX-1 activity was analysed for control, as platelets also express COX-1 [86].

3.4.1 Influence of MK-886 on p12-LO and COX-1 activity in washed platelets

When intact platelets were treated with different concentrations of MK-886, an increase in 12-LO product formation could be seen at a concentration of 10 µM MK-886 in comparison to the solvent control without addition of any stimulus after a pre-incubation of 20 min. This effect vanished when the cells were subsequently stimulated with various agents (Fig. 32.1).

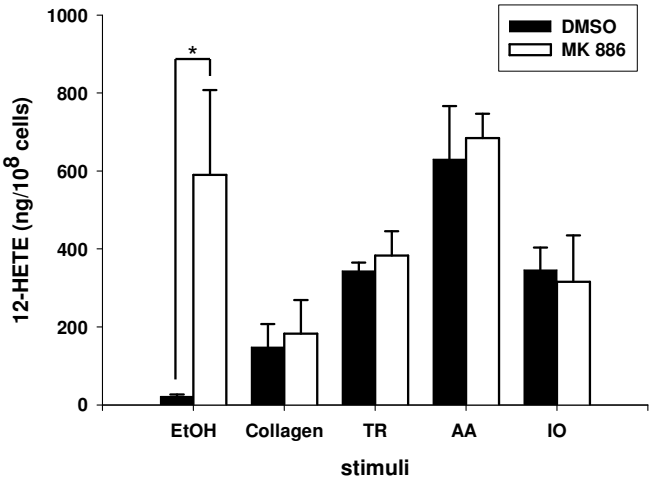

Fig. 32.1 p12-LO activity in intact platelets treated with MK-886 *Washed human platelets from female donors (10^8/ml in PG buffer) were incubated with MK-886 (10 µM) for 20 min at 37°C. Cells were either stimulated with collagen (8 µg/ml), thrombin (TR 1 U/ml), AA (AA 3 µM) or A23187 (IO 3 µM) or ethanol for another 10 min. $CaCl_2$ (1 mM) was added 50 s before MK-886. Reactions were stopped by addition of 1 ml methanol, and samples were analyzed for 12-HETE by RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3).*

For COX-1 product formation a similar stimulatory effect could be observed, although the increase in product formation was not as strong as for p12-LO (Fig. 32.2). However, in stimulated samples COX-1 product formation decreased with increasing MK-886 concentration, corresponding to MK-886 characteristics as a COX-1 inhibitor [157].

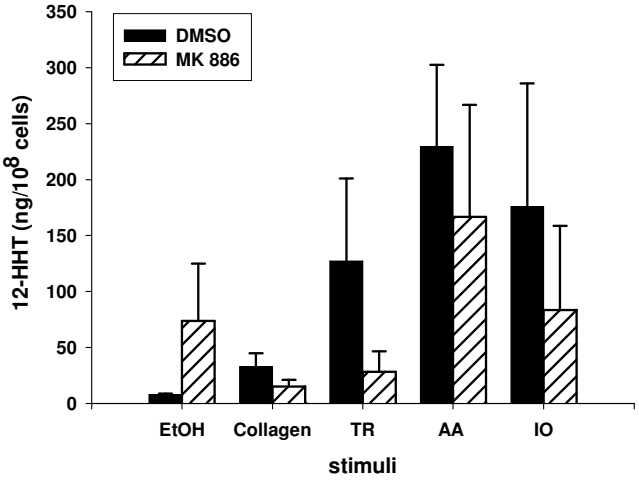

Fig.32.2 COX-1 activity in intact platelets treated with MK-886 *Washed human platelets from female donors (10^8/ ml in PG buffer) were incubated with MK-886 (10 µM) for 20 min at 37°C. Cells were either stimulated with collagen (8 µg/ml), thrombin (TR 1 U/ml), AA (AA 3 µM) or A23187 (IO 3 µM) or ethanol for another 10 min. $CaCl_2$ (1 mM) was added 50 s before MK-886. Reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as mean ng/10^8 cells + S.E. (n = 3). 12-HHT as stable COX-1 metabolite was used for determination of COX-1 activity.*

The efficiency of MK-886 was assessed by performing concentration-response assays. A concentration of 10 µM gave maximal p12-LO product formation and led to a 40-fold increase in comparison to the DMSO control. A comparable effect could be observed for COX-1 product formation as well, where an increase of about 10-fold was observed (Fig. 32.3).

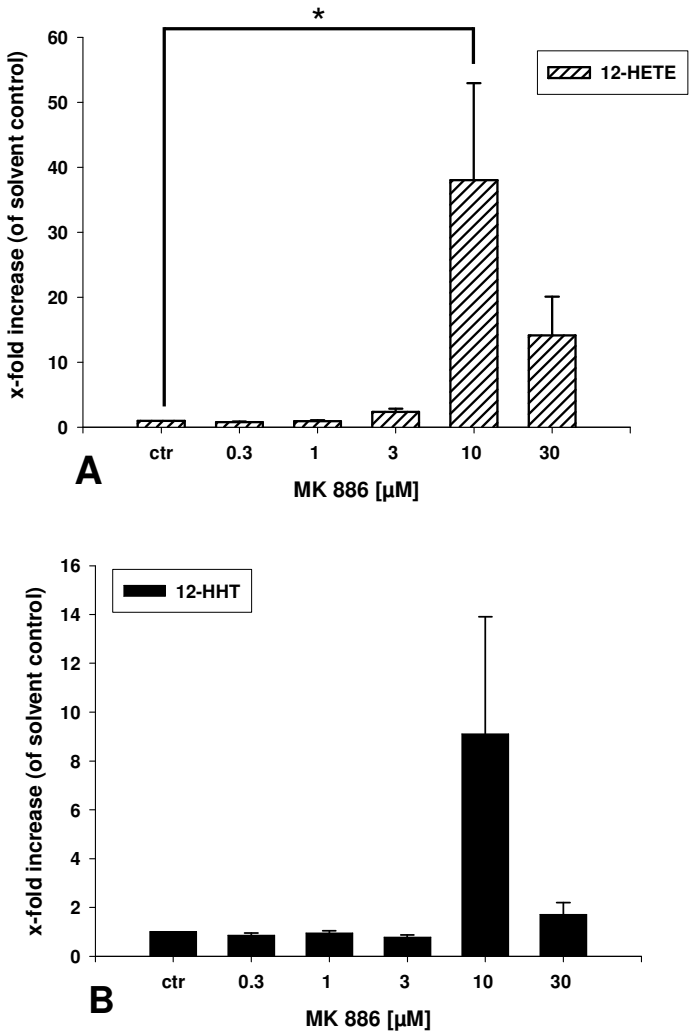

Fig.32.3 Increase of p12-LO and COX-1 product formation in MK-886 treated human platelets without stimulation *Washed human platelets from female donors (10^8/ml in PG buffer) were incubated with the indicated concentrations of MK-886 for 20 min at 37°C. $CaCl_2$ (1 mM) was added 50 s before MK-886. Reactions were stopped by addition of 1 ml methanol and samples were analyzed by RP-HPLC.* ***(A)*** *p12-LO product formation measured as 12-HETE.* ***(B)*** *COX-1 product formation measured as 12-HHT. Data are given as x-fold increase of product in comparison to solvent control + S.E. for both p12-LO and COX-1 (n = 3) *p 0.05 ANOVA + Bonferroni post hoc test*

3.4.2. Effect of MK-886 on p12-LO and COX-1 in AA supplemented platelets and platelet homogenates

Different mechanisms may exist by which MK-886 could exert its effect on p12-LO and COX-1. MK-886 could directly stimulate both enzymes but also may increase the availability of substrate AA for both enzymes. When cells were supplemented with 10 µM AA, no stimulating effect of MK-886 on the activity of both enzymes could be observed. p12-LO product formation remained approximately the same for all MK-886 concentrations, whereas COX-1 activity decreased significantly with rising MK-886 concentrations (Fig. 33.1), as expected [157].

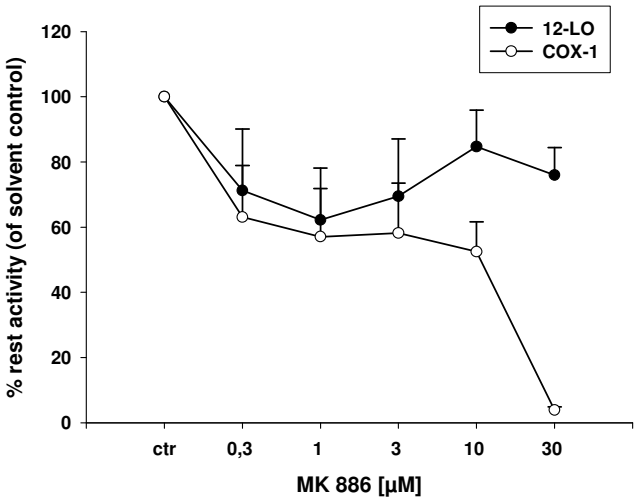

Fig. 33.1 Effect of MK-886 on p12-LO and COX-1 activity in intact human platelets supplemented with arachidonic acid *Washed human platelets (10^8/ ml in PG buffer) were incubated with the indicated concentrations of MK-886 for 20 min at 37°C. $CaCl_2$ (1 mM) was added 50 s before MK-886. Cells were then stimulated for 10 min with arachidonic acid (10 µM). Reactions were stopped with 1 ml methanol and samples were analyzed by RP-HPLC. Data are given as % rest activity of solvent control + S.E. (n = 3) and were determined using 12-HETE as indicator for p12-LO activity and 12-HHT for COX-1 activity.*

In platelet homogenates, MK-886 showed neither a stimulating effect on p12-LO nor on COX-1 activity. No increase in product formation for p12-LO or COX-1 could be observed at any MK-886 concentration in homogenates supplemented with exogenous arachidonic acid (Fig. 33.2). Instead, for COX-1 product formation, a slight decrease in 12-HHT amounts could be observed for rising MK-886 concentrations (Fig. 33.2 B).

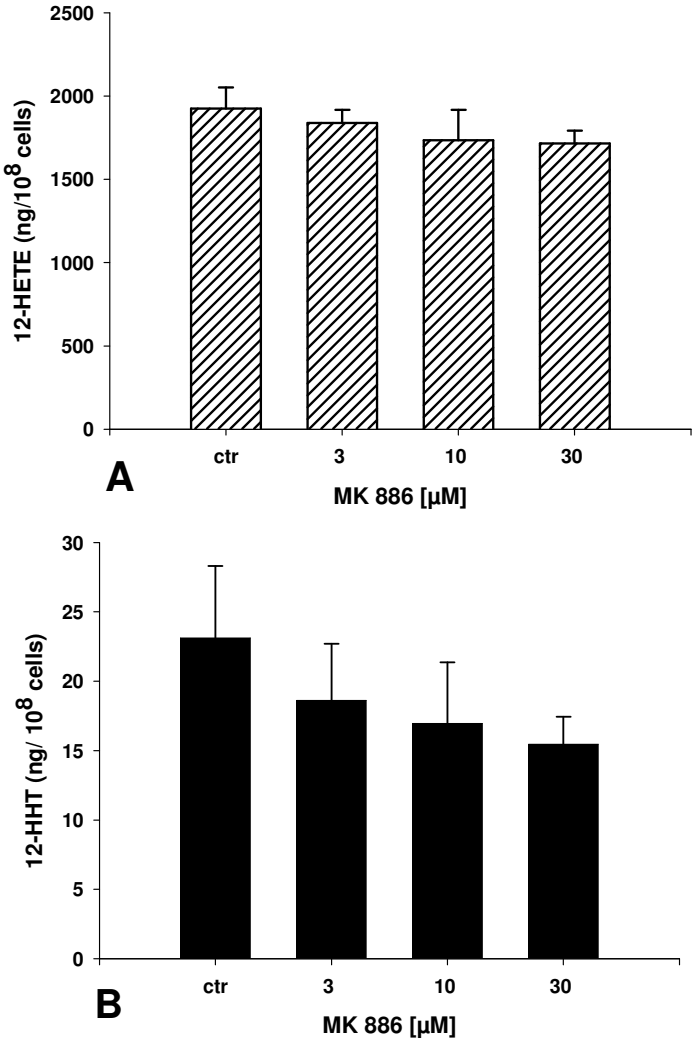

Fig. 33.2 Effect of MK-886 on p12 LO and COX-1 activity in platelet homogenates *Washed human platelets from female donors were homogenized by sonification. Cell rupture was checked under the microscope. Homogenates (1 ml corresponding to 10^8 cells in PBS-EDTA) were incubated with MK-886 in the indicated concentrations for 10 min on ice. Homogenates were then supplemented with AA (10 µM) and incubated for another 10 min at 37°C. $CaCl_2$ (1 mM) was added 50 s before MK-886 on ice. Reactions were stopped with 1 ml methanol, and samples were analyzed by RP-HPLC.* ***(A)*** *12-HETE formation and* ***(B)*** *12-HHT formation were measured as means to determine p12-LO and COX-1 activity respectively. Data are given as mean ng/10^8 cells + S.E. (n = 3).*

In homogenates, where no exogenous arachidonic acid was added, also no activating effect of MK-886 on p12-LO and COX-1 activities could be observed (data not shown).

3.4.3. Influence of MK-886 on AA release in intact platelets and on $cPLA_2$ activity in a cell free system

Treatment of intact platelets with varying concentrations of MK-886 led to an increase in AA release with a maximum at 10 µM MK-886. The AA liberation was approximately 4-fold enhanced in comparison to DMSO control after an incubation time of 20 min (Fig. 34.1).

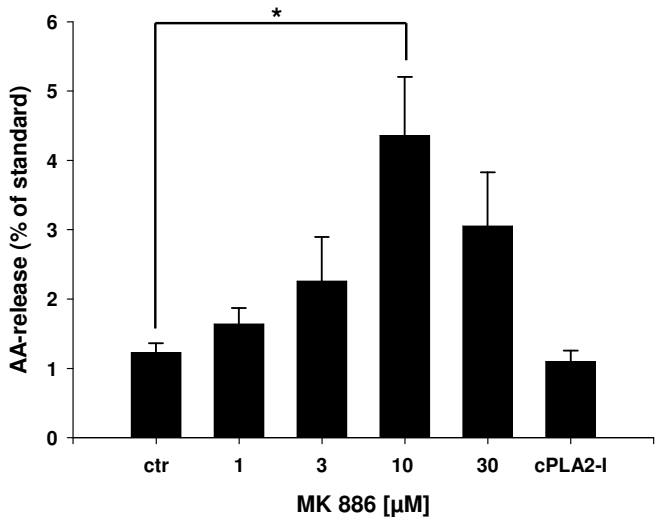

Fig. 34.1 AA release in intact human platelets in response to MK-886*.Washed human platelets (10^8/ml in PG buffer) were incubated with indicated concentrations of MK-886 at 37°C for 20. As control a $cPLA_2$ inhibitor (10 µM) was used. $CaCl_2$ (1 mM) was added 50 s before MK-886. Reactions were stopped with 2 ml methanol, released AA was derivatized, and samples were analyzed by RP-HPLC. Data are given as mean x-fold increase of AA in comparison to solvent control +S.E. (n = 3)*

To investigate whether MK-886 was activating $cPLA_2$ directly, different concentrations of MK-886 were tested in a cell free $cPLA_2$ assay. Recombinantly expressed purified $cPLA_2$ was incubated for 60 min with different concentrations of MK-886 in a solution of AA-containing vesicles. Amounts of released AA were determined by HPLC after a derivatisation step. No direct activation of $cPLA_2$ could be observed (Fig. 34.2).

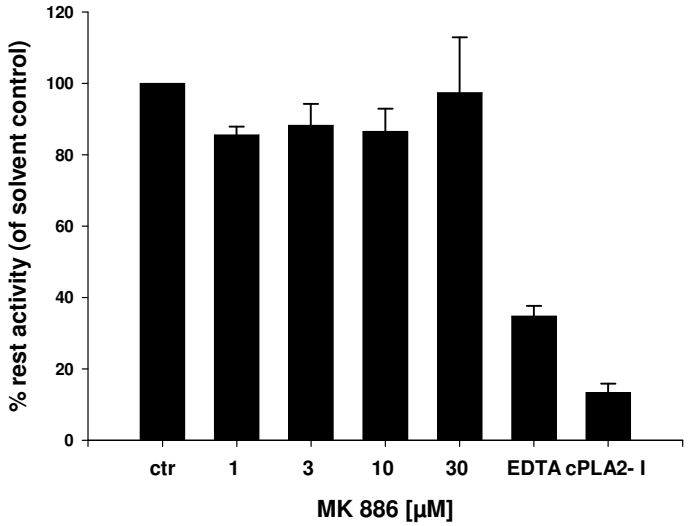

Fig. 34.2 Effect of MK-886 on the activity of $cPLA_2$ in a cell free system
Purified $cPLA_2$ was incubated for 60 min with MK-886 in the indicated concentrations and the activity was determined as described in the methods section. The $cPLA_2$ inhibitor (pyrrolidine-1 10 µM) or DMSO as solvent control (ctr) were added. Reactions were stopped by addition of 2 ml methanol, released AA was derivatized and samples were analyzed by RP-HPLC. Data is given as mean % rest activity + S.E. (n = 3). Measurements were performed by M. Verhoff (University of Tübingen, Germany).

3.4.4. MK-886 induced $[Ca^{2+}]_i$ mobilisation in platelets and PMNL

Recently it has been found that MK-886 is also able to induce $[Ca^{2+}]_i$ influx in PC-3 cells (prostate carcinoma cell line) [168]. As $cPLA_2$ is strongly influenced by $[Ca^{2+}]_i$ levels in the cell and in the surrounding buffer system, $[Ca^{2+}]_i$ mobilisation in response to MK-886 was also analyzed in platelets. At a concentration of 10 µM, MK-886 caused a strong increase in $[Ca^{2+}]_i$ levels (Fig. 35.1) also in platelets.

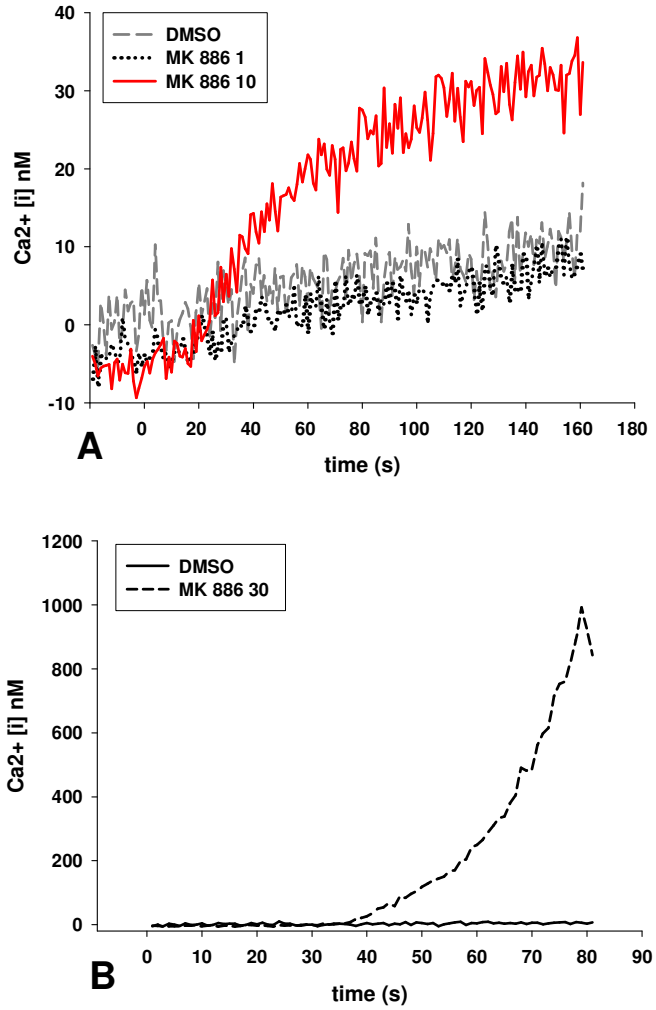

Fig. 35.1 Effect of MK-886 on intracellular $[Ca^{2+}]_i$ levels in intact human platelets *To Fura-2 loaded female platelets (10^8/ml in PG buffer) $CaCl_2$ (1 mM) was added 60 s. prior start of measurement of $[Ca^{2+}]_i$. MK-886 in the indicated concentrations or DMSO 1 µl as vehicle control were added after 30 s. Cells were left non-stimulated and $[Ca^{2+}]_i$. was measured for 180 s. Data are given as $[Ca^{2}+]_i$.in nM/10^8 cells and are representative of 3 individual experiments.*

An even stronger $[Ca^{2+}]_i$ elevation equivalent to agonist stimulation was found when platelets were treated with 30 µM MK-886 (Fig. 35.1 B). To find out whether this was a platelet specific effect, also PMNL were treated with MK-886 at different concentrations, and intracellular $[Ca^{2+}]_i$ elevation

was measured. At a concentration of 30 µM MK-886, a slight increase could be detected shortly after MK-886 addition. This effect was more prominent when a concentration of 50 µM was added to the PMNL (Fig. 35.2). Another FLAP inhibitor, licofelone [185] was tested in the same set-up at a concentration of 50 µM. Also licofelone induced an elevation of $[Ca^{2+}]_i$ in PMNL (data not shown).

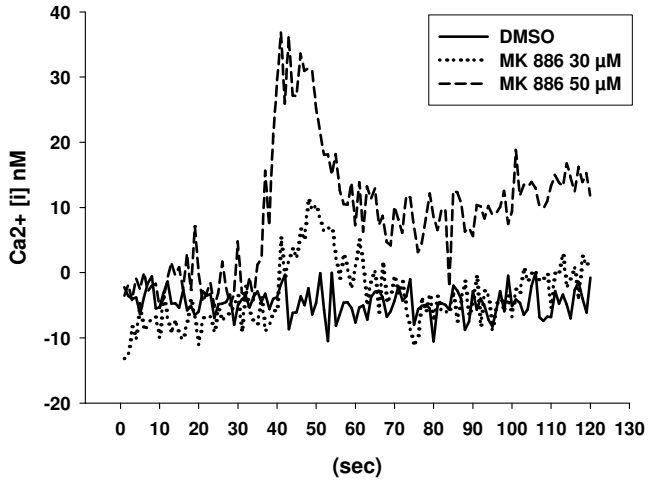

Fig. 35.2 Effect of MK-886 on $[Ca^{2+}]_i$ levels in intact PMNL *To Fura-2 loaded female PMNL (10^6/ml in PG buffer), $CaCl_2$ 1 mM was added 60 s prior start of measurement of $[Ca^{2+}]_i$. MK-886 in the indicated concentrations or DMSO 1 µl as vehicle control were added after 30 s. Cells were left non-stimulated, and $[Ca^{2+}]_i$ was measured for 180 s. Data shown is representative of 3 individual experiments.*

In conclusion as many studies especially concerning studies investigating the role of the 5-LO pathway in apoptosis or mechanisms of cancer development use MK-886 as a pharmacological tool at concentrations of 10 to 50 µM, an effect of MK 866 on $[Ca^{2+}]_i$ levels in cellular systems should always be taken into account when evaluating generated data.

4 Discussion

4.1 Sex differential regulation of and hormonal influence on p12-LO activity

Androgens were recently identified to regulate 5-LO activity in isolated PMNL and whole blood revealing a possible basis for a sex bias in leukotriene (LT) biosynthesis [127]. A different pattern of LT biosynthesis results in a varying predisposition for inflammatory diseases in male and female [127]. p12-LO activity in human platelets was investigated and a similar picture as for 5-LO activity was expected, since p12-LO and 5-LO are related enzymes [57]. Freshly isolated washed platelets from male and female donors were compared in regard to spontaneous and stimulated p12-LO activity, AA release, p12-LO subcellular localisation and p12-LO expression. Surprisingly, no significant differences could be found in any of the points investigated except for AA release in A23187-stimulated cells, in contrast to 5-LO where a lower leukotriene biosynthesis was found in male cells associated with different 5-LO subcellular localisation caused by androgens [127].

In washed platelets from male and female donors, AA release after A23187-stimulation differed slightly but significantly between male and female platelets. This might explain the tendency for minmal higher 12-HETE formation in A23187-stimulated PRP from female donors as compared to male PRP. However, this higher p12-LO activity in PRP was not found to be statistically significant. Debatable is the comparison of p12-LO activity in PRP and AA-release in washed platelets since the experimental conditions vary between the two assays. The physiological relevance of observed differences in AA release between male and female is unclear.

A lower basal p12-LO product formation in non-stimulated female platelets after incubation times of 15 and 120 min was not considered significant. The very low amounts of 12-HETE produced during these incubations will not have an impact on the overall product formation in stimulated platelets, where 12-HETE amounts measured are approx. 10- to 100-fold higher depending on stimulus applied.

Furthermore, no consistent tendency for higher or lower p12-LO activity in stimulated female cells was observed except for this particular incidence in A23187-stimulated platelets. Nevertheless, at least in non-stimulated female platelets a tendency for lower p12-LO product formation could be detected.

COX-1 activity was monitored in each experiment as control. COX-1 activity and product amounts did not differ between male and female donors neither in washed cells nor in PRP incubations.

Experimentation with washed platelets means to handle cells that have been removed from all plasma constituents including sex hormones. To remedy this flaw, the influence of sex hormones was reconstituted by addition of sex hormones to the washed platelets resuspended in buffer. Progesterone impaired p12-LO significantly after 30 min of incubation in non-stimulated cells. Estradiol and DHT proved to be ineffective in comparable concentrations. This rapid progesterone effect, however, was found to be reversible in a very fast manner. As soon as progesterone was removed from the incubated sample p12-LO regained almost full activity (90%). This observation may explain the lack of significant differences for the p12-LO activity between washed platelets from female and male donors since sex hormones had been removed during the cell isolation process.
Astonishingly, no significant difference was found in p12-LO activity in PRP incubations from male and female donors where all hormones are still present and should affect p12-LO activity. Yet effective concentrations of progesterone to impair p12-LO activity were determined to be between 1 and 3 µM. Such high progesterone concentrations are only reached in females during pregnancy, so the missing significant difference between male and female PRP is not surprising since progesterone concentrations in male and female do not strongly differ during most of the days of the normal menstrual cycle and are below 0.1 µM. Still, as mentioned above, a slight trend towards lower p12-LO activity in female PRP compared to male PRP could be observed.
Matching the results obtained from male and nonpregnant female PRP showing no significant difference in p12-LO product formation, PRP from

pregnant donors showed a lower p12-LO activity. However, this lower p12-LO activity can only in part be attributed to a progesterone influence as also COX-1 activity was lower in the samples of pregnant donors. COX-1 activity however was not influenced by progesterone in any concentration in washed platelets. A more complex regulation of p12-LO in pregnant females seems likely since complex changes in blood coagulation and composition have been detected during pregnancy as well [186] and might also change susceptibility of platelets for their agonists and thus the metabolic productivity of platelet-residing enzymes.
Progesterone addition to PRP isolated from male donors did impair p12-LO activity, although very slightly. Subsequent progesterone supplementation of platelets resuspended in plasma prepared from male donors also slightly lowered p12-LO product formation in comparison to controls. Both results support a possible influence of progesterone on p12-LO *in vivo*. However, analyzing p12-LO activity in platelets resuspended in plasma isolated from pregnant donors at different stages of pregnancy showed no conclusive effect. p12-LO activities could not be correlated to different progesterone plasma levels. Again, a more complex regulation depending on several factors seems obvious.

In general, p12-LO activity was lower in plasma or PRP incubations than in platelets incubated in a buffer system. This suggests that plasma may contain some p12-LO inhibitory component. Detection of basal p12-LO activity in PRP or plasma induced by incubation at 37°C was hardly evaluable as product formation was not as clearly discernible as in samples from washed platelets. A very strong plasma binding could easily entail this low product amount traceable in plasma but would not impair physiological function of 12-HETE. Also an apparently low concentration of 12-HETE could be compensated by an “omnipresence” of 12-HETE in a constant concentration around its target cells or receptors thus continuously influencing these possible targets.

p12-LO activity is often linked to the translocation of the enzyme to the platelet membrane [95, 98]. Although a translocation of p12-LO from the

cytosol was observed upon platelet stimulation, the major amount of p12-LO protein present in the cells was already located at the membrane fraction. This finding contradicts previous results while supporting others [70, 92]. However, an increase of p12-LO amount in the membrane fraction upon thrombin or collagen stimulation was not clearly detectable. Translocation of p12-LO also was not complete, a very small fraction remained in the cytosol even upon thrombin stimulation. Since most of the protein was already located at the membranes small increases in the protein concentration (by translocation from the cytosol) would probably go unnoticed. Interestingly, p12-LO also translocates to the membrane fraction without a stimulating impulse just at incubation at 37°C. This spontaneous translocation fits to the results of a spontaneous basal 12-HETE formation that can be observed in non-stimulated cells. However, a stringent connection of stimulus-induced p12-LO translocation and p12-LO activity cannot be deduced from these findings alone.

4.2 Progesterone affects p12-LO activity

Progesterone belongs to the class of steroid hormones, a category of signalling molecules mediating their effects via genomic actions and in the classical sense influencing protein expression in a slow mode of action. Platelets, however, are anucleate cells. Genomic effects in the classical meaning (concerning protein biosynthesis) cannot be monitored in anucleate cells as they have no or only limited ability for *de novo* protein biosynthesis. Progesterone is able to potently inhibit p12-LO activity in intact platelets in a fast and reversible manner, two characteristics that are not exhibited by classic genomic signalling events. Progesterone influences on p12-LO have to be attributed to different modes of action of progesterone in platelets for the reasons mentioned.

So how does progesterone influence p12-LO activity?
Progesterone interference with p12-LO activity could basically originate at several levels. Progesterone could (1) directly inhibit p12-LO, (2) impair substrate supply (AA release), (3) change the subcellular localisation of p12-LO, (4) alter platelet susceptibility towards external stimuli, (5) influence kinase activation states, in particular of PKA.

Progesterone does not inhibit p12-LO activity in cell homogenates, a direct inhibition of p12-LO can therefore be excluded. Progesterone needs an intact cellular environment to mediate its effects. Progesterone influence on p12-LO could not be observed after homogenization of platelets incubated with progesterone prior to cell disruption. Also, progesterone did not affect AA release in intact cells or $cPLA_2$ activity in a cell free system. Thus, a lack of substrate supply can also be excluded as effective mechanism for reduced p12-LO product formation. Translocation and subcellular localisation of p12-LO in platelets and agonist induced aggregation as indicators of platelet reactivity were not impaired or changed by progesterone pre-treatment of platelets. Measurement of aggregation might not be an ideal tool, since progesterone already influences spontaneous p12-LO activity and aggregation events need to be induced by platelet agonists. Alongside progesterone, also estradiol and DHT were tested in varying concentrations up to 10 µM. No other hormone showed an impact on p12-LO activity implying specificity for progesterone.

Progesterone is able to potently influence p12-LO activity in collagen- and to a lesser extent in thrombin-stimulated cells. An interference of progesterone with intracellular kinase signalling pathways generally induced by those two agonists seems likely. One has to keep in mind though, that only non-stimulated cells were tested for an influence of progesterone on kinase phosphorylation states as the strongest impact of progesterone on p12-LO activity was evident in non-stimulated cells. Whether agonist-induced kinase activation could be changed by progesterone remains a subject of further detailed research.

However, none of the kinases normally activated by thrombin or collagen stimulation like ERK1/2, p38 or Akt [13, 14, 17] were involved in progesterone influence, as progesterone neither enhanced nor impaired activation (measured by phosphorylation state) of these kinases in comparison to solvent control. Though all kinases directly investigated for phosphorylation are involved in platelet activation, progesterone exerts an

inhibitory function on p12-LO. Such a negative regulation of p12-LO could be mediated by protein kinase A (PKA), a kinase playing a major role in platelet inhibition [25]. Interestingly, p12-LO does indeed exhibit a phosphorylation motif for PKA assigning p12-LO as a putative PKA target [99]. PGE_2, a PKA activator was capable to induce a significant p12-LO inhibition in platelets. H89, a PKA inhibitor, could partially reverse effects of progesterone, supporting a connection of PKA to p12-LO regulation. A rapid, non-genomic activation of PKA by progesterone has been described in the acrosome reaction of sperm cells [143] and could be the pathway leading to p12-LO regulation in platelets as well.

PKA is regulated by intracellular cAMP levels and therefore also known as cAMP-dependent PKA [25]. Hence, a cAMP analogue, 8-bromo-cAMP was tested for its impact on p12-LO activity. Astonishingly, 8-bromo-cAMP was not able to induce a significant inhibition of p12-LO activity. An induction of rise in intracellular cAMP levels by progesterone seems unlikely. In own studies, even the control PGE_2 failed to induce a significant rise in cAMP levels (data not shown). However, which level of cAMP is actually needed to activate PKA in platelets requests further research. To ascertain p12-LO regulation via PKA pathways also phosphorylation status of other PKA targets in platelets as GPIbβ [25] and of PKA itself should be analysed after progesterone incubation of platelets. Possibly *in vitro* kinase assays studying p12-LO phosphorylation by PKA is a useful tool to reveal the actual pathway. Also a regulatory function of PTP (protein tyrosine phosphatases) involved in inhibitory platelet regulation [28] could be proposed as possible mechanism of PKA activation.

In physiological contexts, p12-LO rather seems to be a constitutively active enzyme with possible “house-keeping” functions rather than a dormant enzyme that is only activated for special occasions by stimulation [76]. Supply of free AA seems sufficient to induce p12-LO product formation. At least in platelets p12-LO is unlikely to be an enzyme derived from *de novo* protein synthesis as platelets only have limited potential for protein biosynthesis [3]. In this study it was observed that 12-HETE amounts

increased as soon as non-stimulated cells were incubated at 37°C, 12-HETE concentration accumulating at a plateau concentration after approximately 2 h. Cells kept on ice for the same period did not produce 12-HETE. More importantly, in stimulated cells a sudden increase of p12-LO activity overlapped with activation of $cPLA_2$ and subsequent AA release by the applied agonists. Strong p12-LO product formation after cell stimulation could well be independent from any activating signalling directly pointed at p12-LO. This very high p12-LO product formation could simply be due to a high substrate supply for p12-LO coinciding with a high capability of p12-LO to excessively metabolise its substrate. Especially with a background of a permanent spontaneous non-stimulated p12-LO activity [75, 76] a negative inhibiting regulation of p12-LO seems more probable than a positive activating regulation.

Progesterone does not inhibit p12-LO completely but suppresses p12-LO activity to a basal level. A certain amount of 12-HETE (approx. 20 ng/10^8 cells) is always measured. A permanent attachment of a p12-LO fraction to the platelet membranes further supports the hypothesis of a constitutive function for p12-LO. Separation of the total p12-LO protein present in platelets in two fractions forms a possibility for differential p12-LO regulation and even could imply different enzyme purposes in the cell. The larger fraction was predominately located at the membranes, the smaller fraction in the cytosol dividing p12-LO in a continuously active fraction at the membrane and a resting fraction in the cytosol that can be stimulated by platelet agonists to high activity. In this way, p12-LO could exert several different functions depending on the subcellular distribution and the activation state of the cell.

p12-LO activity is influenced by progesterone, but neither by direct interactions with p12-LO or $cPLA_2$ nor by overall platelet inhibition. Possibly, even though progesterone might not influence AA release directly it might hamper the access of p12-LO to free AA or influence p12-LO membrane binding at sites where AA is released and thus impede p12-LO access to AA.

Although involvement of $cPLA_2$ was specifically proposed in AA release needed for p12-LO activity [30] different isoforms of PLA_2 could be responsible for supplying AA as substrate for p12-LO in resting and in activated cells. Only $cPLA_2$ activity was considered in the present study. However, other PLA_2 isoforms supplying p12-LO in basal conditions could be influenced by progesterone impairing p12-LO activity.

4.3 Physiological implications of progesterone regulation of p12-LO activity

During pregnancy progesterone plasma concentrations reach levels that could influence p12-LO activity *in vivo*. Especially towards the end of pregnancy in the 3rd trimester, plasma progesterone concentrations rise to 1 µM and even above. These levels are not reached during the normal menstrual cycle. Pregnancy itself is characterized by immense changes of endometrial and uterine tissues. To sustain pregnancy, new tissue and new vessels have to be built up to secure a rich supply of nutrients for the embryo. At the end of pregnancy, angiogenesis and endometrial tissue growth slow down as the body prepares for giving birth. Although the situation during pregnancy cannot be compared with the excessive growth and angiogenesis observed for cancer and tumour growth, certain similarities concerning formation of new blood vessels and the high growth rate of cells can be discovered.

p12-LO has been brought into connection with angiogenesis and a supportive function for high metastatic potential and high growth rate of certain cancers. These properties could be important for angiogenesis during pregnancy to a certain extent supporting the immense changes in the uterus. In fact, a p12-LO is expressed in uterine cervical tissues [187, 188] and could contribute with its activity to angiogenesis and endometrial growth during the first two trimesters of pregnancy. During this time progesterone plasma levels are < 1 µM and therefore too low to significantly affect p12-LO activity. As soon as pregnancy nears full term, progesterone concentrations rise to levels inhibiting p12-LO activity. The diminished output of 12-HETE may then slow angiogenesis and endometrial growth.

Even though plasma levels of 1 µM progesterone are not reached during menstruation, a possible regulation of p12-LO by lower progesterone levels is possible. Induction of endometrial tissue shedding and influence on blood coagulation by progesterone during menstruation could be completed by progesterone regulation of p12-LO activity. Rise in progesterone levels in the follicular phase could lead to a slight suppression of p12-LO activity and onset of endometrial shedding as proliferative effects of 12-HETE are suppressed. During menstruation blood clotting is lower than that of normal blood [189]. Beside other factors, even a slight suppression of p12-LO could possibly contribute to a higher threshold for platelet activation and thus to a lower blood coagulation as p12-LO activity is important for platelet activation [103].

A potential for physiological functions of p12-LO activity lies in the effectiveness of its metabolite 12-HETE as chemotactic agent for leukocytes [116]. Suppression of p12-LO activity by progesterone would lead to lower 12-HETE concentration and therefore to a decreased infiltration of leukocytes into endometrial tissues during the secretory phase of the cycle. After progesterone withdrawal, infiltration of uterine tissues by leukocytes would increase with a resumed activity of p12-LO and higher local 12-HETE concentrations. A stronger presence of leukocytes could probably increase the local concentration of PG that lead to uterus contraction and support endometrial shedding.

How, and if 12-HETE plays a role as chemotactic factor in pregnancy and preparation of birth cannot conclusively be assessed, although a differential effect of progesterone depending on its concentration in the target tissue should be investigated. This proposed biased function of progesterone promoting different actions in different concentrations most probably is accurate for p12-LO regulation by progesterone. On the other hand, opposing functions can be proposed for p12-LO and 12-HETE involvement in pregnancy and the normal menstrual cycle.

Nevertheless, p12-LO functions may contribute only in a moderate manner in pregnancy and menstruation since the majority of progesterone properties affect protein expression and genomic regulations of uterine tissues.

Women do have higher mean plasma levels of progesterone during menstruation. Accordingly, a trend for lower p12-LO product formation in platelets could be observed in the present study.

It is a well known fact that more men (lower mean progesterone levels) are suffering from cardiovascular disease than women. Cardiovascular events are often preceded by a history of high blood pressure and are a result of a higher responsiveness of platelets. p12-LO was connected to platelet aggregation as well as it has recently been found to influence blood pressure [103, 152]. In patients with essential hypertension, an elevated urinary excretion of 12-HETE has been surveyed attributing a possible role for p12-LO activity and its metabolites in hypertension [190]. If progesterone would lower the basal p12-LO activity in females thereby inducing a fundamentally lower blood pressure and a lower susceptibility of platelets for their activating agonists, higher progesterone plasma levels could partly explain the lower incidence of cardiovascular disease in women as compared to men.

More studies on other 12-lipoxgenases possibly influencing blood pressure and platelet aggregation to progesterone have to be conducted.

4.4 Rapid non-genomic effects of sex hormones in platelets

Traditionally, effects of sex hormones are expected to be mediated by genomic regulation of their target cells. For this purpose, a functional nucleus containing genomic DNA and a transcriptional machinery is a prerequisite. Progesterone binds to its receptor, the hormone-receptor complex translocates to the nucleus and induces transcription of DNA after binding to hormone response elements in the nucleus [140]. Recently, also fast, non-genomic effects have been described for sex hormones directly activating kinase cascades or other fast regulatory mechanisms along with

progesterone receptors located in the cell membranes [138, 139]. However, those rapid effects probably mediated by membrane progesterone receptors have so far only been described for nucleated cells or for hormone concentrations far higher (10 µM or more) than the physiological range [180]. Moreover, effects described so far could not be linked to definite intracellular events, i.e., regulation of enzyme functions. In this study an effect of progesterone in a physiological range could be observed in anucleated cells (human platelets) for the first time causing an inhibition of p12-LO activity. This observation opens a completely novel field of research (also see 4.5), especially as platelets are key players in aggregation and also cardiovascular diseases. A regulation of platelet functions by sex hormones is of great interest as a clear bias exists in the incidence of cardiovascular events (also see 4.5).

4.5 Progesterone receptors on platelets

Mediation of progesterone effects is thought to primarily occur via progesterone receptors. Several receptors have been identified so far including the classical receptors PR-A and PR-B transmitting progesterone effects in a genomic manner. Also membrane-located progesterone receptors (mPR) have been identified. They are characterized by smaller size and mediate rapid non-genomic effects. Progesterone actions, probably mediated by binding to these mPR, could not be blocked by the progesterone receptor antagonist mifepristone [140]. In the present study no classical progesterone receptors could unambiguously be identified to be present in platelets whereas estradiol and androgen receptors were identified in platelets before [191]. The progesterone effect monitored in platelets could also not be blocked by mifepristone. A yet undiscovered mPR in platelets responsible for mediating regulation of p12-LO activity by progesterone seems the only possibility for the manifestation of rapid progesterone effects and has yet to be fully revealed.

4.6 p12-LO inhibitor and its pharmacological profile in platelets

CDC was first described as a selective 12-LO inhibitor [147]. Nowadays, it is commonly used as a pharmacological tool to investigate the contribution or involvement of 12-LO and its metabolite 12-HETE in different cell types

and also in animal studies. Here we show that CDC inhibits p12-LO but also COX-1 activity in intact platelets. Accordingly, approaches applying CDC as selective 12-LO inhibitor to elucidate involvement of 12-LO pathways in cancer development or hypertension [153, 192] could easily lead to false results since CDC may act also by suppressing COX activity. To reveal the pharmacological profile of CDC, its effects on intact cells, cell homogenates and cell free enzymes were investigated more closely.

In intact platelets, IC_{50} of 0.3 to 11 µM for p12-LO and COX-1 were determined with lowest IC_{50} for collagen-stimulated cells (Table 4). In A23187-stimulated cells COX-1 was more potently inhibited than p12-LO indicating different points of attack for COX-1 activity and for p12-LO in the cell.

Enzyme / Stimulus	Thrombin	Collagen	A23187	AA
p12-LO	1 µM	0.3 µM	11 µM	> 30 µM
COX-1	1 µM	2 µM	3 µM	10 µM

Table 4 *IC_{50} of CDC determined for p12-LO and OCX-1 in intact platelets*

Surprisingly, supplementation of AA led to a complete loss of CDC inhibitory potential for p12-LO. This may indicate that CDC blocks endogenous AA supply. On the other hand, COX-1 activity was still potently impaired in the presence of exogenously added AA. Since CDC was also able to inhibit ROS formation in intact platelets in thrombin- and collagen-stimulated platelets, an additional influence on platelet functions in certain scenarios seems plausible.

CDC may act via suppression of $cPLA_2$ to impair AA supply. p12-LO activity is most likely regulated by substrate supply, which is provided by exogenous addition of AA or by strong stimulus (e.g., A23187) explaining the loss of CDC inhibitory potential in AA-supplemented cells and the higher IC_{50} for A23187-stimulated cells. COX-1 activity however is also closely regulated by intracellular redox state and $[Ca^{2+}]_i$ levels and might

thus be stronger influenced by CDC than p12-LO. p12-LO might also receive AA from a different PLA_2 isoform than COX-1 (see 4.2) and be less affected by $cPLA_2$ inhibition.

In thrombin- and collagen-stimulated cells a major inhibitory mechanism for CDC is probably mediated by deprivation of substrate AA for p12-LO and COX-1. CDC potently blocks $[Ca^{2+}]_i$ mobilisation in thrombin- and collagen-stimulated cells. Although $cPLA_2$, responsible for AA release in stimulated platelets, is not directly inhibited unless at very high concentrations (30 µM), inhibition of $[Ca^{2+}]_i$ mobilisation by CDC keeps $[Ca^{2+}]_i$ levels low and prevents effective $cPLA_2$ activation. Both p12-LO and COX-1 are influenced by CDC in this way mainly via substrate withdrawal.

In case of AA supplementation, p12-LO activity is only scarcely impaired whereas COX-1 activity is strongly inhibited. CDC was able to potently prevent ROS formation in platelets changing the intracellular redox state of the cells. This second influence alters COX-1 metabolic activity despite ample exogenous substrate supply but not p12-LO activity. An impact on intracellular ROS formation was previously described for CDC although a connection was only made to 12-LO inhibition and not to a direct inhibitory effect of CDC on ROS formation itself [193]. Again, a different regulatory pattern for both enzymes emerges. p12-LO seems to be less or even not dependent on intracellular redox tone as compared to COX-1.

A direct inhibition of COX-1, COX-2 and $cPLA_2$ by CDC was not found in cell-free assays. On the other hand, COX-1 was still potently inhibited in platelet homogenates suggesting an important function for lipid matrices (membrane fractions present in platelet homogenates) for CDC inhibition on the respective enzymes. Inhibition of p12-LO in homogenates implies direct influence of p12-LO activity by CDC possibly via redox activity. Nonetheless, also for p12-LO inhibition multiple factors may contribute to the inhibitory effect seems likely.

Inhibitory properties of CDC culminate in its ability to influence general platelet activation displayed by its impairment of collagen-stimulated platelet aggregation. A concentration of 30 µM CDC inhibited platelet aggregation even more potently than the control inhibitor indomethacin. Thrombin-, U46619- and AA-stimulated aggregation however, was not impaired even when applying high concentrations (30 µM) of CDC. In thrombin-stimulated platelets CDC inhibits COX-1 activity and therefore an effect of CDC on aggregation in thrombin-stimulated cells should be observed. Apparently, CDC interacts with kinase signalling induced by collagen but not with signalling events induced by the other platelet agonists. ROS formation, $[Ca^{2+}]_i$ mobilisation and p12-LO activation in collagen-stimulated platelets are most potently affected supporting a proposed interference with kinase signalling pathways induced by collagen in platelets. CDC also inhibits TXB_2 formation in intact washed platelets and whole blood reflecting the lower COX-1 activity.

Further studies employing CDC as a pharmacological tool to distinguish the involvement of 12-LO and/or COX enzymes in intracellular signalling events have to take into account additional cellular effects of CDC (e.g., $[Ca^{2+}]_i$ signalling, redox state, etc.). Reasonable development of CDC as a possible anti-platelet drug should also be considered as CDC interferes with certain properties typical for platelet inhibitors.

4.7 FLAP inhibitor MK-886 and its effect in intact platelets

MK-886 is a very potent FLAP inhibitor (2.5 nM) impairing 5-LO activity and leukotriene biosynthesis [157]. It is often used as a pharmacological tool to investigate the role of the 5-LO pathway in many inflammatory diseases and also applied in studies related to cancer, as MK-886 is able to induce apoptosis of cancer cells in concentrations up to 50 µM [163].

In platelets however, MK-886 has a completely different effect that has not been described for this cell type, yet. MK-886 strongly increased the formation of AA metabolites in platelets. It also potently induced $[Ca^{2+}]_i$ mobilisation in a concentration range of 10 to 50 µM. A similar effect on

$[Ca^{2+}]_i$ mobilisation has been already described for PC-3 and other cells [168, 169].

The $[Ca^{2+}]_i$ mobilisation in platelets possibly accounts for the strong increase in free AA triggering a high product formation of COX-1 and especially p12-LO. The strongest effect is produced at a concentration of 10 µM MK-886. The effect of MK-886 is not observed in cells exogenously supplemented with 10 µM AA or in cell homogenates. Therefore, an impact of MK-886 on AA release and the need for an intact cellular environment is obvious. Moreover, 10 µM MK-886 induced $[Ca^{2+}]_i$ mobilisation in platelets. Hence, an activation of $cPLA_2$ by increased $[Ca^{2+}]_i$ levels seemed the most evident explanation for this phenomenon. On the other hand, the achieved increase in $[Ca^{2+}]_i$ at 10 µM MK-886 seemed too weak to explain this strong increase in p12-LO and COX-1 product formation simply by $cPLA_2$ activation. At 30 µM MK-886, $[Ca^{2+}]_i$ influx induced is comparable with $[Ca^{2+}]_i$ mobilisation induced by platelet agonists like thrombin. Therefore, whether MK-886 causes p12-LO activity by elevation of $[Ca^{2+}]_i$ is questionable.

FLAP is able to bind and transfer AA to 5-LO [156], and MK-886 as a FLAP inhibitor interferes with this function. Therefore, a proposed interference of MK-886 with reincorporation of AA into the platelet membranes offers a more suitable explanation for the MK-886 effect in intact platelets. An accumulation of AA instead of an active increased release would explain the enhanced p12-LO activity. 30 µM MK-886, although inducing a stronger $[Ca^{2+}]_i$ mobilisation, does not increase p12-LO and COX-1 activities but rather reduces product amounts in comparison to values at 10 µM MK-886. MK-886 potently inhibits COX-1 activity [157] and possibly also p12-LO activity or induces additional effects at those high concentrations.

MK-886 also affects $[Ca^{2+}]_i$ levels in PMNL expressing 5-LO, an enzyme of the AA cascade strongly dependent on $[Ca^{2+}]_i$ as cofactor. However, effective concentrations for $[Ca^{2+}]_i$ elevation are found at a concentration of about 50 µM MK-886, concentrations that are not applied in studies

concentrating on 5-LO activity and leukotriene signalling. Licofelone, another FLAP inhibitor [185], caused $[Ca^{2+}]_i$ mobilisation in PMNL at concentrations between 30 and 50 µM (data not shown). Structural similarities between MK-886 and licofelone probably are the connecting element for effects observed for both substances.

$[Ca^{2+}]_i$ signalling and $[Ca^{2+}]_i$ levels are very strongly affecting intracellular processes in almost every cell type, whether kinase phosphorylation or metabolic regulation is concerned. A pharmacological tool strongly influencing $[Ca^{2+}]_i$ signalling when applied in common concentrations should not be used without consideration of possible side effects.

5 Conclusion

Platelet-type 12-lipoxgenase was the first lipoxygenase to be identified in mammalian tissues [44]. In spite of ongoing research to expand the knowledge on regulation of p12-LO activity, no signalling pathways controlling p12-LO activity have been identified so far. p12-LO is involved in the development of certain cancers [105] and takes part in hypertension and platelet aggregation [103, 190]. A detailed pharmacological profile enclosing regulatory factors and possible inhibitors could pose new starting-points for medical treatments of disorders where p12-LO plays a potential role. In particular, cardiovascular diseases seem to be influenced by p12-LO activity. Research of sex influences on p12-LO activity was enclosed in this study since a clear sex bias in the incidence of cardiovascular events has been a long known fact [128].

Basic research regarding possible gender differences in the regulation of p12-LO was issued in this study. p12-LO activity is not significantly different between isolated male and female platelets. Progesterone, however, impairs p12-LO activity by a pathway possibly involving PKA as effector kinase. A trend for a lower p12-LO basal activity in females could be observed. This very small difference could support a lower susceptibility of women for cardiovascular disease before menopause mediated by a slightly higher progesterone level in women as compared to men. A possible role for progesterone regulation of p12-LO in pregnancy and menstruation could be connected to angiogenesis, endometrial growth and shedding, although further investigations will have to be performed.
A mechanism of hormonal regulation in platelets by rapid, non-genomic actions of progesterone led to a proposition of a new progesterone receptor located in platelet membranes.

p12-LO could be defined as a permanently active enzyme dependent on inhibitory regulation rather than on activating agonists for a regulation of its activity. Possible physiological functions for a constitutive p12-LO activity *in vivo* seem likely as p12-LO activity is not completely suppressed by progesterone as a proposed *in vivo* regulator.

CDC and MK-886, commonly and widely used as tools to connect 12-LO or the 5-LO pathway to respective functionality, were identified to be suboptimal pharmacological tools if applied without further thought. Both inhibitors show broad and unspecific pharmacological effects including influence on $[Ca^{2+}]_i$ mobilisation and ROS formation. Both these events influenced by CDC and MK-886 outside their inhibitory range have a strong impact on several other intracelular functions. For example, changes in $[Ca^{2+}]_i$ levels are often a precursor reaction to major signalling events in the cell. Impact of pharmacological tools on $[Ca^{2+}]_i$ and ROS formation imply an influence of these tools on many more functions than their indented targets and complicate an interpretation of observed events.

In conclusion, p12-LO poses an interesting subject for investigation of mechanisms underlying sex related incidences in certain diseases. Especially in anucleate platelets hormone regulation of intracellular events seemed unlikely. Interestingly, those cells and their enzymes are targets for rapid non-genomic effects giving interesting new insights in intracellular regulation events in platelets. Finally, p12-LO should be considered as an interesting target for new agents impeding p12-LO activity in cancer treatment and in therapy of hypertension and cardiovascular diseases taking into account the sex hormonal status of the respective subject.

6 Summary

The first mammalian lipoxygenase to be discovered in 1974 was platelet-type 12-lipoxygenase (p12-LO) which was named for the site of its discovery. It belongs to a large family of non-heme dioxygenases including 5-lipoxygenase responsible for leukotriene biosynthesis. Although p12-LO was the first lipoxygenase in mammals to be discovered and research has been going on since the discovery, only very little is known about regulation of activity. Involvement in cancer, angiogenesis, hypertension and platelet activation could be attributed to p12-LO activity and its metabolite 12-HETE and suggests the necessity of intensified research. For this study a focus was put on probable regulation in a sex dependent manner, as androgens had recently been found to be involved in regulation of 5-LO and leukotriene biosynthesis. Additionally, in particular for cardiovascular diseases a gender bias with higher incidence in male has been a long established fact, so a possible sex dependent regulation of p12-LO was proposed.

To explore p12-LO regulation in general and to elucidate possible sex dependent regulation in particular, a characterisation of p12-LO regulation in platelets was attempted in this work with two different approaches.

One, gender differences in regulation of p12-LO and possible influences of sex hormones were investigated. Two, common pharmacological tools, CDC and MK-886, were used to elucidate possible properties of p12-LO in intact cells.

Platelet 12-lipoxygenase is not differently active between the two sexes whether in non-stimulated and stimulated platelets. No differences were manifest in release of AA, subcellular localisation, translocation pattern or protein amount of p12-LO compared in platelets from male and female donors. However, a slight tendency towards lower product formation in female platelets was observed.

A strong spontaneous p12-LO activity was measured in non-stimulated platelets and metabolic activity continued over log time periods. p12-LO activity could strongly be enhanced by platelet stimulation with thrombin, collagen, A23187 and arachidonic acid. However, a direct induction of p12-

LO activity by these agonists seems improbable. Platelet stimulation rather strongly increases the amount of available substrate for p12-LO as $cPLA_2$ is activated and AA released than activates p12-LO itself.

p12-LO activity was potently and selectively suppressed to a basal level by addition of 1 to 3 µM progesterone in a fast and non-genomic manner. Application of the other sex hormones estradiol and DHT failed to show an effect on p12-LO activity. Only p12-LO was affected by progesterone, other enzymes metabolizing AA as $cPLA_2$ and COX-1 were not affected in any circumstances. $[Ca^{2+}]_i$ levels, platelet aggregation, subcellular localisation and translocation pattern of p12-LO were not influenced by progesterone. A direct inhibition of p12-LO by progesterone could furthermore be excluded as no progesterone effect could be observed in homogenate incubations. p12-LO activity is not complete blocked by progesterone but only suppressed to a basal level supporting evidence for p12-LO role as a "house-keeping" enzyme in physiological contexts.

Surprisingly, the hormone progesterone normally acting on genomic levels affects its target enzyme p12-LO in anucleate cells, in platelets. Progesterone impairs p12-LO activity in a rapid, reversible manner as opposed to classical genomic actions. This progesterone effect is possibly mediated by a novel progesterone receptor (mPR) localized in the platelet membranes. Regulation pathways could be identified to involve activation of PKA and ascertain a negative, deactivating regulation of p12-LO activity in stimulated and in non-stimulated cells. PKA most likely converts its influence on p12-LO possibly by phosphorylation of the enzyme as p12-LO contains a phosphorylation motif for PKA.

In vivo progesterone levels reached during pregnancy overlap with effective progesterone concentrations for an inhibition of p12-LO *in vitro* (0.3 to 1 µM). Attempts to transfer *in vitro* findings to *in vivo* conditions showed that progesterone could slightly suppress p12-LO activity in male PRP. Moreover, p12-LO activity in PRP prepared from pregnant females was lower than in controls from male and female. Both findings support an *in*

vivo relevance for the progesterone regulation of p12-LO activity although progesterone probably contributes only marginally to regulation of p12-LO, so more complex mechanisms adding to this effect should be expected.

CDC revealed many more complex properties than just a 12-LO inhibitor was suspected to display. CDC not only inhibited p12-LO activity, but affected platelet functions on levels of ROS formation, $[Ca^{2+}]_i$ mobilisation and aggregation and also potently inhibited COX-1 activity in platelets. MK-886 as FLAP inhibitor should not affect p12-LO activity in intact platelets at all, as platelets do not express FLAP or a likewise enzyme. However, 10 µM MK-886 potently increases p12-LO activity approximately 40-fold probably due to an inhibition of AA reincorporation into the cellular membrane increasing substrate amounts available for p12-LO metabolism. An increase of available AA leads to an increase of p12-LO activity sustaining p12-LO properties as enzyme mostly independent from stimulatory agents but dependent on negative, inhibitory regulation.

To conclude: p12-LO activity is not significantly different in male and female platelets, although a slight tendency to lower activity in female was proposed. Regulation of p12-LO activity by progesterone gave a surprising yet promising novel effect of sex hormones conveyed in anucelate cells. This contributes to (1) a better insight in p12-LO activation and regulation and (2) understanding rapid non-genomic actions of sex hormones on intracellular events. Fundamental properties of p12-LO could be clearly defined identifying p12-LO as permanently active enzyme with putative "house-keeping" functions *in vivo*.
For treatment of various diseases connected to p12-LO activity a better understanding of p12-LO is vital and understanding of its basic functions and regulation needs to be enforced.
CDC and MK-886 are useful pharmacological tools with a far wider range of possibilities and effects than it was known before. They should only be applied considering those new findings carefully for their probable impact on events investigated.

7 Zusammenfassung

Die erste in Säugetieren gefundene 12-Lipoxygenase wurde 1974 entdeckt und nach ihrem Entdeckungsort als Thrombozyten- oder platelet-type 12-Lipoxygenase (p12-LO) bezeichnet. Sie gehört zu einer großen Familie von non-heme Dioxygenasen, die unter anderem die 5-LO einschließt. Diese ist an der Leukotrienbiosynthese maßgeblich beteiligt. Obwohl die p12-LO die erste entdeckte Säugetier-Lipoxygenase war und die Forschung seit der Entdeckung nicht nachgelassen hat, ist bisher nur wenig über die Regulation ihrer Aktivität bekannt. Die Rolle der p12-LO und ihres Hauptproduktes 12-HETE in der Krebsentstehung, bei der Angiogeneseregulation und der Entstehung von Bluthochdruck sowie in der Regulation der Thrombozytenaktivierung macht die Notwendigkeit für intensive Forschung deutlich. Für die vorliegende Arbeit wurde ein besonderer Augenmerk auf die mögliche geschlechtsabhängige Regulation der p12-LO gerichtet, da kürzlich für das verwandte Enzym 5-LO eine Aktivitätsregulation und damit eine Regulation der Leukotrienbiosynthese durch Androgene beschrieben wurde. Gerade für kardiovaskuläre Erkrankungen, bei denen der Einfluss der p12-LO postuliert wird, ist ein geschlechtsabhängiges Ungleichgewicht in der Inzidenz bekannt. Deshalb wurde auch die p12-LO auf eine mögliche geschlechtsabhängige Regulation untersucht.

Um die Regulation der p12-LO im Allgemeinen und mögliche Einflüsse des Geschlechts auf die p12-LO im Speziellen zu untersuchen, wurden zwei Herangehensweisen ausgewählt. Zum einen wurden geschlechtsspezifische Unterschiede der p12-LO Aktivitätsregulierung und mögliche direkte Einflüsse von Geschlechtshormonen auf die p12-LO untersucht. Zum anderen kamen zwei typische pharmakologische Werkzeuge – CDC und MK-886 – zum Einsatz, um mögliche Regulationsmechanismen und Eigenschaften der p12-LO in intakten Thrombozyten zu beleuchten.

Die p12-LO zeigte keine Aktivitätsunterschiede zwischen Thrombozyten von männlichen und weiblichen Spendern, ob in unstimulierten oder stimulierten Zellen. Weiterhin konnten beim Vergleich von männlichen und weiblichen Zellen weder Unterschiede im Bezug auf AA-Freisetzung noch

bei subzellulärer Lokalisation, Translokationsmustern oder Proteinmenge der p12-LO festgestellt werden. Allerdings ließ sich eine leichte Tendenz zu geringerer Produktbildung in weiblichen Zellen feststellen.
In unstimulierten Thrombozyten wurde eine ausgeprägte spontane Aktivität der p12-LO gemessen, die kontinuierlich über längere Zeiträume anhielt. Diese basale Aktivität wurde durch Stimulation der Zellen mit Thrombin, Collagen, A23187 Ionophor oder AA deutlich verstärkt. Allerdings scheint eine direkte Aktivierung der p12-LO unwahrscheinlich, vielmehr erhöht die Thrombozytenaktivierung das Angebot an freier AA und damit die Aktivität der p12-LO deutlich, da durch die Stimulation die $cPLA_2$ zur AA-Freisetzung angeregt wird.

Die Aktivität der p12-LO wird durch Zugabe von 1 bis 3 µM Progesteron wirksam und selektiv auf einem basalen niedrigen Niveau gehalten. Der beobachtete Effekt tritt – anders als genregulatorische Effekte – schnell ein und ist reversibel. Behandlung der Zellen mit anderen Geschlechtshormonen wie Estradiol und DHT ergab keinen Effekt auf die p12-LO. Außerdem wird nur die p12-LO von Progesteron in seiner Aktivität beeinflusst, andere Enzyme des AA-Stoffwechsels wie $cPLA_2$ und COX-1 werden in ihrer Aktivität nicht eingeschränkt. Auch intrazelluläre Calciumkonzentration, Thrombozytenaggregation, subzellulare Lokalisation und Translokation der p12-LO wurden von Progesteron nicht verändert. Eine direkte Interaktion der p12-LO mit Progesteron konnte ausgeschlossen werden, da durch Progesteronzusatz zu Thrombozytenhomogenaten keine Inhibition der p12-LO beobachtet wurde.
Die p12-LO Aktivität wird durch Progesteron nicht vollkommen unterdrückt, sondern nur auf ein basales Niveau reduziert. Diese Tatsache unterstützt die Vermutung, dass p12-LO wahrscheinlich als „house-keeping enzyme“ im physiologischen System eine Rolle spielt.

Überraschenderweise kann das Hormon Progesteron, das gemeinhin seine Funktion auf genomischer Ebene erfüllt, sein Zielenzym auch in kernlosen Zellen, den Thrombozyten, beeinflussen – und zwar in schneller, reversibler Weise, ganz anders als für klassische genomische Effekte zu erwarten wäre.

Diese Wirkung des Progesterons wird vermutlich über einen neuartigen Progesteronrezeptor (mPR) vermittelt, der in den Membranen der Thrombozyten lokalisiert sein könnte. Als Endpunkt der angestoßenen Signalwege konnte die PKA als mögliche Effektorkinase identifiziert werden, die eine negative, deaktivierende Regulation der p12-LO in stimulierten und unstimulierten Thrombozyten sicherstellt. Höchstwahrscheinlich übt PKA ihren Effekt auf p12-LO durch Phosphorylierung aus, die Sequenz der p12-LO enthält ein entsprechendes Erkennungsmotiv für die PKA.

Progesteron-Plasmaspiegel *in vivo* erreichen während der Schwangerschaft Werte, die sich *in vitro* als effektive Konzentrationen zur p12-LO-Inhibition erwiesen hatten. Um mögliche *in vivo* Wirkungen des Progesterons abschätzen zu können, wurde Progesteron zu PRP von männlichen Spendern zugesetzt und die Aktivität der p12-LO vermessen. Eine leichte Hemmung der Aktivität wurde beobachtet. In PRP von schwangeren Spenderinnen konnte ebenfalls eine geringere Aktivität der p12-LO festgestellt werden. Beide Ergebnisse zeigen, dass auch *in vivo* eine Regulation der p12-Lo durch Progesteron in Betracht gezogen werden sollte. Allerdings ist zu erwarten, dass Progesteron nur einen kleinen Beitrag im Regulationsgeschehen leistet und darüber hinaus komplexere Mechanismen an der Regulation der p12-LO beteiligt sind.

CDC offenbarte sich als deutlich vielseitiger als von einem p12-LO-Inhibitor zu erwarten gewesen wäre. CDC hemmte nicht nur die p12-LO-Aktivität, sondern beeinflusste die Thrombozytenfunktion im Bezug auf ROS-Produktion, Calciummobilisierung und Aggregation. Außerdem wurde auch COX-1 in Thrombozyten potent in seiner Aktivität gehemmt. Für MK-886 als FLAP-Inhibitor wurde keinerlei Effekt auf die p12-LO Aktivität erwartet, da Thrombozyten höchstwahrscheinlich kein FLAP oder ein verwandtes Protein exprimieren. Nichtsdestotrotz steigerte MK-886 in einer Konzentration von 10 µM die p12-LO Aktivität etwa 40-fach. Aller Wahrscheinlichkeit nach inhibiert MK-886 die Reinkorporation freigesetzter AA in die Zellmembran und führt so zu einer Akkumulation an AA in den

Zellen. Allein dieses erhöhte Angebot an Substrat (AA) erhöht die p12-LO Aktivität, was die Unabhängigkeit der p12-LO von stimulierenden Agonisten stärker herausstellt und eine eher negative Regulation der Aktivität wahrscheinlich macht.

Zusammenfassend lässt sich sagen, dass keine signifikanten Unterschiede in der p12-LO-Aktivität zwischen männlichen und weiblichen Thrombozyten bestehen, obwohl eine Tendenz zu geringere Aktivität in weiblichen Zellen beobachtet wurde. Die Regulation intrazellulärer Vorgänge durch Geschlechtshormone in kernlosen Zellen war ein überraschender und zugleich viel versprechender neuer Effekt, der dazu beiträgt, (1) einen besseren Einblick in die Regulation der p12-LO zu erhalten und (2) schnelle nicht-genomische Effekte von Geschlechtshormonen und ihren Eingriff in intrazelluläre Vorgänge besser zu verstehen. Grundsätzliche Eigenschaften der p12-LO konnten klar definiert werden, wie zum Beispiel die mögliche Funktion als „house-keeping enzyme“ *in vivo* aufgrund der kontinuierlichen Aktivität.
Um Therapiemöglichkeiten für bestimmte Krankheiten, an deren Entstehung die p12-LO beteiligt ist, optimieren zu können, ist ein besseres Verständnis der p12-LO unerlässlich und die Erforschung ihrer Grundfunktionen und Aktivitätsregulation sollte vorangetrieben werden.
CDC und MK-886 sind nützliche pharmakologische Werkzeuge mit weit größeren Möglichkeiten und Wirkungen als bisher gedacht. Sie sollten nur nach sorgfältiger Abwägung eventueller weitergehender Wirkung auf den zu untersuchenden Effekt eingesetzt werden.

8 References

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9 Akademische Lehrer

Prof. G. Brehm

Prof. T. Clark

Prof. hc. W. Dressendörfer

Prof. P. Gmeiner

Prof. B. Hinz

Prof. W. Kreis

Prof. W. Kretschmer

Prof. G. Lee

Prof. R. Troschütz

Prof. G. Tiegs

Prof. R. van Eldik

Prof. O. Werz

10 Curriculum Vitae

- Name: Ulrike Elisabeth Bühring
- Geburtstag: 19.07.1981 in München
- Staatsangehörigkeit: deutsch
- Familienstand: ledig

Ausbildung

- ***05/2000*** allgemeine Hochschulreife, Gymnasium Vaterstetten (jetzt Humboldt-Gymnasium), Vaterstetten
- ***10/2000*** Pharmaziestudium an der FAU Erlangen – Nürnberg, 1. und 2. Staatsexamen
- ***11/2004 – 04/2005*** 1. Halbjahr PJ, Apotheke des städtischen Klinikums Ludwigshafen
- ***05/2005 – 10/2006*** 2. Halbjahr PJ, Löns-Apotheke, Celle
- ***12/2005*** 3. Staatsexamen Pharmazie, Approbation zur Apothekerin
- ***03/2006 – 12/2009*** Doktorarbeit im Fach Pharmazeutische Chemie am Pharmazeutischen Institut der Eberhard - Karls - Universität Tübingen bei Prof. Dr. O. Werz

  Thema „Regulation der Aktivität der platelet-type 12-Lipoxygenase in Thrombozyten unter besonderer Berücksichtigung geschlechtsspezifischer Einflüsse“

Beruflicher Werdegang

- ***07 – 09/2000*** Praktikum bei der Firma Metabion GmbH in München- Martinsried
- ***seit 03/2006*** Betreuung des Praktikums „Pharmazeutische Chemie II, Arzneibuchanalytik“, Studiengang Pharmazie, Universität Tübingen, seit ***04/2009*** stellvertretende Praktikumsleiterin
- ***06/2006 – 09/2009*** Apothekerin in der Gartenstadtapotheke, Reutlingen
- ***09/2007*** Chefvertretung in der Römerapotheke Rottenburg
- ***03/2006 – 12/2009*** Weiterbildung zum Fachapotheker für pharmazeutische Analytik