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An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis

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ABSTRACT

Background: Subcellular distribution of 5-lipoxygenase (5-LO) to the perinuclear region and interaction with the 5-LO-activating protein (FLAP) are assumed as key steps in leukotriene biosynthesis and are prone to FLAP antagonists.
Methods: FLAP and/or 5-LO were stably expressed in HEK293 cells, 5-LO products were analyzed by HPLC, and 5-LO and FLAP subcellular localization was visualized by immunofluorescence microscopy.
Results: 5-LO and FLAP were stably expressed in HEK293 cells, and upon Ca^{2+}-ionophore A23187 stimulation exogenous AA was efficiently transformed into the 5-LO products 5-hydro(pero)xyeicosatetraenoic acid (5-H(p)ETE) and the trans-isomers of LTB_4. A23187 stimulation caused 5-LO accumulation at the nuclear membrane only when FLAP was co-expressed. Unexpectedly, A23187 stimulation of HEK cells expressing 5-LO and FLAP without exogenous AA failed in 5-LO product synthesis. HEK cells liberated AA in response to A23187, and transfected HEK cells expressing 12-LO generated 12-HETE after A23187 challenge from endogenous AA. FLAP co-expression increased 5-LO product formation in A23187-stimulated cells at low AA concentrations. Only in cells expressing FLAP and 5-LO, the FLAP antagonist MK886 blocked FLAP-mediated increase in 5-LO product formation, and prevented 5-LO nuclear membrane translocation and co-localization with FLAP.
Conclusion: The cellular biosynthesis of 5-LO products from endogenously derived substrate requires not only functional 5-LO/FLAP co-localization but also additional prerequisites which are dispensable when exogenous AA is supplied; identification of these determinants is challenging.
General significance: We present a cell model to study the role of FLAP as 5-LO interacting protein in LT biosynthesis in intact cells and for characterization of putative FLAP antagonists.

1. Introduction

Leukotrienes (LT) are potent lipid mediators that are released during many human pathologic processes such as acute and chronic inflammation, allergy, cancer, and cardiovascular diseases [1]. They derive from arachidonic acid (AA) released by cytosolic phospholipase A_2 ($cPLA_2$) from phospholipids within the nuclear membrane. 5-Lipoxygenase (5-LO), a non-heme dioxygenase, metabolizes AA by stereospecific hydrogen abstraction at C-7, followed by radical migration and antarafacial oxygen insertion at C-5, generating 5(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE). In a second step of 5-LO catalysis, the enzyme abstracts hydrogen at C-10 of 5-HpETE with subsequent radical migration to C-6 and double bond rearrangement to a conjugated triene. The radical relocates, dehydrates, and forms the instable epoxide leukotriene A_4 (LTA_4) [2,3]. Subsequently, LTA_4 can be further converted by other lipoxygenases to lipoxins [4], by LTA_4 hydrolase (LTA_4H) to leukotriene B_4 (LTB_4) [5], or conjugated with reduced glutathione by leukotriene C_4 synthase (LTC_4S) to LTC_4 [6]. Additionally, LTA_4 is non-enzymatically degraded to trans-isomers of LTB_4.

In contrast to other lipoxygenases, 5-LO is activated and regulated after cell stimulation by diverse factors including Ca^{2+}, phosphorylations, and stabilization by binding to coactosin-like protein (CLP) [7,8]. Activated 5-LO in the cytosol or soluble compartment of the nucleus migrates to the perinuclear region, where AA is released by $cPLA_2$ and believed to be delivered to 5-LO by the membrane bound 5-lipoxygenase-activating protein (FLAP). As far as is known, 5-LO is the only lipoxygenase that requires FLAP, or any transmembrane-bound helper protein, for full activity in the cell. It has been shown that FLAP

Abbreviations: 5-LO, 5-lipoxygenase; 5-H(p)ETE, 5-hydro(pero)xy-6-trans 8,11,14-cis-eicosatetraenoic acid; 12-LO, 12-lipoxygenase; AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; HEK cells, human embryonic kidney cells; LT, leukotriene; LTB_4, leukotriene B_4

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binds AA, and inhibition by the FLAP antagonist MK886 blocks LT formation and prevents 5-LO translocation in intact activated human leukocytes [9–11]. FLAP belongs to the superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) [12]. However, it differs from other members in lacking any enzymatic activity and the ability to conjugate glutathione. Despite intense investigations during the last two decades and its unquestioned importance for 5-LO activity [10], whether and how FLAP interacts with 5-LO and how FLAP facilitates the transport of AA from $cPLA_2$ to 5-LO are incompletely understood.

Since FLAP does not possess enzymatic activity and the 5-LO/FLAP functional interaction is limited to the cellular context, it is challenging to study the function of FLAP outside a cellular environment independent of 5-LO. So far, functional interference with FLAP by antagonists can essentially be assessed only by means of decreased 5-LO product formation in intact cells but not in cell-free preparations where FLAP is dispensable for 5-LO activity. Nevertheless, the affinity of putative FLAP antagonists towards FLAP can be analyzed by a radio ligand binding assays using human leukocyte membrane preparations as FLAP source [13]. However, this method requires competitive binding of the inhibitor and excludes allosteric inhibition of FLAP. Moreover, studies on recombinant purified FLAP have limited applicability, as the protein needs a bilayer surrounding to express full functionality.

Previous studies have utilized mammalian cell lines such as osteosarcoma cells for expression of 5-LO and FLAP in order to study the interaction and functional coupling for LT biosynthesis [9,14]. However, the cell lines used were transiently transfected with 5-LO or artificial 5-LO-GFP constructs were investigated [15] that eventually limited the reproducibility and biological relevance, respectively. In this study, we established a HEK cell model stably expressing 5-LO with or without FLAP that may allow for detailed analysis of the functional role of FLAP for 5-LO cell biology. Such a cellular model may constitute a robust experimental system for studying directed protein mutations in order to reveal the critical residues involved in the dynamic 5-LO/FLAP interaction. Finally, application of this HEK cell model may provide new possibilities to design and characterize novel 5-LO/FLAP inhibitors.

2. Experimental procedure

2.1. Materials

Phorbol-12-myristate-13-acetat (PMA) was from Applichem (Darmstadt, Germany) and Rotiszint® eco plus from Roth (Karlsruhe, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, trypsin/EDTA, and geneticin were from PAA Laboratories (Coelbe, Germany). Lipofectamine LTX Reagent Plus, 10% non-immune goat serum, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, diamidino-2-phenylindol (DAPI), hygromycin B, pcDNA3.1/Hygro (−) vector kit, and chemically competent *Escherichia coli* cells (OneShot Top10) were from Invitrogen (Darmstadt, Germany). Phusion High fidelity polymerase, restriction enzymes, and GenJet-plasmid midiprep kit were from Fermentas (Darmstadt, Germany). The mouse anti-5-LO monoclonal antibody, the FLAP-cDNA-containing plasmid pSG5_FLAP, and the pcDNA3.1/neom (+)_5-LO-vector were generous gifts by Dr. Dieter Steinhilber (Goethe University Frankfurt, Germany). The rabbit anti-FLAP polyclonal antibody was from Abcam (Cambridge, UK) and the mouse anti-12-LO monoclonal antibody was from Santa Cruz. Tritium labeled [5,6,8,9,11,12,14,15-^{3}H]AA was from Biotrend Chemicals GmbH (Cologne, Germany). The FLAP inhibitor MK886 was from Cayman Chemicals (AnnArbor, US), zileuton (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea) was from Sequoia Research Products (Oxford, UK) and the $cPLA_2\alpha$ inhibitor RSC-3388 (N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl) as well as mowiol was from Calbiochem (Bad Soden, Germany). Oligonucleotides were from Tib Molbiol (Berlin, Germany). HPLC solvents were from Merck (Darmstadt, Germany). AA, Ca^{2+}-ionophore A23187, prostaglandin (PG)B_1, dNTPs, and all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

2.2. Cells

HEK293 cells were cultured as monolayers at 37 °C and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK cell lines stably expressing 5-LO and/or FLAP were selected using 400 μg/ml geneticin and/or 200 μg/ml hygromycin B, respectively.

Human neutrophils and monocytes were freshly isolated from leukocyte concentrates obtained from the Institute of Transfusion Medicine, University Hospital Jena, as described [16]. In brief, neutrophils were isolated by dextran sedimentation, centrifuged on lymphocyte separation medium (LSM 1077, PAA, Coelbe, Germany) and subjected to hypertonic lysis of erythrocytes.

Monocytes were separated by adherence to culture flasks from peripheral blood mononuclear cells, as described [17]. Neutrophils and monocytes were resuspended in PBS plus glucose (0.1%) to a final cell density of 5×10^6 and 2×10^6 cells/ml, respectively.

2.3. Cloning of FLAP and platelet-type 12(S)-LO

Standard protocols for molecular biology were applied. The pSG5-FLAP vector was used as a template for amplification of the FLAP coding sequence, using primers extended by EcoRV and HindIII restriction sites at the 5′ and 3′ ends, respectively (forward 5′-gatatcatggatcaagaaactg, reverse 5′-gatgctattgctttatttgta). The cDNA was ligated between the EcoRV and HindIII cloning sites of pcDNA3.1/Hygro (−), generating the pcDNA3.1/Hygro (−)_FLAP expression vector. The cDNA of 12*(S)*-LO was amplified in two partly overlapping fragments of 1217 bp (nucleotides 1–1211) and 1337 bp (nucleotides 661–1989). A KpNI and XhoI restriction site was introduced in front of the ATG (5′ ggtaccATG) and after the stop codon (5′ ctcgagTCA) by PCR technology, respectively. The fragments were sequentially ligated (KpNI-EcoRI and EcoRI-XhoI) into the pcDNA3.1/Neom (+) vector, generating the pcDNA3.1/Neom(+)_12-LO expression vector.

2.4. Stable expression of 5-LO, FLAP and 12(S)-LO in HEK293

Transfection of HEK cells was performed using lipofectamine according to the instructions of the agent (Invitrogen, Darmstadt, Germany). Briefly, HEK cells were grown until ~60% confluency and 2 h prior transfection the medium was replaced by medium without antibiotics ("reduced medium"). Transfection mix composed of "reduced medium", 40 μg of purified pcDNA3.1/neom (+)_5-LO or pcDNA3.1/neom (+)_12-LO, and lipofectamine was added drop wise onto the cells. After 24 h, the medium was replaced by complete medium (with antibiotics) and cultured for additional 24 h. Then, cells expressing 5- or 12-LO were selected by 400 μg/ml geneticin. Stable transfectants were screened by activity tests and expression was verified by immunoblotting. Once a stable 5-LO-expressing cell line was generated, cells were co-transfected with a FLAP-coding construct (pcDNA3.1/Hygro (−) _FLAP) and selected using 200 μg/ml hygromycin B.

2.5. Determination of LO product formation in transfected HEK293 cells

In order to determine 5-LO product formation in intact cells, HEK cells expressing 5-LO +/− FLAP were harvested by trypsinization, centrifuged (1200 rpm; 10 min; 4 °C), and resuspended in PGC buffer (PBS/0.1% glucose/1 mM $CaCl_2$). Cells (1×10^6 /ml) pre-incubated with inhibitors or vehicle (0.1% DMSO) at 37 °C for 15 min and subsequently stimulated with 2.5 μM A23187 plus the indicated concentrations of AA at 37 °C. The reaction was stopped after 10 min by

addition of 1 ml ice cold methanol. The internal standard (200 ng PGB_1) and acidified PBS were added and subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO metabolites were eluted by methanol and analyzed by RP-HPLC using C-18 Radial-PAK column (Waters, Eschborn, Germany), as described before [18]. 5-LO products include the all-*trans*-isomers of LTB_4 and 5-H(p)ETE.

For determination of 5-LO products in cell homogenates, cells were harvested as described above, resuspended in ice cold PBS containing 1 mM EDTA, and sonicated for 3 × 10 sec, at 4 °C. Samples (corresponding to 1×10^6 cells/ml) were pre-incubated with inhibitors or vehicle (0.1% DMSO) at 4 °C for 15 min before addition of 2 mM $CaCl_2$ and indicated concentrations of AA for 10 min at 37 °C. 5-LO products were extracted and analyzed as described for intact cells. To determine 12-LO activity in intact cells, HEK cells stably expressing 12-LO were harvested as described above and stimulated with 2.5 μM A23187 at 37 °C with or without exogenous AA, as indicated. The reaction was stopped at the indicated time points by addition of 1 ml methanol and 12(S)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid (12(*S*)-H(p)ETE) was analyzed by RP-HPLC, as described above.

2.6. Determination of LO product formation in neutrophils and monocytes

Intact neutrophils or monocytes were resuspended in 1 ml PGC buffer and stimulated with 2.5 μM A23187 with or without the indicated concentrations of AA for 10 min at 37 °C. The reaction was stopped with 1 ml methanol on ice and acidified PBS as well as the internal standard (200 ng PGB_1) was added to each sample. LO metabolites were isolated by solid phase extraction and analyzed by RP-HPLC, as described above.

2.7. Analysis of subcellular localization by immunofluorescence microscopy

HEK cells expressing 5-LO +/− FLAP were seeded onto acid-washed (50% sulfuric acid) and poly-L-lysine (0.01%)-coated glass coverslips and cultured for 48 h at 37 °C until ~60% confluency. Cells were pre-incubated with test compounds or vehicle (0.1% DMSO) for 10 min before stimulation with 2.5 μM A23187 for 5 min at 37 °C. The cells were fixed with 4% paraformaldehyde solution, followed by addition of 50 mM ammonium chloride to reduce fixative-induced autofluorescence. Acetone (3 min, 4 °C) was used to permeabilize the cells, before blocking with 10% non-immune goat serum. The cells were incubated for 1 h with mouse monoclonal anti-5-LO antibody (1:100) and rabbit polyclonal anti-FLAP antibody (5 μg/ml). The coverslips were intensively washed with PBS before staining with the fluorophore-labeled secondary antibodies Alexa Fluor 488 goat anti-rabbit (1:500) and Alexa Fluor 555 goat anti-mouse (1:500) for 20 min in the dark. DNA was stained with 0.1 μg/ml DAPI. The coverslips were mounted on glass slides with mowiol containing 2.5% n-propyl gallate. The cells were visualized by a Zeiss Axiovert 200 M microscope, and a Plan Neofluar × 100/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany). An AxioCam MR camera (Carl Zeiss) was used for image acquisition.

2.8. Aracidonic acid release

Cells (2.5×10^6 /ml) were resuspended in DMEM without additives and incubated with 0.5 μCi/ml ^{3}H-labelled AA (specific activity 200 Ci/mmol) for 2 h at 37 °C and 6% CO_2. Cells were washed twice with PBS containing 0.1% glucose and 0.2% fatty acid-free BSA. The cell number was adjusted to 1×10^6 /ml and 1 mM $CaCl_2$ was added to the incubation buffer (PBS/0.1% glucose/1 mM $CaCl_2$/0.2% fatty acid-free BSA). The cells were pre-incubated with 1 μM $cPLA_2\alpha$ inhibitor RSC-3388 or vehicle (0.1% DMSO) at 37 °C. After 15 min, vehicle (0.1% DMSO) or 0.1 μM PMA was added, incubated for additional 10 min at 37 °C, and subsequently stimulated with 5 μM A23187 for 30 min at 37 °C. Samples were placed on ice (10 min) and centrifuged (500 ×g, 10 min, and 4 °C). Aliquots (300 μl) of the supernatants were combined with 2 ml Rotiszint® eco plus and assayed for radioactivity by scintillation counting (Micro Beta Trilux, Perkin Elmer, Waltham, MA).

2.9. SDS-page and Western blot analysis

Cell lysates, corresponding 1×10^6 cells, were separated on 10% and 16% polyacrylamide gels, respectively, and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Freiburg, Germany), as described [19]. The membranes were incubated with primary antibodies (mouse anti-5-LO, 1:1000; rabbit anti-FLAP, 0.1 μg/ml and mouse anti-12-LO, 1:1000) with subsequent detection using IRDye 800CW-labeled anti-rabbit and/or anti-mouse antibodies (1:10,000 each). Immunoreactive bands were visualized by an Odyssey infrared imager (LI-COR Biosciences).

2.10. Statistics

Results are expressed as means ± standard error of the mean (SEM) of *n* observations, where *n* represents the number of experiments performed at different days in duplicates unless stated otherwise. The IC_{50} values were determined by interpolation on semi-logarithmic graphs with GraphPad Prism 4 and validated with GraphPad InStat (Graphpad Software Inc., San Diego, CA). Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. Where appropriate, Student's *t* test was applied. A *P*-value of <0.05 (*) was considered significant.

3. Results

3.1. 5-LO product formation in HEK293 cells stably expressing 5-LO with or without FLAP

HEK cells were transfected with cDNA of either 5-LO (HEK-5-LO), 5-LO and FLAP (HEK-5-LO/FLAP) or 12-LO (HEK-12-LO). Colonies that stably express LOs and FLAP were selected by geneticin and hygromycin B, respectively. Expression of 5-LO, 12-LO and FLAP at the protein level in the respected cells was verified by immunoblotting (Fig. 1A,B). To our surprise, activation of HEK-5-LO/FLAP and HEK-5-LO with A23187 caused no significant formation of the 5-LO products (<2 ng/10^6 cells), while control incubations with A23187-stimulated human neutrophils or monocytes caused substantial 5-LO product synthesis under the same incubation conditions (Fig. 1C), as reported before [17,20]. In contrast, HEK cells transfected with 12-LO produced 12-H(p)ETE as AA-derived 12-LO metabolite upon A23187 stimulation (Fig. 1D). Note that 12-H(p)ETE formation was continuously formed for more than 15 min after a lag phase of around 2 min (Fig. 1E), which was surprising since it has been shown that platelet-type 12-LO metabolizes endogenously released AA extremely fast (within seconds) after A23187 stimulation [21].

Interestingly, upon addition of 3 or 10 μM AA, A23187-activated HEK-5-LO/FLAP and HEK-5-LO cells produced massive and even superior amounts of 5-LO products (5-H(p)ETE and trans-isomers of LTB_4) as compared to neutrophils and monocytes treated the same way (Fig. 1C). Because exogenous AA was necessary for cellular 5-LO product synthesis in A23187-activated HEK-5-LO and HEK-5-LO/FLAP cells, it appeared possible that these cells fail to provide sufficient AA as substrate for 5-LO/FLAP from endogenous sources, i.e., $cPLA_2$-mediated hydrolysis of membrane phospholipids. However, a significant increase in AA release from ^{3}[H] AA-labelled HEK cells (regardless of transfection) was evident after A23187 stimulation, which could be blocked by the $cPLA_2$ inhibitor RSC-3388. Together, our data show that endogenously released AA in HEK cells expressing both 5-LO and FLAP cannot be converted to 5-LO products even though 12-LO transforms endogenous AA. However, exogenous AA is substantially converted by 5-LO in HEK cells with or without of FLAP.

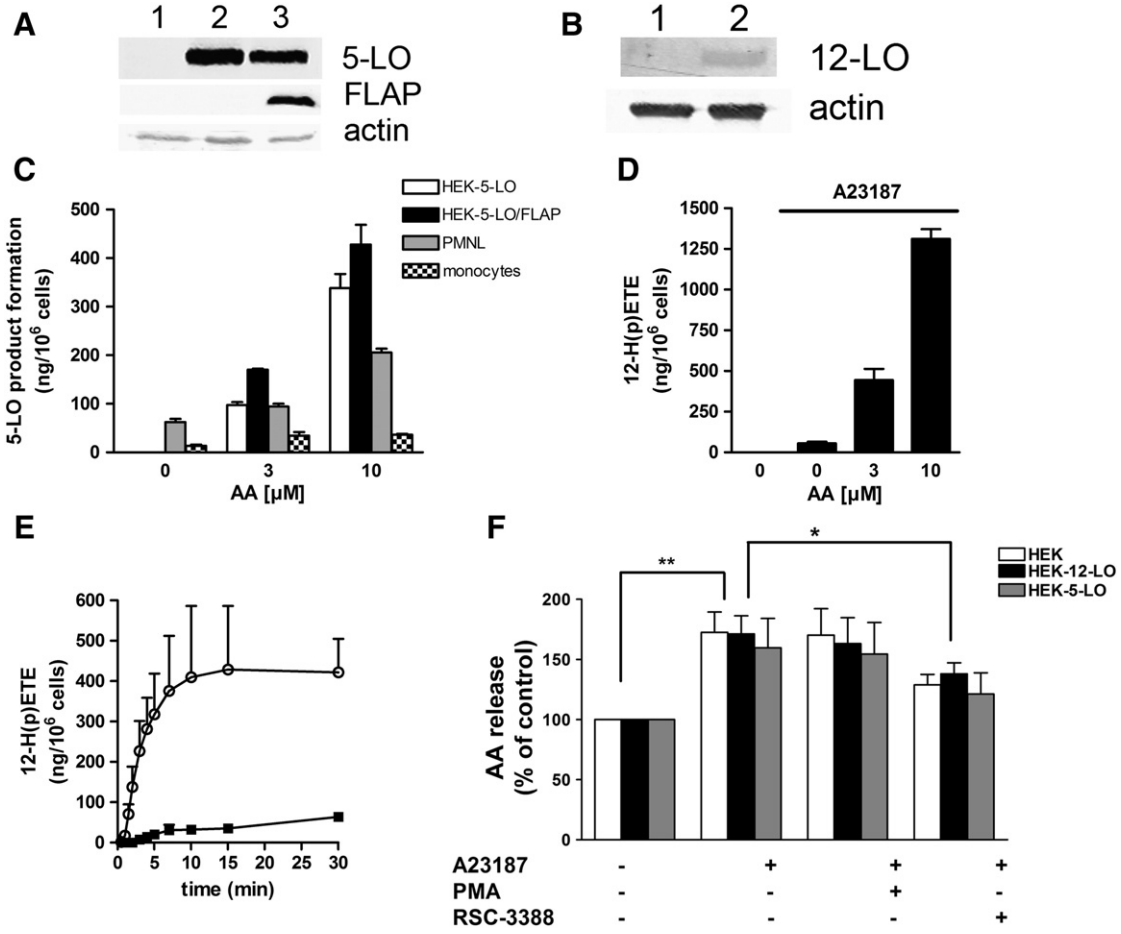

Fig. 1. Expression of 5-LO, FLAP, and 12-LO, and analysis of cellular eicosanoid biosynthesis in HEK293 cells. (A) Cell lysates (1×10^6 cells) of untransfected HEK cells (control) in lane 1, 5-LO-expressing HEK-5-LO in lane 2, and 5-LO/FLAP co-expressing HEK cells in lane 3. LOs and FLAP were resolved on 10% and 16% polyacrylamide gels, respectively. (B) Western blot analysis for 12-LO. Cell lysates of 12-LO-expressing HEK cells (lane 2) versus untransfected cells (lane 1). Images are representative for three independent blots and normalized against actin. (C) 5-LO product formation in intact HEK cells upon A23187 stimulation with or without AA, as indicated. HEK-5-LO, white; HEK-5-LO/FLAP, black; neutrophils, grey; monocytes, pattern. Data are given as means ± SEM, $n = 3$–5. (D) 12-H(p)ETE formation in cells stably expressing 12-LO with and without exogenous AA. (E) Time course of 12-LO product formation in intact HEK cells stably expressing 12-LO upon A23187 stimulation (-■-) and A23187 stimulation plus 3 μM exogenous AA (-○-). Data are representative for three comparable experiments. (F) AA release in untransfected HEK293 cells as well as in 5-LO and 12-LO expressing cells. Data are means ± SEM, $n = 3$–4, duplicates; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs unstimulated control or stimulated vs. inhibitor respectively, ANOVA plus Bonferroni.

Next, the concentration of exogenously added AA was varied. Addition of 3 μM AA together with A23187 revealed a significant higher product formation in HEK-5-LO/FLAP as compared to HEK-5-LO cells (Fig. 2A). However, this difference in 5-LO product formation was abolished at 10 or 30 μM of exogenous AA (Fig. 2A). Note that in homogenates of HEK-5-LO/FLAP and HEK-5-LO cells the formation of 5-LO product was not significantly different, irrespective of the AA concentration (i.e., 3, 10 or 30 μM AA) (Fig. 2B).

Of interest, the 5-LO product profile (5-H(p)ETE and trans-isomers of LTB_4) differed significantly between HEK-5-LO and HEK-5-LO/FLAP cells (Fig. 2C/E). Thus, A23187-stimulated HEK-5-LO/FLAP cells converted exogenous AA (at 3 μM AA) to the final product LTA_4 to a greater extent than HEK-5-LO cells. The ratio of 5-H(p)ETE versus trans-isomers of LTB_4 decreased from 6:1 to 2:1 when FLAP was co-expressed (Fig. 2C/E). However, in homogenates the 5-LO product profile is equal in cells with or without FLAP independent of the AA concentration (Fig. 2D/F), as expected. Together, the data suggest that the presence of FLAP stimulates 5-LO product formation at low substrate concentrations (i.e., at 3 μM AA) and shifts the ratio from 5-H(p)ETE towards LTA_4 metabolites in intact HEK cells, whereas in cell-free assays based on experiments with HEK cell homogenates FLAP is not operative. This indicates that FLAP preferentially promotes the 5-LO-mediated conversion of 5-HpETE to LTA_4 (i.e., the synthase activity of 5-LO).

3.2. MK886 differentially inhibits 5-LO product formation in HEK cells depending on the presence of FLAP

Inhibitor studies using the FLAP antagonist MK886 that suppresses cellular LT biosynthesis with IC_{50} values in the low nanomolar range [22,23], and the direct 5-LO inhibitor zileuton [24] were performed in the described transfected HEK cell lines. When HEK-5-LO/FLAP cells were stimulated with A23187 plus 3 μM AA, MK886 reduced 5-LO product formation by ~35% at 30 nM. Higher concentrations of MK886 up to 3 μM did not further suppress 5-LO product synthesis leading to a plateau of the curve (Fig. 3A). At rather high concentrations of MK886 (i.e., 10 μM) 5-LO product formation was further impaired (by ~80%). As expected, in HEK-5-LO cells MK886 at nanomolar concentrations failed to inhibit 5-LO product formation, and only at high concentrations (3 and 10 μM) a similar reduction was observed as for HEK-5-LO/FLAP cells (Fig. 3A). Note that the elevated 5-LO product formation in HEK-5-LO/FLAP versus HEK-5-LO cells stimulated by A23187 plus 3 μM AA was reduced by 30 nM MK886 down to the level of cells lacking FLAP (Fig. 3C). In contrast, 30 nM MK886 failed to diminish 5-LO product synthesis in HEK-5-LO cells that lack FLAP (Fig. 3C). In homogenates, MK886 was not able to reduce 5-LO product formation regardless of the presence of FLAP (Fig. 3B). In contrast to MK886, the 5-LO inhibitor zileuton reduced 5-LO product formation in HEK cells

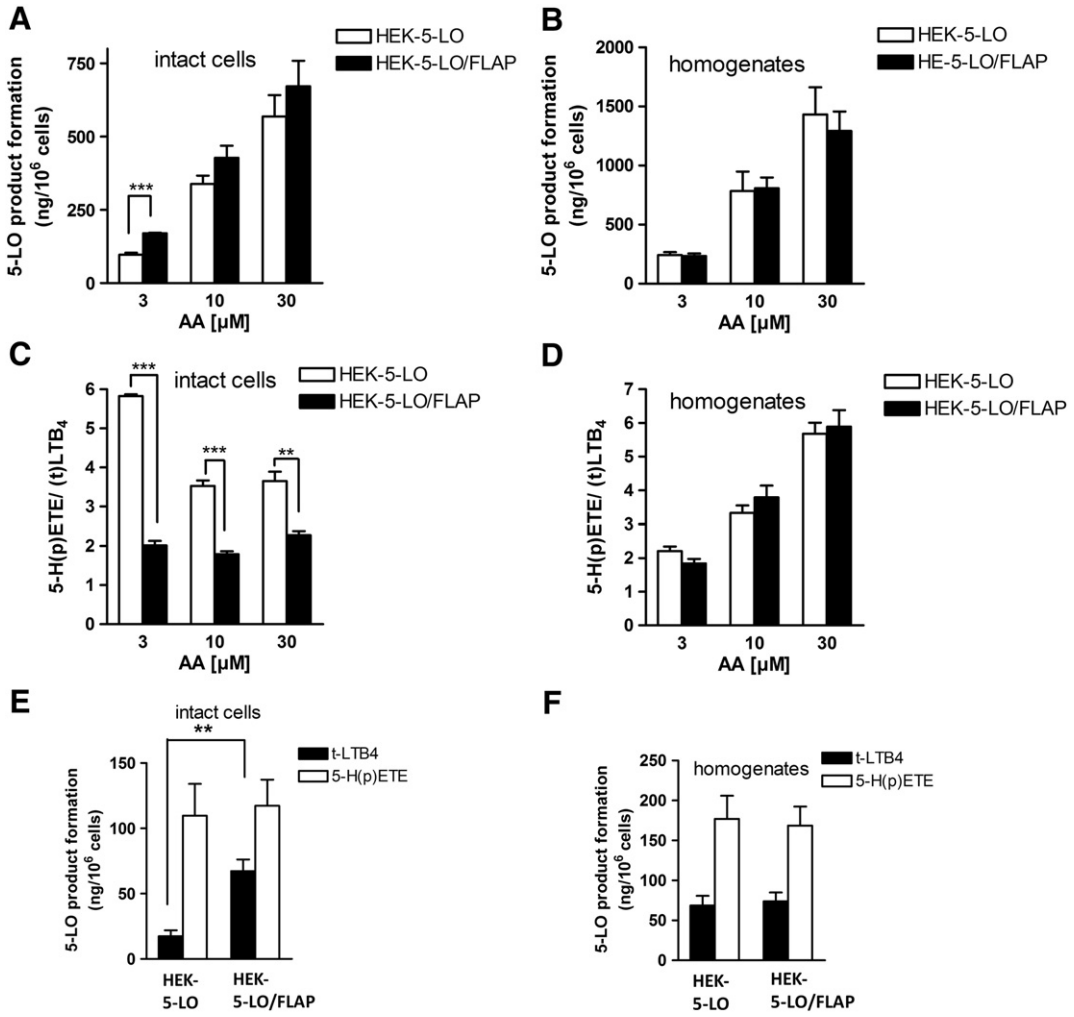

Fig. 2. 5-LO product synthesis in cell-based and cell-free assays using transfected HEK293 cells as source for 5-LO enzyme. Absolute amounts of 5-LO products in intact cells (A) and homogenates (B) after addition of different concentrations of exogenous AA. Cells were incubated with 2.5 μM A23187 and the indicated concentrations of AA. After 10 min at 37 °C, 5-LO products formed were analyzed by RP-HPLC. Ratio of 5-H(p)ETE to the trans-isomers of LTB_4 (trans-LTB_4) in intact cells (C) and homogenates (D). Formation of the trans-isomers of LTB_4 (trans-LTB_4) and 5-H(p)ETE by HEK-5-LO and HEK-5-LO/FLAP in (E) intact cells and (F) in cell homogenates. Data are expressed in means ± SEM; $n = 4$, duplicates; $^{***}p < 0.001$, $^{**}p < 0.01$; HEK-5-LO vs. HEK-5-LO/FLAP, Student's *t* test.

irrespective of FLAP expression with comparable potencies, in intact cells (Fig. 3E) and in homogenates (Fig. 3F) with IC_{50} values as described before for human leukocytes [10].

Since FLAP apparently promotes 5-LO-mediated conversion of 5-HpETE to LTA_4, we investigated whether MK886 may differentially suppress formation of LTA_4 metabolites (trans-isomers of LTB_4) versus 5-H(p)ETE. As shown in Fig. 3D, MK886 increased the ratio of 5-H(p)ETE/trans-isomers of LTB_4 from 2:1 to 2.6:1 at a concentration of 10 nM and 1 μM, respectively. In contrast, the 5-LO inhibitor zileuton did not change the ratio between 5H(p)ETE and the trans-isomers of LTB_4 (Fig. 3D). Therefore, inhibition of FLAP by MK886 seems to impair the second step in 5-LO catalysis, that is, the formation of LTA_4 from 5-HpETE.

3.3. FLAP is required for accumulation of 5-LO in the perinuclear region of HEK cells

It was proposed that in intact cells, translocation of 5-LO from the cytosol to the perinuclear region is necessary to access endogenously released AA by $cPLA_2$ from the nuclear membrane via FLAP [25]. Since 5-LO product formation in HEK-5-LO/FLAP could only be detected when exogenously AA was added, it appeared possible that 5-LO translocation and association with FLAP after A23187 stimulation are not properly operative. Analysis of 5-LO in HEK-5-LO cells by immunofluorescence microscopy revealed a rather homogenous (soluble) intranuclear staining in resting cells (Fig. 4A). Upon stimulation, 5-LO essentially remained within the nucleus but seemingly accumulated in certain intranuclear districts without marked association with the nuclear membrane. In sharp contrast, in HEK-5-LO/FLAP cells, intranuclear 5-LO translocated to the perinuclear region and clearly co-localized with FLAP (Fig. 4B). Next, we investigated if the FLAP antagonist MK886 blocks co-localization of 5-LO with FLAP. Indeed, MK886 (300 nM) prevented co-localization of 5-LO and FLAP, and 5-LO subcellular localization resembled the pattern of unstimulated HEK-5-LO/FLAP cells and/or the pattern of A23187-activated HEK-5-LO cells (Fig. 4B).

4. Discussion

We present here a cellular model using HEK293 cells that are stably transfected with 5-LO together or without FLAP for studying the functional interaction between the two proteins. Although 5-LO and FLAP are markedly expressed in HEK-5-LO/FLAP cells and stimulation with A23187 plus exogenous AA leads to substantial biosynthesis of 5-LO products comparable to neutrophils or monocytes, A23187 alone is insufficient to evoke 5-LO product synthesis in HEK-5-LO/FLAP cells. This is in sharp contrast to primary neutrophils and monocytes [26].

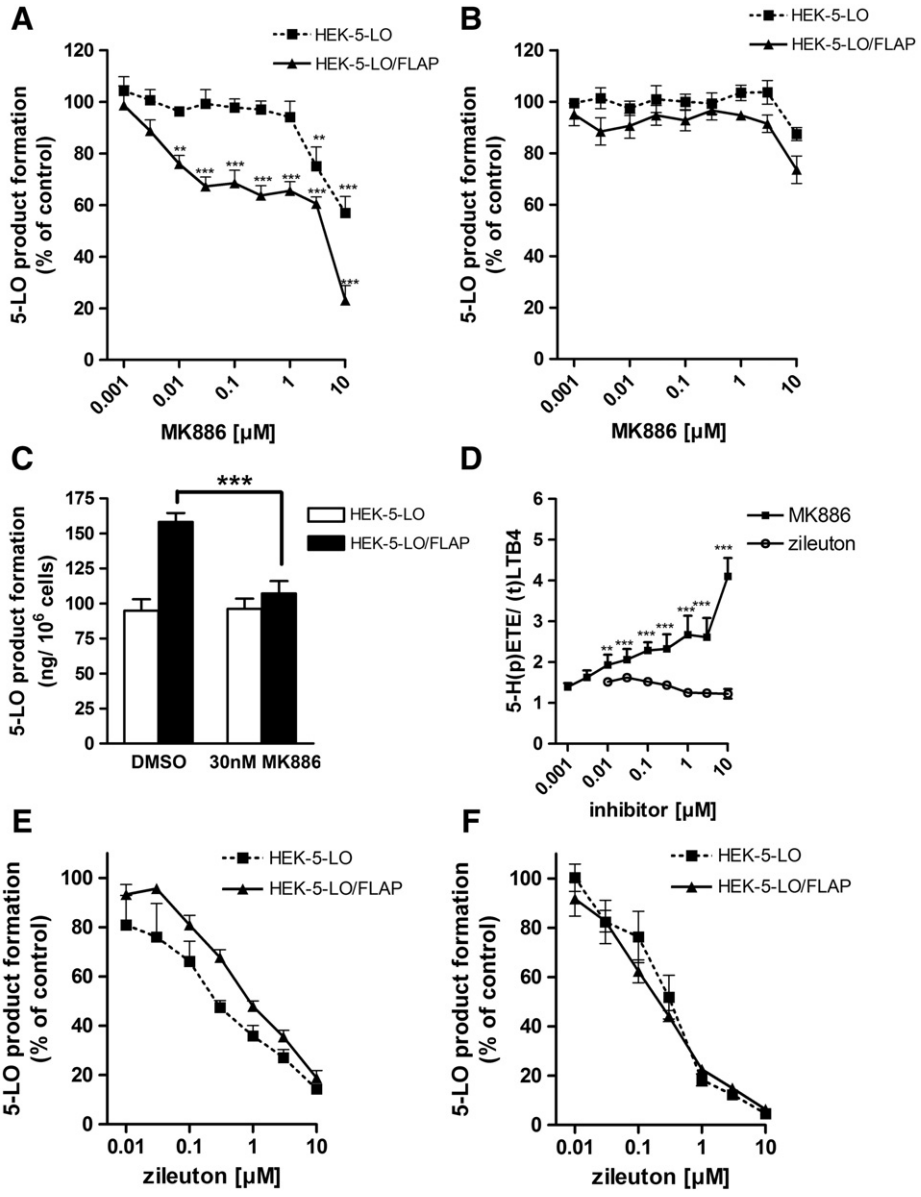

Fig. 3. MK886 inhibits 5-LO product formation in intact HEK293 cells expressing 5-LO and FLAP. (A) Intact HEK cells and (B) homogenates were preincubated with MK886 or vehicle (0.1% DMSO) for 15 min at 37 °C prior to stimulation with 2.5 μM A23187 plus 3 μM AA. After another 10 min at 37 °C 5-LO products formed were analyzed by RP-HPLC. (C) Leukotriene formation in HEK-5-LO and HEK-5-LO/FLAP cells preincubated with vehicle or 30 nM MK886 and stimulated by 2.5 μM A23187 and 3 μM AA. D) Ratios of 5-H(p)ETE to all trans-isomers of LTB_4 (trans-LTB_4) in HEK-5-LO/FLAP, incubated with increasing concentrations of MK886 (-■-) and zileuton (-○-). Intact cells (E) and homogenates (F) were preincubated for 15 min with zileuton, stimulated and analyzed as described above. Data are expressed in means ± SEM; $n = 3$–5, duplicates; $^{***}p < 0.001$, $^{**}p < 0.01$; inhibitor vs. vehicle (DMSO) control, ANOVA + Bonferroni test.

On the other hand, FLAP promoted 5-LO product formation in HEK cells in particular towards LTA_4 at moderate AA levels (i.e., 3 μM), and this FLAP-dependent increase in 5-LO product formation was efficiently blocked by the FLAP antagonist MK886 at nanomolar concentrations. Moreover, in HEK-5-LO/FLAP cells 5-LO clearly associated with FLAP at the perinuclear region upon A23187 challenge. Of interest, p12-LO was able to convert endogenously released AA, which demonstrates AA liberation in A23187 activated HEK cells. In conclusion, our study provides evidence for the existence of a more complex and fine-tuned relationship among 5-LO, FLAP and $cPLA_2$ in LT synthesis, which is operative in primary neutrophils and monocytes but seemingly cannot be completely recapitulated in transfected HEK cells.

Among the various LOs, the 5-LO exhibits extraordinary properties regarding activation, stability, and the essential nuclear membrane translocation process in order to reach FLAP upon stimulation [25]. FLAP is considered to be absolutely essential for LT biosynthesis *in vivo*, as either FLAP gene deletion or pharmacological interference with FLAP (e.g. by MK886) in mice completely abolished LT formation [27]. Also in human whole blood or isolated cells, FLAP inhibitors efficiently repress 5-LO product synthesis [28]. FLAP is distributed on both the inner and outer nuclear membranes and the existence of two pools of FLAP has been proposed [29]. Thus a complex of FLAP together with 5-LO and membrane-bound LTC_4S is formed in RBL-2H3 cells at the outer nuclear membrane that promotes the formation of cysLTs, whereas on the inner membrane (which is devoid of LTC_4S), the complex may consist only of 5-LO and FLAP in murine neutrophils and presumably interacts with nuclear soluble LTA_4H to produce LTB_4 [30]. FLAP can bind AA and certain other fatty acids [31,32], and may transport AA to 5-LO for efficient metabolism. In fact, formation of the 5-LO/FLAP complex in intact cells depends on the presence of AA [33]. Meanwhile, the structures of both 5-LO and FLAP are known [34,35]. However, even though there is substantial evidence for the necessity

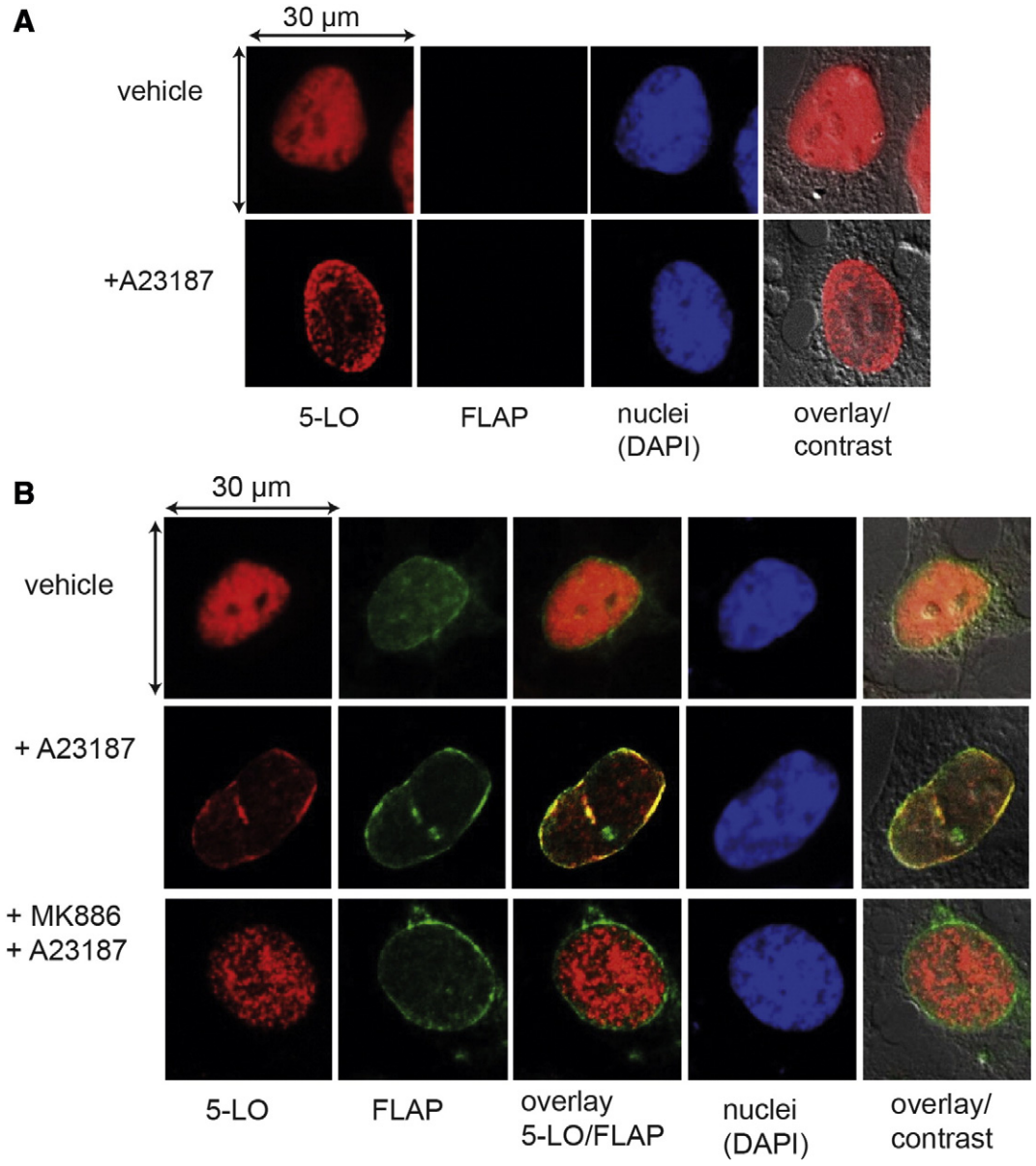

Fig. 4. Subcellular localization of 5-LO and FLAP in transfected HEK293 cells. Subcellular localization of 5-LO, analyzed by immunofluorescence microscopy, in resting cells and upon A23187 stimulation (2.5 µM; 5 min, 37 °C) is shown for cells expressing 5-LO (A) and 5-LO and FLAP (B). Cells co-expressing 5-LO and FLAP were preincubated with MK886 (300 nM, 10 min, 37 °C) or vehicle (0.1% DMSO) as indicated. Results are representative of three independent experiments. Images show single staining for 5-LO (Alexa Fluor 555, red), FLAP (Alexa Fluor 488, green), overlay of 5-LO and FLAP, DNA (DAPI, blue) and overlay with contrast; scale bar = 30 µm.

of FLAP for LT formation and for the establishment of 5-LO/FLAP complexes at various sites in the perinuclear region, how FLAP and 5-LO interact with each other and how FLAP stimulates 5-LO product synthesis remain elusive.

In this report, we developed an experimental cell model that may allow studying the functional interaction between 5-LO and FLAP to get deeper insights into the putative role of FLAP in the modulation of cellular 5-LO product formation. Thus, in contrast to previous models using primary cells or cell lines endogenously expressing FLAP and 5-LO [27], our cell-based model allows to investigate the consequences of site-directed mutation of 5-LO and/or FLAP in stably expressed in HEK cells. Reasonable candidate residues might be F177 and Y181 in 5-LO that appear to preclude AA entry into the 5-LO active site [34]. Indeed, substantial and reproducible amounts of FLAP and 5-LO protein were expressed in HEK-5-LO/FLAP cells, and AA given exogenously to intact A23187-activated cells or corresponding homogenates was effectively converted to the direct 5-LO products trans-isomers of LTB_4 and 5-H(p)ETE.

An unexpected major drawback in the course of our studies was, however, the failure of HEK-5-LO/FLAP cells to generate 5-LO products from endogenously released AA upon A23187 stimulation. HEK cells were shown to express $cPLA_2$ [36] and we confirmed AA release from A23187-activated HEK cells as well as transformation of endogenously liberated AA by 12-LO. These data exclude an absolute lack of free AA in HEK cells after stimulation with A23187. However, compared to human primary monocytes that respond with pronounced AA release upon A23187 stimulation (studied in parallel in our laboratory [37]), the liberation of AA from HEK cells is minute and rather delayed which might be insufficient to enable 5-LO product formation. Nevertheless, this moderate AA release in HEK293 cells seemingly affords generation of 12-H(p)ETE from endogenous substrate by stably transfected p12-LO. Note that only after exogenous addition of 3 µM AA, 5-LO

products were clearly detectable in HEK cells. Interestingly, comparison of 5-LO activity at low AA concentrations (3 μM) revealed significantly higher amounts of 5-LO products formed in cells expressing FLAP, indicating that FLAP was only operative under these conditions. In the HEK-cell model, at high concentrations of AA (10 μM and 30 μM) the FLAP-mediated increase in 5-LO product formation was not significant. This coincides with the reduced potency of FLAP inhibitors in intact human leukocytes with increased exogenous AA [38,39]. The functionality of FLAP is also supported by the fact that HEK-5-LO cells metabolized AA essentially to 5-H(p)ETE whereas co-expression with FLAP promoted the synthase activity of 5-LO with an increased formation of LTA_4 reflected by a higher level of trans-isomers of LTB_4. These data are consistent with observations in leukocytes and transfected osteosarcoma cells [40]. Note that under conditions where FLAP is dispensable for 5-LO product synthesis, as for example in cell homogenates or when excess of substrate (10 or 30 μM AA) is presented, the ratio between 5-H(p)ETE and LTA_4 metabolites were the same for cells with or without FLAP.

The role of FLAP for 5-LO product synthesis in HEK cells is also reflected from experiments with MK886, a potent FLAP antagonist with IC_{50} values in the low nanomalar range [9]. MK886 up to 1 μM failed to reduce 5-LO activity in cells expressing only 5-LO, but under conditions where FLAP seemingly stimulated 5-LO product formation (i.e., HEK-5-LO/FLAP cells activated with A23187 plus 3 μM AA) MK886 reduced it by 35% at low nanomolar concentrations. Thus, MK886 solely antagonized the 5-LO-stimulatory FLAP effect. Strong evidence that FLAP channels the first intermediate 5-HpETE towards LTA_4 is provided when examining the metabolite profile under MK886 treatment. MK886 but not the 5-LO inhibitor zileuton reduced the formation of LTA_4 as visualized by lower LTA_4 metabolites versus 5-H(p)ETE. FLAP is known to supply 5-LO with AA and is proposed to function as a membrane anchor for 5-LO [11,31], but here we demonstrate an additional activity, that is, promoting 5-LO synthase activity leading to LTA_4 at the expense of 5-HETE. It is conceivable that FLAP retains AA inside the substrate cavity of 5-LO to support hydrogen abstraction at C-7 with dioxygenation at C-5 and subsequent hydrogen abstraction at C-10 and thus LTA_4 formation.

Results from the IF microscopy analysis confirm the current understanding of 5-LO subcellular redistribution and co-localization with FLAP at the nuclear membrane [27,30,33]. Our data show that 5-LO translocated from the nucleoplasm and cytosol to the nuclear membrane in HEK-5-LO/FLAP cells after challenge with A23187 and co-localized with FLAP at the perinuclear region. As expected, 5-LO co-localization with FLAP was prevented by MK886 in cells expressing both proteins which is in agreement with MK886 actions in human leukocytes after A23187 stimulation [10,41].

In conclusion, we present a cell model that may help to study the role of FLAP as 5-LO interacting protein in LT biosynthesis in intact cells and the characterization of putative FLAP antagonists. Our data clearly show that co-expression of 5-LO and FLAP and their co-localization at the nuclear membrane together with concomitant release of AA are not sufficient for cellular LT formation from endogenous substrate, even though supplementation of low amounts of exogenous AA leads to tremendous 5-LO product synthesis. It will be challenging in future studies to reveal the respective missing piece of the puzzle. Furthermore, putative FLAP inhibitors can be better examined in such model as interference with FLAP within LT biosynthesis can be distinguished from interference with 5-LO. In fact, novel FLAP antagonists are currently evaluated in clinical trials as potential therapeutics for LT-related disease [42] and our presented cellular model may facilitate hit identification in early preclinical studies.

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