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A 5-lipoxygenase-specific sequence motif impedes enzyme activity and confers dependence on a partner protein

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1. Introduction

The biosynthesis of leukotrienes (LT), lipid mediators of the inflammatory response, is initiated by 5-lipoxygenase (5-LOX) [1,2]. 5-LOX translocates to the nuclear membrane upon intracellular Ca²⁺ mobilization [3,4], along with phospholipase A₂ (PLA₂). PLA₂ cleaves arachidonic acid (AA) from phospholipids that form the nuclear bilayer so that it is then available for 5-LOX to be converted to LTA₄. Ad-ditionally, 5-LOX requires a helper protein, termed 5-lipoxygenase-activating protein (FLAP), a nuclear transmembrane protein. In lipoxygenases the main chain carboxylate of the C-terminus is a ligand for the non-heme iron and thus part of the catalytic center. We investigated the role of a lysine-rich sequence (KKK⁶⁵³-⁶⁵⁵) 20 amino acids upstream of the C-terminus unique to 5-LOX that might displace the main-chain carboxylate in the iron coordination sphere. A 5-LOX mutant in which KKK⁶⁵³-⁶⁵⁵ is replaced by ENL was transfected into HEK293 cells in the absence and presence of FLAP. This mutant gave ~20-fold higher 5-LOX product levels in stimulated HEK cells relative to the wild-type 5-LOX. Co-expression of the enzymes with FLAP led to an equalization of 5-LOX products detected, with wild-type 5-LOX product levels increased and those from the mutant enzyme decreased. These data suggest that the KKK motif limits 5-LOX activity and that this attenuated activity must be compensated by the presence of FLAP as a partner protein for effective LT biosynthesis.
into a WT-5-LOX expression construct (yielding the so-called Triple-K-Mutant-5-LOX, TKM-5-LOX) to investigate the role of this KKK motif. We observed that HEK cells expressing TKM-5-LOX display a ~20-fold increase of 5-LOX product formation relative to the wild-type enzyme. In parallel experiments with Ca²⁺-ionophore-stimulated cells we see an accelerated loss of the TKM-5-LOX, being subject to turnover-based inactivation like WT-5-LOX [16]. However, when co-expressed with FLAP, 5-LOX products detected in HEK cells for the mutant and wild-type enzyme are equivalent. Our data suggest that the KKK motif is a governor of 5-LOX enzyme activity and the helper protein FLAP is required to sufficiently relieve its auto-suppression for effective LT synthesis. Moreover, substrate sequestering and/or handoff by FLAP may limit the rate of LTA₄ synthesis by the hyperactive TKM-mutant.

2. Results

2.1. Substitution of the KKK653–655 motif stabilizes 5-LOX against proteolysis

In order to establish that the mutation of KKK to ENL did not inadvertently disrupt the 5-LOX protein fold, we subjected both the purified wild-type and mutant enzymes to limited proteolysis by chymotrypsin. The enzymes were incubated with the protease in the absence and presence of Ca²⁺. TKM-5-LOX exhibited a higher resistance to cleavage by chymotrypsin than WT-5-LOX when the proteins were assessed with two distinct antibodies: one specific for the C-terminal twelve amino acids ("Tail") and the other for the amino acids 130–149 ("Body") (Fig. 2, antibody epitopes highlighted in Fig. 1). Essentially, little to none of the full-length enzyme was detected by either antibody when purified WT-5-LOX was incubated with chymotrypsin, implying its completed proteolysis. In contrast, in these same conditions the TKM-5-LOX remained largely intact. The major band detected for the TKM-5-LOX after exposure to protease is likely to be full-length enzyme given that it is recognized by an antibody raised to the C-terminal peptide and migrates at the same molecular weight as the purified enzyme.

The TKM-5-LOX is also more resistant to chymotrypsin in crude cell lysates. Intact HEK cells expressing WT- or TKM-5-LOX (but not FLAP) were first treated with (or without) Ca²⁺-ionophore A23187. Cell lysates were prepared and incubated with and without chymotrypsin. The proteins (fragments) of the incubations were analyzed by Western blot using the Tail antibody. It is important to note that the cleavage bands observed in samples incubated without chymotrypsin demonstrate the enzyme's susceptibility to endogenous cellular proteases upon cell lysis, as the experiments were free of protease inhibitors. Fragments detected for WT-5-LOX are more pronounced than in the lysates of TKM-5-LOX-expressing cells (Fig. 3).

2.2. Co-localization of 5-LOX and FLAP at the nuclear membrane appears unaffected by substitution of the KKK motif

Immunofluorescence microscopy studies indicate that like the wild-type enzyme [11,27], the TKM-5-LOX variant translocates to the nuclear membrane upon stimulation with Ca²⁺-ionophore, both in the absence and presence of FLAP (Fig. 4). Detection by both "Body" and "Tail" antibody demonstrated that TKM-5-LOX colocalizes with FLAP, as seen for the wild-type enzyme (Fig. S2). Additionally, the TKM-5-LOX displays the same cytoplasmic localization as WT-5-LOX when unstimulated. Representative immunofluorescence images are presented in Fig. 4.

2.3. HEK cells expressing TKM-5-LOX yield elevated levels of 5-LOX products

TKM-5-LOX was expressed in HEK293 cells at levels comparable to the WT-5-LOX, as judged by mRNA and protein levels, detected by qPCR (Fig. S3) and Western blotting, respectively (vida infra, Section 2.5). 5-LOX-expressing HEK cells devoid of FLAP were incubated with
In the absence of FLAP, TKM-5-LOX-expressing cells were first treated with or without Ca\(^{2+}\)-ionophore (A23187), lysed, incubated with or without chymotrypsin, and then developed with the primary antibody. Lanes labelled ‘mwm’ and ‘UT’ correspond to the molecular weight markers and lysates of untransfected cells, respectively. Note the intensity of the lower molecular weight band generated by chymotrypsin for the WT-5-LOX relative to the full-length protein band.

Fig. 3. Chymotrypsin cleavage patterns of WT-5-LOX or TKM-5-LOX in lysates of HEK cells. Cells were co-expressing FLAP and WT- or TKM-5-LOX. Ultra Performance Liquid Chromatography (UPLC)-MS/MS analysis was performed with cells stimulated with ionophore and AA (added at t0), and 5-LOX products sampled at 30, 60, 120, 180, 360 and 600 s. Again, the presence of FLAP led to similar levels of products generated by both cell types, with no significant differences detected at early (< 300 s) or late (600 s) time points when total 5-LOX products are summed (Fig. 6). Both cell lines reached the plateau for 5-LOX products at the same time points (180 s), indicating no differences in their ability to metabolize AA. When 5-HETE and LTB\(_4\)-breakdown products are plotted individually, the kinetics of 5-HETE production in the two cell lines agree, while TKM-5-LOX is strikingly more resistant. TKM-5-LOX displays susceptibility to chymotrypsin cleavage, while TKM-5-LOX is strikingly more resistant.

In parallel experiments and in agreement with previous findings [27], co-expression of FLAP in HEK cells together with 5-LOX caused a ~4 fold increase in product formation by WT-5-LOX [27] (Fig. 5). In contrast, co-expression of FLAP reduced product formation of TKM-5-LOX down to the levels of WT-5-LOX (TKM 53 ng 5-HETE, 28 ng LTs; WT 43 ng 5-HETE; 23 ng LTs). Thus, the presence of FLAP elevates the activity of WT-5-LOX but attenuates the increased activity of the TKM mutant in these assay conditions.

In addition, we compared the kinetics of product formation in HEK cells co-expressing FLAP and WT- or TKM-5-LOX. Ultra Performance Liquid Chromatography (UPLC)-MS/MS analysis was performed with cells stimulated with ionophore and AA and 5-LOX products sampled at 30, 60, 120, 180, 360 and 600 s. Again, the presence of FLAP led to similar levels of products generated by both cell types, with no significant differences detected at early (< 300 s) or late (600 s) time points when total 5-LOX products are summed (Fig. 6). Both cell lines reached the plateau for 5-LOX products at the same time points (180 s), indicating no differences in their ability to metabolize AA. When 5-HETE and LTB\(_4\)-breakdown products are plotted individually, the kinetics of 5-HETE production in the two cell lines agree more than those of LT production, with the latter at higher levels in the WT-5-LOX-expressing cells. However, again at 10 min the difference between the LT levels in the two cell types does not appear significant (Figs. 5, 6). These data support the conclusion that the presence of FLAP reduces TKM-5-LOX product formation to WT-5-LOX levels.

2.5. The substitution of KKK promotes loss of enzyme activity in conditions in which substrate is available

We next monitored 5-LOX protein levels in the HEK cells in order to investigate whether the increased product levels of the TKM-5-LOX might be a consequence of increased enzyme longevity (Fig. 7). Using a method adapted from that described by Dai et al. [28], 5-LOX protein levels (relative to the house-keeping protein GAPDH) in the presence of the protein synthesis inhibitor cycloheximide were monitored in HEK cells for 4 h by Western blot. These experiments were performed in the presence or absence of Ca\(^{2+}\)-ionophore: in the presence of Ca\(^{2+}\)-ionophore, the levels of WT-5-LOX and TKM-5-LOX cleavage patterns with “Body” (left) or “Tail” (right) antibody development, ± Ca\(^{2+}\) and/or chymotrypsin. WT-5-LOX displays susceptibility to chymotrypsin cleavage, while TKM-5-LOX is strikingly more resistant.

In the absence of Ca\(^{2+}\)-ionophore, the levels of WT- and TKM-5-LOX remained roughly stable up to 4 h, regardless of the presence of FLAP. In these conditions, the WT-5-LOX appears to be expressed at slightly elevated levels versus TKM-5-LOX (Fig. 7) (5-LOX/GAPDH ratios at 0.6 ± 0.20 for WT-5-LOX and 0.3 ± 0.15 for TKM-5-LOX). Upon Ca\(^{2+}\)-ionophore stimulation of cells devoid of FLAP, the levels of the
TKM-5-LOX declined within 90 min, while those for the WT-5-LOX much less so. The loss of TKM-5-LOX is consistent with its enhanced enzyme activity and turnover-based inactivation as previously described for 5-LOX [16]. In contrast, when co-expressed with FLAP, where 5-LOX product levels of WT- and TKM-5-LOX are comparable, the levels of TKM-5-LOX remain stable. These data suggest that the increase in product yields from TKM-5-LOX (without its partner FLAP) is not a consequence of a protracted half-life for TKM-5-LOX, despite the enhanced in vitro stability. Moreover, TKM-5-LOX levels decline in conditions in which it can produce 5-HETE or LTA4.

Previous work indicated that 5-LOX products are only detected in the 5-LOX expressing HEK293 cells when exogenous arachidonic acid is added [27]. However, we confirmed the presence and availability of endogenous AA by increasing the sample size ($5 \times 10^6$ vs. $1 \times 10^6$ cells). The UV spectrum displayed in Fig. 8 is consistent with the presence of LTA4 hydrolysis products in extracts of HEK293 cells co-
expressing FLAP and TKM-5-LOX and stimulated with Ca^{2+}-ionophore.

3. Discussion

3.1. Structural impact of the triple-lysine motif

The KKK^{653-655} sequence is located 20-amino acids up stream of the C-terminal Ile^{673}, which ligates the active site iron of 5-LOX via its main chain carboxyl. This unique sequence motif appears to conflict with salt-link and π-cation interactions observed in other LOX structures. K{sup 655} of the KKK motif in other LOXs is typically a conserved Leu. A highly conserved Arg^{651} lies one turn away on the C-terminal helix and participates in a conserved salt-link with Asp^{473} and a π-cation interaction with Phe^{469} [24]. Thus, a Lys at 655 in WT-5-LOX may disrupt these interactions and impact the positioning of the C-terminal Ile^{673} carboxyl in the Fe^{2+} coordination sphere. The structure of a bacterial LOX that harbors a positively charged amino acid at the position corresponding to 5-LOX Lys^{655} confirmed this prediction: the invariant Arg (Arg^{651}) that participates in a conserved salt link has been displaced in this enzyme [29].

The mutation of the KKK to ENL was performed to engineer a 5-LOX variant for crystallographic studies, and it was observed that the mutation increased the melting temperature of the mutant protein [24]. This led us to suggest that the KKK motif might contribute to an

Fig. 6. Time-dependent 5-LOX product analysis. HEK-WT-5-LOX + FLAP and HEK-TKM-5-LOX + FLAP were stimulated with 2.5 μM A23187 plus 3 μM AA at 37 °C and 5-LOX products were quantitated at the indicated time points. Formed lipid mediators were isolated by solid phase extraction and analyzed by UPLC-MS-MS and are shown as ng/1 × 10^6 cells. (A) Total 5-LOX products (B) 5-HETE, (C) LTA{sub 4} breakdown products. Results were analyzed in n = 3 independent experiments.

Fig. 7. In the absence of de novo protein synthesis WT 5-LOX levels remain constant in HEK 293 cells. A. Western blots of WT- and TKM-5-LOX. WT-5-LOX and TKM-5-LOX ± FLAP expressing HEK cells were incubated with cycloheximide over a four-hour period. Western blots for both 5-LOX and the housekeeping enzyme GAPDH (lower panel) at time points 0, 60, 90, 120, 150, 180, 210, and 240 min in the absence (top) and presence (bottom) of Ca^{2+}-ionophore A21387 were developed with "Tail" antibody. Both mutant and WT-5-LOX levels remain stable in the absence of Ca^{2+}-ionophore. Post-stimulation TKM-5-LOX levels decline when FLAP is absent. Representative Westerns are depicted for each of the experiments. B. Triplicate/duplicate plots (black, gray and white circles) of the ratio of 5-LOX to GAPDH levels, as determined from band densities. Although differences in expression levels are observed between duplicate and triplicate experiments, the trends are equivalent. Cycloheximide is not stable for the entire time period and was replenished at 120 min. Quantification of 5-LOX variants was measured relative to GAPDH density with ImageJ. Only TKM-5-LOX, when expressed in the absence of FLAP, displays a significant decline in enzyme detected in the first 90 min after stimulation.
Fig. 8. TKM-5-LOX FLAP expressing cells produce LT from endogenous AA. (Left) The UV-spectrum of the gray fraction is consistent with LTA₄ hydrolysis products. (Right) Overall proportions of extracted oxylipins from TKM-5-LOX FLAP cells stimulated with Ca²⁺-ionophore A23187.

It was noted that FLAP cells showed higher 5-LOX activity than TKM-5-LOX in the absence of FLAP as FLAP increases the LT biosynthetic capacity of the WT-5-LOX-expressing cells, the TKM-5-LOX-expressing cells display a significantly reduced LT biosynthetic efficiency when FLAP is present. Interestingly, the pronounced increase in product formation by TKM-5-LOX is only apparent in incubations with intact cells, as assays of cell homogenates have similar product yields (Fig 5c). Similarly, the impact of FLAP on 5-LOX product formation is only observed in intact cells [7]. The presence of an intact nuclear membrane may be essential for both processes.

It is tempting to speculate on how the presence of FLAP equalizes the product levels for WT- and TKM-5-LOX. Our data may suggest that the transfer of AA from FLAP to 5-LOX is the rate-limiting step of the transformation of AA to LTA₄. For the WT-5-LOX, FLAP increases its access to substrate. However, the more robust activity of TKM is obscured when it must access FLAP-sequestered AA. FLAP serves as a gatekeeper. An alternative model is that a FLAP-induced conformational change in 5-LOX abrogates the effect of the KKK to ENL mutation by constraining the conformations it can sample in catalysis. For example, any effect of the mutation on the Fe²⁺ coordination sphere may be negated once 5-LOX and FLAP interact. Either model is consistent with the suggestion that a specific FLAP:5-LOX interaction allows FLAP to rescue 5-LOX mutants with impaired enzyme and membrane-binding activities [11].

In intact cells FLAP is able to compensate for the lower activity of the WT-5-LOX to some extent. Thus, we suggest that the KKK motif confers the requirement for FLAP for rapid initiation of LT biosynthesis. This regulatory mechanism is reminiscent of the cyclin/cyclin-dependent kinase scenario, where an enzyme (the kinase) requires interaction with a binding partner to promote catalytic competence [30]. Overproduction of LTs as pro-inflammatory mediators is detrimental to the organism, thus the tempered activity of 5-LOX that relies on alleviation with a protein partner limits this possibility. WT-5-LOX alone is particularly inefficient at producing lipid mediators but stimulated by FLAP it still does not reach the levels produced by the TKM-5-LOX expressed without FLAP. The fact that the presence of FLAP obviates the marked increase in 5-LOX product formation by TKM-5-LOX suggests that the KKK motif is not required for interaction with FLAP.

3.3. Turnover based inactivation

By monitoring 5-LOX protein levels in the presence of an inhibitor of protein biosynthesis, we observed a turnover-dependent loss of TKM-5-LOX. In the absence of Ca²⁺-ionophore, both WT- and TKM-5-LOX exhibited stable protein levels. However, in the presence of Ca²⁺-ionophore there is a decline in the level of TKM-5-LOX detected in intact cells after 90 min (Fig. 7). The cycloheximide analysis was performed without addition of exogenous AA. However, we can detect the products of TKM-5-LOX activity in Ca²⁺-ionophore-stimulated HEK cells without addition of exogenous AA (Fig. 8) and confirm that endogenous AA is released from the phospholipids upon stimulation. The presence of endogenous phospholipid-esterified AA that might provide a source of substrate is supported by data from other groups. Dawaliby et al. established that HEK293 cell membranes contain C20 containing phospholipids [31]. More specifically, Zhu et al. [32] report the presence of 20:4n-6 fatty acids in the cell membrane phospholipids of HEK293 cells. Additionally, HEK cells transfected to express 12-LOX generate product without the addition of exogenous AA when cultured in DMEM [27]. Therefore, the elevated product levels detected in HEK cells expressing TKM-5-LOX in the presence of exogenous AA and the disappearance of TKM-5-LOX, once stimulated with Ca²⁺-ionophore to promote release of endogenous PI-esterified AA, are consistent with turnover-based inactivation.
3.4. Intrinsic regulators

The KKK motif might be an integral deactivation sequence that acts as a regulator of LT biosynthesis. Internal enzyme fail-safe mechanisms are one of many regulatory mechanisms that keep the intricate web of biosynthetic pathways in check. An intriguing auto-inactivation mechanism of another LT biosynthetic enzyme has been described for LTA4 hydrolase. In this enzyme, the presence of Tyr378 makes it susceptible to covalent modification by its reactive substrate. Substitution of Tyr378 with Gln or Ala leads to a variant that is less susceptible to covalent modification, and consequently inactivation, by the substrate [33–35]. Thus, both 5-LOX and LTA4 hydrolase display auto-suppression of catalytic activity, but through entirely different mechanisms.

Other amino acids in 5-LOX have been demonstrated to play significant roles in tempering a robust rate of protein formation. Rakonjac et al. examined the significance of a salt-link between residues Arg101 and Asp166 of the regulatory and catalytic domains, respectively. Disruption of this interaction leads to an increase in enzyme activity [36].

Our studies do not reveal exactly how the presence of the KKK motif modulates 5-LOX activity. Computational methods, as summarized by O’Rourke et al., can be combined with high-resolution X-ray data to reveal possible internal protein communication networks [37]. However, the resolution of the crystal structure of Stable-5-LOX makes this approach inaccessible at this point. In any case, it is apparent that the KKK motif significantly contributes to tight regulation of 5-LOX activity as the initiator of LT biosynthesis. This motif, which initially we studied as a possible mechanism to limit protein half-life, appears to be part of a more intricate, finely-tuned control mechanism to regulate LT biosynthesis in conjunction with FLAP.

4. Materials and methods

4.1. Materials

The “Tail” antibody was generated by Genscript (Piscataway) to a peptide with the C-terminal 12 amino acids of 5-LOX: CSPDRIPNSVAL. Specificity was confirmed by its ability to detect full-length bacterially expressed Stable-5-LOX, but not a truncated version of the construct. 5-LOX “Body” was purchased from Abcam and is raised to the sequence E.R. Schexnaydre et al. (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2% Triton-×100) before being incubated with ionophore A23187 for 5 min at 37 °C then incubated to 10 min with 3 M chymotrypsin (2:1 protein to protease ratio) for 1 min on ice. Samples were run on an SDS-PAGE gel and transferred to a PVDF membrane with a Bio-Rad Trans-Blot system for Western development. The primary antibodies were rabbit anti-5-LOX “Body” (amino acids 130–149) and rabbit anti-5-LOX “Tail” (12 amino acids from the C-terminus). The secondary antibody utilized was goat-antibody AlexaFlour647. Western blots were imaged with a Typhoon 9410 imager using channel 670 30 BP.

4.2. 5-LOX purification

His-tagged WT-5-LOX and TKM-5-LOX were expressed in Rosetta cells with a pET14B vector. Bacteria were cultured at 37 °C and 220 rpm for 4 h and then at 20 °C for an additional 27 h. Cells expressing WT-5-LOX were lysed by sonication and the lysate was clarified by centrifugation at 36,000 × g for 30 min. To the supernatant was added ammonium sulfate to 50% saturation. The precipitant was pelleted and washed with 100 mM Tris, 100 mM NaCl, 2 mM TCEP, 10 mM imidazole. After concentration of the eluant, protein concentration was determined with a Nanodrop spectrophotometer. Protein purity was confirmed by SDS-PAGE. Purified protein was frozen dropwise and stored in liquid N2.

4.3. TKM-5-LOX purification

Bacterial cell cultures prepared as above were lysed by French press. After clarification of the lysate by centrifugation at 36,000 × g, the supernatant was applied to a Co2+-affinity column. The column was washed with 100 mM Tris, 500 mM NaCl, 20 mM imidazole. The immobilized enzyme was eluted with 100 mM Tris, 500 mM NaCl, 200 mM imidazole. After concentration of the eluent, protein concentration was determined with a Nanodrop spectrophotometer. Protein purity was confirmed by SDS-PAGE. Purified protein was frozen dropwise and stored in liquid N2.

4.4. Expression of 5-LOX and FLAP in HEK293 cells

HEK293 cells were transfected with WT-5-LOX, TKM-5-LOX, and FLAP using the pcDNA3.1 vector for stable protein expression as described by Gerstmeier et al. [27]. Cells were cultured at 37 °C, 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Atlanta Biologicals), in the presence of selection antibiotic (G418, 5-LOX; and hygromycin, FLAP (both from Sigma)). Successful protein expression was confirmed by Western blotting.

4.5. Proteolytic cleavage

4.5.1. Purified enzyme

Purified WT- and TKM-5-LOX were diluted to 9.8 μM in 100 mM Tris, 100 mM NaCl. The purified protein solutions were incubated with or without 2.5 μM CaCl2 for 5 min. The solutions were then incubated with or without 5 μM chymotrypsin (2:1 protein to protease ratio) for 1 min on ice. Samples were run on an SDS-PAGE gel and transferred to a PVDF membrane with a Bio-Rad Trans-Blot system for Western development. The primary antibodies were rabbit anti-5-LOX “Body” (amino acids 130–149) and rabbit anti-5-LOX “Tail” (12 amino acids from the C-terminus). The secondary antibody utilized was goat-antibody AlexaFlour647. Western blots were imaged with a Typhoon 9410 imager using channel 670 30 BP.

4.5.2. Cell lysates

HEK293 cells expressing WT- or TKM-5-LOX were collected and resuspended at 1 × 106 cells/ml in phosphate-buffered saline (PBS) plus 0.1% glucose. Cells were incubated with or without 2.5 μM Ca2+-ionophore A23187 for 5 min in a 37 °C water bath. The cells were spun down and resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2% Triton-×100) before being incubated with 100 mM chymotrypsin for 1 min on ice. Sample protein concentrations were determined with a BCA protein assay kit (Pierce). Equivalent amounts of protein were loaded onto 10% SDS-PAGE gels. After blotting onto PVDF membranes, the resulting Western blots were developed as described above.

4.6. 5-LOX HPLC product analysis of HEK cells

HEK293 cells expressing WT- or TKM-5-LOX with or without FLAP were collected and resuspended at 1 × 106 cells/ml in phosphate-buffered saline (PBS) plus 0.1% glucose. Each sample was incubated with or without 2.5 μM Ca2+-ionophore A23187 for 5 min at 37 °C then incubated for 10 min with 3 μM AA (Cayman). Modest substitutions have been shown to effect the kinetics of 5-LOX translocation to the nuclear membrane [11] and pre-incubation with ionophore was necessary to control for any differences in membrane trafficking that might result from the substitution of the KKK motif. Incubations were stopped with the addition of 1 ml (1 volume) of methanol. Prior to solid phase extraction with C18 cartridges (UCT CLEAN-UP C18 EC18112Z), 0.5 ml PBS, 30 μl of 1 M HCl, and 10 μl of 50 ng/μl prostaglandin B1 (Cayman, PGB1) were added. The products were eluted with methanol. The methanol was evaporated off under N2 gas and the residue was resuspended in 60% acetonitrile, 0.1% formic acid, the HPLC mobile phase. Samples were treated with triphenylphosphine as a reducing agent prior to HPLC analysis. Isocratic reverse-phase HPLC was performed with a Supelco Discovery
4.7. 5-LOX product analysis of HEK cells analyzed by UPLC-MS-MS

HEK-WT-5-LOX + FLAP and HEK-TK5-LOX + FLAP cells (1 × 10⁶ cells/ml) were incubated in PBS containing 1 mM CaCl₂. Cells were stimulated with 2.5 μM A23187 plus 3 μM AA at 37 °C and sampled at 0, 30, 60, 120, 180, 360 and 600 s. Ice-cold methanol (2 ml) was added to the samples to stop the reaction along with 10 μl of deuterium-labelled internal standards (200 nM d8-5HETE, d4-LTB₄, d4-HETE) to facilitate quantification and sample recovery. Sample preparation was conducted by adapting published criteria [38,39]. Samples were kept at −20 °C for 60 min to allow protein precipitation. After centrifugation (1200 × g, 4 °C, 10 min) 8 ml acidified H₂O was added (final pH = 3.5) and the sample was subjected to solid phase extraction. Solid phase cartridges (Sep-Pak® Vac 6 cc 500 mg/6 ml C18; Waters, Milford, MA) were equilibrated with 6 ml methanol and 2 ml H₂O before samples were loaded onto columns. After washing with 6 ml H₂O and additional 6 ml hexane, lipid mediators were eluted with 6 ml methyl formate. Finally, the samples were brought to dryness using an evaporation system (TurboVap LV, Biotage, Uppsala, Sweden) and resuspended in 100 μl methanol-water (50/50, v/v) for UPLC-MS-MS automated injections. Lipid mediator profiling was analyzed with an Acquity™ UPLC system (Waters, Milford, MA, USA) and a QTRAP 5500 Mass Spectrometer (ABSciex, Darmstadt, Germany) equipped with a Turbo V™ Source and electrospray ionization (ESI). Lipid mediators were eluted using an ACQUITY UPLC® BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Eschborn, Germany) at a flow rate of methanol-water-acetic acid of 42:58:0.01 (v/v/v) that was ramped to 86:14:0.01 (v/v/v) over 12.5 min and then to 98:2:0.01 (v/v/v) for 3 min [40]. The QTrap 5500 was operated in negative ionization mode using scheduled multiple reaction monitoring (MRM) coupled with information-dependent acquisition. The scheduled MRM window was 60 s, optimized lipid mediator parameters were adopted (CE, EP, DP, CXP), and the curtain gas pressure was set to 35 psi. The retention time and at least six diagnostic ions for each lipid mediator were confirmed by means of an external standard (Cayman Chemicals/Biomol, Hamburg Germany). Quantification was achieved by calibration curves for each of the lipid mediators analyzed. Linear calibration curves were obtained that gave r² values of 0.998 or higher (for fatty acids 0.95 or higher).

4.8. Immunofluorescence imaging of 5-LOX and FLAP in HEK293 cells

HEK293 cells expressing WT- or TK5-LOX with or without FLAP were seeded out at 200 cells/ml in each well of an Ibidi 12-well slide. After 24–48 h at 37 °C 5% CO₂, 2.5 μM Ca²⁺-ionophore A23187 was added to stimulate the cells, which were then incubated for 5 min at 37 °C 5% CO₂. The cells were fixed with 4% paraformaldehyde then washed 3 times with PBS. The cells were then incubated with a 50 mM NH₄Cl solution, followed by a wash cycle and subsequently blocked for an hour with 10% donkey serum, 0.1% Tween-20 in PBS. The samples were then incubated overnight at 4 °C with a primary antibody solution consisting of either rabbit anti-5-LOX “Body” ≥ goat anti-FLAP or rabbit anti-5-LOX “Tail” ≥ goat anti-FLAP in 0.1% Tween-20 PBS solution. After a wash cycle with 0.1% Tween-20 PBS the cells were incubated with a secondary antibody solution consisting of donkey anti-rabbit AlexaFluor488 ± donkey anti-goat AlexaFluor647 in a 1:1 Tween-20 PBS solution for 20 min. After another wash cycle, the cells were incubated with 2 μg/μl DAPI solution for 3 min followed by another wash cycle. Invitrogen Prolong Gold antifade reagent and a coverslip were then added to the slide. The slide was imaged with a Leica DM6B upright microscope using DAPI, GFP (5-LOX), and Cy5 (FLAP) filters.

4.9. HEK cell protein time-course in the absence of protein synthesis

HEK cells expressing WT- or TK5-LOX with or without FLAP were collected and resuspended at 1 × 10⁶ cells/ml in PBS plus 0.1% glucose. The cells were incubated with or without 2.5 μM Ca²⁺-ionophore A23187 for 5 min and then treated with 200 μM cycloheximide at 37 °C and analyzed at eight time points (0, 60, 90, 120, 150, 210, 240 min). An extra 200 μM cycloheximide was added after 120 min to ensure continuous inhibition of protein synthesis. At each time point 2 × 10⁶ cells were treated with lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, 1 μM pepstatin, leupeptin and PMSF). Sample protein concentrations were determined with a BCA assay and equal amounts of protein were loaded onto 10% SDS-PAGE gels. The samples were then analyzed by Western blot using rabbit anti-5-LOX “Tail” and goat anti-GAPDH primary antibodies and donkey anti-rabbit AlexaFluor488 and donkey anti-goat AlexaFluor647 secondary antibodies. Western blots were imaged with a Typhoon 9410 imager using channels 526 SP and 670 30 BP. For quantitative analysis of western bands we used ImageJ software.

4.10. qPCR HEK 5-LOX mRNA quantification

4.10.1. RNA isolation

Samples of 1 × 10⁷ cells were collected for each of the four cell lines, i.e., WT- or TK5-LOX with and without FLAP. After spinning down cells and removing the media, the cells were resuspended in Trizol Reagent and incubated for 5 min at room temperature. Then, a 50/50 mixture of chloroform and isooamyl alcohol was mixed with the samples and allowed to incubate for 3 min at room temperature. After centrifugation, the upper aqueous phase of the samples was transferred to a fresh tube. Isopropanol was mixed with aqueous phase and incubated for 10 min at room temperature. The samples were again centrifuged and the RNA pellet was washed twice with 75% ethanol and then let to air dry. The RNA pellets were then dissolved in diethylpyrocarbonate (DEPC) water before undergoing DNase treatment where the samples were incubated with DNase I at 37 °C for 30 min then with the addition of 10 mM EDTA incubated at 70 °C for 10 min. Next, the samples were incubated with 300 mM sodium acetate and 2.5 volumes of ethanol at −80 °C for 15 min before being centrifugation at 4 °C for 10 min. The RNA pellets were dissolved in DEPC water and concentration readings were taken by Nanodrop.

4.10.2. cDNA synthesis and gel analysis

The isolated RNA (10 μg) served as a template for cDNA synthesis using the Goscript reverse transcriptase kit (Promega). Forward and reverse oligos for 5-LOXs and the GAPDH control were made by Integrated DNA Technologies (IDT) (Fig. S4). Samples were then treated with RNaseH before undergoing PCR. The PCR samples were run on an Agarose gel and analyzed visually. For quantitative analysis we used ImageJ software.

Abbreviations

- 5-LOX: 5-lipoxygenase
- FLAP: 5-lipoxygenase activating protein
- PLA₂: phospholipase A2
- AA: arachidonic acid
- HETE: hydroxyeicosatetraenoic acid
HYETE hydro-peroxyeicosatetraenoic
PGTB1 prostaglandin B1
LT leukotriene
TCEP tris(2-carboxy-ethyl)phosphine
DAPI 2-(4-Amidino-phenyl)-1H-indole-6-carboxamidine
UPLC Ultra Performance Liquid Chromatography

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2018.09.011.

References