IMPACT OF MYELOPEROXIDASE-DERIVED OXIDANTS ON THE PRODUCT PROFILE OF HUMAN 5-LIPOXYGENASE

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Highlights

- Myeloperoxidase-derived oxidants changed the product profile of 5-lipoxygenase.
- The ratio of 5-HpETE to 6-*trans*-LTB₄ increased with increasing HOCl and HOBr.
- Epoxidation of 5-HpETE was more strongly affected than conversion of arachidonic acid.
- 5-Lipoxygenase also produced 8- and 12-HpETE after incubation with HOCl and HOBr.
- The whole myeloperoxidase system showed effects similar to those of HOCl and HOBr.

Abstract

Human 5-lipoxygenase (5-LOX) oxidizes arachidonic acid to 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE) and leukotriene (LT) A₄. In neutrophils, LTA₄ is further converted to the potent chemoattractant LTB₄. These cells also contain the heme enzyme myeloperoxidase (MPO), which produces several potent oxidants such as hypochlorous acid (HOCl), which are involved in pathogen defense and immune regulation. Here, we

addressed the question whether MPO-derived oxidants are able to affect the activity of 5-LOX and the product profile of this enzyme. Human 5-LOX was incubated with increasing amounts of HOCl or HOBr. Afterward, arachidonic acid metabolites of 5-LOX were analyzed by reverse-phase high-performance liquid chromatography as well as by liquid chromatography-electrospray ionization-tandem mass spectrometry. The incubation of 5-LOX with the MPO-derived oxidants significantly changed the product profile of 5-LOX. Thereby, HOCl and HOBr increased the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ in a concentration-dependent manner. At low oxidant concentrations, there was a strong decrease in the yield of 6-*trans*-LTB₄, whereas 5-HpETE did not change or increased. Additionally, the formation of 8-HpETE and 12-HpETE by 5-LOX rose slightly with increasing HOCl and HOBr. Comparable results were obtained with the MPO-H₂O₂-Cl⁻ system when glucose oxidase and glucose were applied as a source of H₂O₂. This was necessary because of a strong impairment of 5-LOX activity by H₂O₂. In summary, MPO-derived oxidants showed a considerable impact on 5-LOX, impairing the epoxidation of 5-HpETE, whereas the hydroperoxidation of arachidonic acid was unaffected. Apparently, this was caused by an oxidative modification of critical amino acid residues of 5-LOX. Further work is necessary to assess the specific type and position of oxidation in the substrate-binding cavity of 5-LOX and to specify whether this interaction between 5-LOX and MPO-derived oxidants also takes place in stimulated neutrophils.

Keywords

Eicosanoids, Arachidonic acid, HOCl, HOBr, Neutrophils, Free radicals

Introduction

During the innate immune response, polymorphonuclear leukocytes, also called neutrophils, are the first effector cells that will be recruited from the peripheral blood to inflammatory loci. These cells are equipped with an arsenal of protecting systems able to recognize, inactivate, and destroy foreign microorganisms. Enzymes and signaling systems will be sequentially activated in neutrophils during their interaction with the inflamed endothelium, during diapedesis and migration of these cells through the endothelial layer and adjacent space, as well as finally during recognition and phagocytosis of microorganisms [Kolaczowska and Kubes 2013].

In this respect, 5-lipoxygenase (5-LOX), a non-heme-iron dioxygenase that uses arachidonic acid (AA) as substrate, is crucial for the chemotactic movement of neutrophils. This enzyme is also present in eosinophils, monocytes/macrophages, dendritic cells, and mast cells [Radmark *et al.* 2015]. Equimolar amounts of hydroperoxides can activate 5-LOX by oxidation of the inactive ferrous iron to the ferric form [Rouzer and Samuelsson 1986]. Afterward, the lipoxygenase reaction is started by stereoselective hydrogen abstraction at C7 of AA. Then, the resulting radical migrates to the C5 position, where it reacts with dioxygen forming a peroxy radical. In a subsequent interaction with the ferrous form of 5-LOX, a peroxy anion is generated yielding 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE) after protonation [Haeggstrom and Funk 2011]. Human 5-LOX also possesses a secondary leukotriene A₄ (LTA₄) synthase activity [Rouzer *et al.* 1986]. Thereby, a hydrogen at C10 of 5-HpETE is abstracted followed by a radical migration to the C6 position, rearrangement of double bonds, and formation of an epoxide moiety [Maas *et al.* 1982]. In neutrophils the unstable LTA₄ is thereafter metabolized to leukotriene B₄ (LTB₄) by the action of LTA₄ hydrolase [Newcomer and Gilbert 2010].

Leukotriene B₄, a potent autocrine chemoattractant to these cells, is released at the moving site of neutrophils where it acts on specific receptors and causes an actin contraction in the adjacent cell compartment [McDonald *et al.* 1994].

The heme protein myeloperoxidase (MPO) is highly specific to neutrophils. It has also been found to a lesser extent in monocytes. Myeloperoxidase is present in the azurophilic granules that will fuse with phagocytosed bacteria. Constituents of azurophilic granules contribute to the inactivation and killing of microorganisms [Klebanoff *et al.* 2013]. Myeloperoxidase is known to be activated by hydrogen peroxide producing an arsenal of active oxidants including hypochlorous acid (HOCl) and hypobromous acid (HOBr) [Arnhold and Flemmig 2010]. Under physiological conditions especially HOCl is generated in neutrophils. Typical reaction partners of HOCl are thiols, thioethers, and amino groups [Hawkins *et al.* 2003]. Therefore, HOCl is principally able to oxidize amino acid residues of proteins, such as cysteine, methionine, tyrosine, tryptophan, and lysine residues [Pattison and Davies 2001]. Furthermore, various studies showed chemical modifications of certain proteins [Bouriche *et al.* 2007; Malle *et al.* 2006; Salavej *et al.* 2006].

Both enzymes, 5-LOX and MPO, get sequentially activated during migration of neutrophils to inflammatory loci. An increased Ca²⁺ level during neutrophil activation leads to translocation of cytosolic 5-LOX to the nuclear membrane and in consequence to the synthesis of LTB₄, promoting chemotaxis of these cells [Radmark *et al.* 2015]. At the final destination of neutrophils, HOCl production via MPO is activated through stronger stimulation. Here, the question arises whether HOCl, a potent amino acid-oxidizing agent, could affect the activity or the product profile of 5-LOX. Previously, effects of a possible interaction of HOCl with AA-derived hydroperoxides were investigated [Zschaler and Arnhold 2014]. Here, the hydroperoxide moiety was not affected by HOCl, and chlorohydrins were formed instead. However, in the presence of a potential reaction partner of HOCl, such as the thioether group of methionine, the formation of chlorohydrins was disturbed [Zschaler and Arnhold 2014]. Thus, 5-LOX could be a better target site of HOCl.

The aim of this study was to assess the effects of HOCl on the activity and product profile of 5-LOX. Analysis was performed by reverse-phase high-performance liquid chromatography (RP-HPLC) as well as by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Furthermore, hydrogen peroxide, also generated during neutrophil activation; hypobromous acid, the equivalent of HOCl in eosinophil granulocytes; and finally activated MPO were evaluated according to their impact on 5-LOX. We could demonstrate that HOCl, either present as a reagent or formed by the myeloperoxidase-hydrogen peroxide-chloride system, and HOBr considerably affect the product profile of 5-LOX, without, however, disturbing the lipoxygenase activity.

Material and methods

Material

Human 5-LOX was purchased from MyBioSource (supplier A, Catalog No. MBS537729, San Diego, CA, USA) or Cayman Chemical distributed by Biomol (supplier B, Catalog No. Cay60402, Hamburg, Germany). In both cases the recombinant enzyme was isolated from a baculovirus overexpression system in Sf21 cells and the S100 supernatant was used after centrifugation (100,000 g). A dialysis was performed against phosphate buffer (50 mM, pH 7.4) to replace the Tris buffer of the commercial 5-LOX solution using a Slide-A-Lyzer dialysis cassette (3500 Da molecular mass cut-off, Thermo Scientific).

The chemicals and further enzymes used were obtained from the following sources: human neutrophil myeloperoxidase from Planta (Vienna, Austria); glucose oxidase from bovine erythrocytes (G6137) from Sigma (Taufkirchen, Germany); HPLC standards 5-HpETE, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 5S,12R-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid (5,12-DiHETE), 12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HpETE), 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HpETE), 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HpODE), and AA were from Cayman Chemical (distributed by Biomol); HPLC solvents were from Carl Roth (Karlsruhe, Germany); and all other chemicals were from Sigma.

Working solutions of HOCl and H₂O₂ were prepared by dilution of the corresponding stock solutions. Their concentrations were determined spectrophotometrically using $\epsilon_{292} = 350 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 12 in 1 N sodium hydroxide solution for ⁻OCI [Morris 1966] and $\epsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$ for H₂O₂ [Beers and Sizer 1952]. The synthesis of HOBr was performed by the reaction of HOCl with bromide, whereby 40 mM NaBr was mixed with 20 mM HOCl in phosphate buffer (50 mM, pH 7.4). The concentration of ⁻OBr was measured spectrophotometrically using $\epsilon_{329} = 332 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 12 in 1 N sodium hydroxide solution [Kumar and Margerum 1987]. The solutions were essentially stable for 1 h at 4 °C and were used within this time.

Incubation of human 5-LOX with various oxidants

Dialyzed recombinant human 5-LOX (6 U) was incubated with various oxidants (HOCl, HOBr, or H₂O₂) at 20 °C in phosphate buffer (50 mM, pH 7.4) for 5 min (V = 80 µl) under shaking conditions (40 rpm). Afterward, 5-LOX activity assay was performed by addition of final 100 µM AA, 5 µM 13-HpODE, 100 µM EDTA, 300 µM Ca²⁺ and 200 µM ATP and incubation for further 10 min (V_{final} = 100 µl). The reaction was terminated by the addition of 200 µl ice-cold acetonitrile and 3 µl acetic acid (100%) and the precipitate was separated by centrifugation (10 min, 4 °C, 10,000 g). The supernatants were stored at -80 °C and analyzed by RP-HPLC within 24 h.

Incubation of human 5-LOX with the MPO-H₂O₂-Cl⁻ system

Dialyzed recombinant human 5-LOX (6 U) was incubated with 200 nM MPO, 140 mM NaCl, 400 mU glucose oxidase, and 30-140 µM glucose at 20 °C in phosphate buffer (50 mM, pH 7.4) for 15 min (V = 80 µl) under shaking conditions (40 rpm). Glucose oxidase and glucose were added to ensure a steady supply of low amounts of H₂O₂ and consequently activate MPO to produce HOCl. The synthesized HOCl was measured using a 3,3',5,5'-tetramethylbenzidine (TMB)-based assay [Dyppbukt *et al.* 2005]. Afterward, the 5-LOX

activity assay was performed (see *Incubation of human 5-LOX with various oxidants*). Finally, samples were stored at -80 °C and analyzed by RP-HPLC within 24 h.

Analysis of 5-LOX products by RP-HPLC

The samples were analyzed by RP-HPLC using a C₁₈ column (Supelcosil LC-18-DB, 25 cm × 4.6 mm i.d., 5 µm), with an isocratic eluent consisting of acetonitrile/H₂O/acetic acid (60/40/0.2, v/v/v) with a flow rate of 1 ml/min. The eluate was monitored at 234 nm (quantification of conjugated dienes) and 270 nm (quantification of conjugated trienes). The HPLC consisted of a Shimadzu liquid chromatographic system equipped with a Shimadzu LC-10ATvp isocratic solvent delivery system, Shimadzu SPD-10Avp dual-wavelength absorbance detector, Shimadzu CTO-10ASvp column oven (35 °C), and Rheodyne injector with 20 µl loop volume. The concentrations of the LOX products (5-H(p)ETE, 6-*trans*-LTB₄, 12-HpETE) were quantified using a calibration curve (13-point calibration) of the appropriate synthetic standards. In the case of 8-HpETE no synthetic standard was available, therefore the 12-HpETE calibration curve was used instead. The identification of 8-HpETE was performed by MS/MS analysis after RP-HPLC separation. Therefore, a mixture of hydroperoxides of AA was synthesized using controlled α-tocopherol-inhibited autooxidation of AA [Peers and Coxon 1983]. Afterward, this mixture was separated by RP-HPLC as described above. Thereby 5-HETE showed a shoulder at a lower retention time. This peak was collected and analyzed by mass spectrometry. According to its fragmentation pattern this peak could be attributed to 8-HpETE [MacMillan and Murphy 1995].

Analysis of HOCl-modified 5-LOX products by LC-ESI-MS/MS

Human 5-LOX (6 U) was incubated with HOCl as described under *Incubation of human 5-LOX with various oxidants*. The reaction was terminated by dilution (1:500 and 1:5000) in phosphate buffer (50 mM, pH 7.4) and addition of 50 µl internal standard solution and 400 µl precipitation reagent containing methanol:zinc sulfate 4:1 (v/v) to 200 µl of the corresponding sample. After 2 min vortexing the samples were centrifuged for 5 min at 10,000 g and transferred in HPLC vials for storage at -80 °C. LC-ESI-MS/MS was performed according to Kortz *et al.* [Kortz *et al.* 2013]. In brief, 200 µl of the sample solution was injected onto a Strata-X extraction column (20 × 2 mm i.d., 25 µm, Phenomenex, Aschaffenburg, Germany) for online solid-phase extraction and chromatographically separated on a Kinetex C18 column (100 × 2.1 mm i.d., 2.6 µm, Phenomenex). A 5500 QTrap mass spectrometer (AB Sciex, Darmstadt, Germany) in negative ionization mode was applied for MS/MS analysis with scheduled multiple-reaction monitoring (MRM) experiments. For detection of the 5-LOX metabolite 6-*trans*-LTB₄ at the retention time of 4.3 min the MRM *m/z* 335.2/194.9 was included in the experiment. Quantification of 6-*trans*-LTB₄ was performed using the LTB₄ calibration curve (internal standard: LTB₄-d₄).

Results

HOCl affects the product profile of human 5-LOX

Arachidonic acid was converted by human 5-LOX into two major products, 5-HpETE and LTA₄. 5-HpETE resulted from the dioxygen insertion at the C5 position of AA and can be reduced spontaneously or *in vitro* by a peroxidase-catalyzed reduction into 5-HETE due to the instability of the hydroperoxide group. Leukotriene A₄ was rapidly nonenzymatically hydrolyzed into 6-*trans*-leukotriene B₄ (6-*trans*-LTB₄). Accordingly, chromatographic analysis of AA oxidation by 5-LOX revealed 5-HpETE (*c* = 28.7 μ M), 5-HETE (*c* = 6.1 μ M), and 6-*trans*-LTB₄ (*c* = 20.1 μ M) (Figure 1 A and B, black line) as main products.

Afterward, the influence of HOCl on the enzyme activity and product profile of 5-LOX was assessed. Thus, 5-LOX was incubated with increasing amounts of HOCl before the substrate mixture was added (Figure 1).

Figure 1: Recombinant human 5-LOX (6 U, 46.5 U/mg, supplier A) was incubated with HOCl at 20 °C in phosphate buffer (50 mM, pH 7.4) for 5 min. Afterward, 100 μ M AA, 5 μ M 13-HpODE, 100 μ M EDTA, 300 μ M Ca²⁺, and 200 μ M ATP were added and incubated for a further 10 min. The reaction was terminated by protein precipitation and the samples were analyzed by RP-HPLC at (A) 234 nm and (B) 270 nm. The concentrations of (C) 5-HpETE, 5-HETE, and 6-*trans*-LTB₄ and 8-HpETE, 12-HpETE, and 15-HpETE were calculated using a calibration curve of synthetic standards. pos/neg, positive/negative; two-tailed *t* test against positive control, **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001; *n* = 3 or 4.

A significant increase in the 5-HpETE peak was observed at HOCl concentrations up to 200 μ M (Figure 1 A and C). Only at HOCl concentrations of 400 μ M or higher, the yield of 5-HpETE was lower than in the absence of HOCl. Interestingly, the peaks of 6-*trans*-LTB₄ and 5-HETE decreased over the whole HOCl concentration range (Figure 1 A, B, and C). Surprisingly, two further AA-derived products were detected after incubation of 5-LOX with HOCl, resulting from the oxygenation at C12 and C8 of AA (Figure 1 A, inset; Figure 1 C).

However, the maximal concentrations of 12-HpETE ($c_{\max} = 1.3 \mu\text{M}$) and 8-HpETE ($c_{\max} = 2.3 \mu\text{M}$) were rather low.

The AA solution was also measured as a negative control without the addition of 5-LOX (Figure 1 C, white bar). Here, low intense peaks for 15-HpETE and 5-HpETE were detected owing to nonspecific oxidation of the AA stock solution. Further nonspecific oxidation products such as 12/8-HpETE were not detected. A peroxide-free AA could not be used because of antioxidative additives interfering with HOCl. However, in the untreated 5-LOX samples 15-HpETE was not detected as a product (Figure 1 C, black bar). Possibly, low amounts of AA hydroperoxides were consumed during 5-LOX activation in addition to the applied 13-HpODE. After HOCl addition, low intense peaks were detected representing 15-HpETE ($c_{\max} = 0.7 \mu\text{M}$). It could not be evaluated whether this 15-HpETE was produced by 5-LOX or derived from the AA stock solution.

The HOCl-induced changes in 5-LOX products revealed drastic differences between 5-HpETE on one hand and 5-HETE and 6-*trans*-LTB₄ on the other (Figure 2 A). After application of 150 μM HOCl a 1.76-fold increase in the 5-HpETE concentration took place, whereas a 1.27- and 1.86-fold decrease occurred for 5-HETE and 6-*trans*-LTB₄, respectively. Apparently, low amounts of HOCl already blocked further conversions of 5-HpETE and caused, thus, a rise in 5-HpETE production.

Figure 2: HOCl-induced alterations in the product profile of human 5-LOX as described for Figure 1. (A) The HOCl-induced changes in the main products are related in percentage to the applied HOCl concentrations. Two-tailed *t* test against 5-HpETE, * $p \leq 0.05$, ** $p \leq 0.01$. (B) The ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was related to the applied HOCl concentration. $n = 3$ or 4.

Taking into account that 5-H(p)ETE derived from the first oxygenation of AA by 5-LOX, and 6-*trans*-LTB₄ from the subsequent oxygenation, the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ indicates the prevalence of the respective step (Figure 2 B). There was a linear increase in the 5-H(p)ETE to 6-*trans*-LTB₄ ratio from 1.8:1 to 15.6:1 when 5-LOX was incubated with increasing HOCl concentrations. Hence, especially 5-HpETE was the dominating product of HOCl-modified 5-LOX, whereas the further conversion of 5-HpETE to LTA₄ was strongly impaired.

Products of HOCl-modified 5-LOX analyzed by LC-ESI-MS/MS

The product profile of 5-LOX after treatment with HOCl was further analyzed by LC-ESI-MS/MS. This method enables a fast quantification of a broad spectrum of oxidized metabolites. In addition to the main products 5-H(p)ETE and 6-*trans*-LTB₄, other further AA-derived oxygenation products were detected, namely 5,6-DiHETE, 8-HETE, 12-HpETE, and 5-oxo-ETE (Figure 3 A). However, their concentrations were rather low. 12-HpETE and 8-HpETE were also measured using RP-HPLC (see *HOCl affects the product profile of human 5-LOX*). For 8-HpETE no commercial standard was available, therefore it could not be measured with the applied LC-ESI-MS/MS. Nevertheless, the reduction product 8-HETE indicates the presence of 8-HpETE. Both 5,6-DiHETE and 5-oxo-ETE are further nonenzymatic hydrolysis products of LTA₄ even though they emerged in lower amounts than the 5,12-DiHETE form (6-*trans*-LTB₄) [Borgeat and Samuelsson 1979; Gravel *et al.* 1993]. Both products were also detected using RP-HPLC; however, their concentrations were negligible compared to 6-*trans*-LTB₄.

The most significant HOCl-induced changes in the 5-LOX products arose for 6-*trans*-LTB₄, 5-HETE, and 5,6-DiHETE (Figure 3 A), where a strong decrease occurred. Also a decline was obvious for 5-oxo-ETE, albeit not to the same extent. These alterations are comparable to the previous experiment, also with regard to the further LTA₄ hydrolysis products, 5,6-DiHETE and 5-oxo-ETE.

Figure 3: The incubation of 5-LOX (6 U, 162.9 U/mg, supplier A) with HOCl and the following activity assay were performed as described for Figure 1. The samples were analyzed by LC-ESI-MS/MS. (A) Concentrations of the main products were calculated using a calibration curve of synthetic standards. (B) The ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was related to the applied HOCl concentration. n = 3 or 4.

The HOCl-induced effects on 5-HpETE correspond only partly to the results of the RP-HPLC analysis. The amount of 5-HpETE remained relatively stable after treatment of 5-LOX with HOCl. A comparable increase in the concentration at low HOCl did not occur. Also 12-HpETE showed only a modest HOCl-induced increase. However, here 12-HpETE belonged also to the untreated 5-LOX product profile. In the previous results 5-LOX produced 12-HpETE only after HOCl incubation (see *HOCl affects the product profile of human 5-LOX*). This slightly changed product profile can be explained with another enzyme charge used. Interestingly, for 8-HETE an initial rise in the concentration was detected, decreasing with higher HOCl amounts.

It has to be mentioned that there was a strong quantitative difference. Using the RP-HPLC a six times higher concentration of 5-HpETE was measured compared to the LC-ESI-MS/MS procedure. Also for 5-HETE and 6-*trans*-LTB₄ lower amounts were detected with LC-ESI-MS/MS. Apparently, the different precipitation procedure, necessary for LC-MS/MS measurement, affected the yield of 5-HpETE and diminished the effects of the HOCl-induced increase. Nevertheless, when 5-LOX was incubated with HOCl in a concentration range from 10 to 250 μ M HOCl the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ showed a comparable strong increase, from 1.9:1 to 15.7:1 (Figure 3 B). In the previous experiments, HOCl was tested in a concentration range between 50 and 500 μ M leading to an increase in the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ from 1.8:1 to 15.6:1 (Figure 1). These differences in HOCl concentration can be attributed to the different enzyme charges used, affecting the required HOCl amount for a comparable effect.

Inactivation of human 5-LOX by H₂O₂

The product profile of HOCl-treated 5-LOX was extensively changed, especially concerning the ratio of 5-HpETE to LTA₄. However, it is not clear if a slight inactivation of the enzyme happens after HOCl incubation. Therefore, the effect of HOCl on the product profile of 5-LOX was compared to H₂O₂ as a further reactive oxygen species. For this purpose, 5-LOX was incubated with H₂O₂ under conditions comparable to those of HOCl. Afterward, the substrate mixture was added and the product profile of 5-LOX was assessed by RP-HPLC (Figure 4).

Figure 4: Recombinant 5-LOX (6 U, 34.4 U/mg, supplier B) was incubated with H₂O₂ at 20 °C in phosphate buffer (50 mM, pH 7.4) for 5 min. Afterward, an activity assay of 5-LOX and chromatographic quantification of the product profile was performed and (A) H₂O₂-induced changes in percentage of the product profile of human 5-LOX were calculated. (B) The ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was related to the applied H₂O₂ concentration. n = 3 or 4.

With increasing amounts of H₂O₂ both 5-HpETE and 5-HETE, as well as 6-*trans*-LTB₄, displayed a substantial decrease especially in the range from 5 μ M to 20 μ M H₂O₂ (Figure 4 A). For 6-*trans*-LTB₄ a considerable decline of 77% was observed but also concentrations of 5-HETE and 5-HpETE were markedly reduced by 39 and 49%, respectively. Higher H₂O₂ concentrations up to 80 μ M resulted in only slightly further changes. Interestingly, 12-HpETE and 8-HpETE, detected as clear signals with the enzyme preparation used at 3.2 and 2.9 μ M, respectively, completely diminished up to 20 μ M H₂O₂. This highlights

clearly the different actions of HOCl and H₂O₂ in relation to 5-LOX, where both these oxidation products increased in their concentration after HOCl treatment. The H₂O₂-induced changes in the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ were also determined (Figure 4 B). It became evident that also for H₂O₂-treated 5-LOX this ratio increased from 5.5:1 to 17.4:1. This could be explained by the faster decrease in 6-*trans*-LTB₄ compared to 5-H(p)ETE. However, for HOCl a linear correlation between HOCl and 5-H(p)ETE to 6-*trans*-LTB₄ occurred, whereas H₂O₂ induced an exponential rise. This was apparently caused by the stronger effect in the range between 5 and 20 µM H₂O₂.

HOBr affects the product profile of human 5-LOX

A further prominent hypohalous acid produced from peroxidases is hypobromous acid. HOBr was also assessed according to its effects on the product profile of 5-LOX. Here, HOBr was incubated in the range from 10 to 250 µM with 5-LOX before the enzyme activity was determined (Figure 5).

Figure 5: Recombinant 5-LOX (6 U, 34.4 U/mg, supplier B) was incubated with HOBr at 20 °C in phosphate buffer (50 mM, pH 7.4) for 5 min. Afterward, an activity assay of 5-LOX and chromatographic quantification of the product profile were performed and HOBr-induced changes in percentage of the product profile of human 5-LOX were calculated for (A) 5-H(p)ETE and 6-*trans*-LTB₄ and (B) 8-HpETE and 12-HpETE. (C) The ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was related to the applied HOBr concentration. Two-tailed *t* test against control, **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001; *n* = 4.

The lowest HOBr concentration induced an increase in all metabolites. However, higher amounts of HOBr led to a significant reduction in 6-*trans*-LTB₄ and 5-HETE, whereas for 5-HpETE a significantly higher concentration was still present (Figure 5 A). For example, 125 µM HOBr induced a 1.17-fold increase in 5-HpETE and a decrease of 49 and 62% for 5-HETE and 6-*trans*-LTB₄, respectively. Interestingly, a distinctive HOBr-induced augmentation was observed for 8-HpETE and 12-HpETE. Here, 125 µM HOBr caused a 1.67- and 2.16-fold increase in 8-HpETE and 12-HpETE, respectively (Figure 5 B). Furthermore, the ratio of

5-H(p)ETE to 6-*trans*-LTB₄ was determined (Figure 5 C) showing a linear dependence on the applied HOBr concentration. Unmodified 5-LOX had a ratio of 5-H(p)ETE to 6-*trans*-LTB₄ of 4.4:1, growing up to 10.1:1 after addition of 250 μ M HOBr. This was comparable to the HOCl-induced 5-LOX effects. However, for unmodified 5-LOX (see *HOCl affects the product profile of human 5-LOX*) a lower ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was determined, which can be attributed to the different enzyme charges used.

Interaction of activated MPO and human 5-LOX

The heme-containing protein MPO produces HOCl in neutrophil granulocytes after activation by hydrogen peroxide. The MPO-H₂O₂-Cl⁻ system was now applied to modify the product profile of human 5-LOX. As demonstrated above H₂O₂ easily inactivates 5-LOX (see *Inactivation of human 5-LOX by H₂O₂*). Therefore, a simple addition of H₂O₂ was not possible for MPO activation in the presence of 5-LOX. The glucose oxidase/glucose (GOD/Glu) system was used instead, to ensure a steady supply of low amounts of H₂O₂. Different glucose concentrations were added to glucose oxidase to achieve different degrees of MPO activation and subsequently variable HOCl concentrations. The efficiency of the MPO-GOD/Glu-Cl⁻ system was tested by HOCl quantification using a TMB-based assay. Thereby, half of the applied glucose was converted into HOCl (data not shown). Afterward, 5-LOX was incubated with MPO and the GOD/Glu system (Figure 6). As controls Cl⁻ was omitted as MPO substrate or the GOD/Glu system alone was added to 5-LOX. Here, a strong decrease in the concentrations of all metabolites was observed. For example, addition of 100 μ M glucose to the 5-LOX/MPO/GOD samples devoid of Cl⁻ resulted in a 3.3- and 10.9-fold decrease for 5-HpETE and 6-*trans*-LTB₄, respectively. This was comparable to the H₂O₂ effect seen earlier (*Inactivation of human 5-LOX by H₂O₂*). Also 12-HpETE and 8-HpETE produced from unaffected 5-LOX completely disappeared in these controls. The addition of Cl⁻ to 5-LOX, MPO, and the GOD/Glu system also resulted in a reduction in 5-HETE and 6-*trans*-LTB₄. Also the amount of 5-HpETE was attenuated; however, only to a minor degree compared to 6-*trans*-LTB₄. Here, 100 μ M glucose led to a 1.2- and 3.1-fold decrease for 5-HpETE and 6-*trans*-LTB₄, respectively. The small changes in the 5-HpETE concentration might be due to the H₂O₂-driven impairment of 5-LOX activity. This could superimpose the HOCl-induced 5-HpETE increase. Interestingly, the amount of 12-HpETE and 8-HpETE was unaffected in the 5-LOX, MPO, Cl⁻, and GOD/Glu samples, clearly distinguishing them from the negative controls in which 5-LOX was influenced by the H₂O₂-generating system.

Figure 6: Recombinant human 5-LOX (6 U, 162.9 U/mg, supplier A) was incubated with 200 nM MPO, 400 mU glucose oxidase, and various amounts of glucose at 20 °C in phosphate buffer (50 mM, pH 7.4) for 15 min ($V = 80 \mu\text{l}$). Afterward an activity assay of 5-LOX and chromatographic quantification of the product profile were performed. The concentrations of (A) 5-HpETE, 5-HETE, and 6-*trans*-LTB₄ and (B) 8-HpETE, 12-HpETE, and 15-HpETE were calculated using a calibration curve of synthetic standards. (C) HOCl-induced changes in percentage of the product profile of human 5-LOX after incubation with activated MPO (MPO + Cl⁻ + GOD) and further negative controls with MPO + GOD in the absence of Cl⁻ and sole addition of the GOD/Glu system to 5-LOX. pos/neg, positive/negative; GOD, glucose oxidase; Glu, glucose, $n = 3$.

As shown in the previous experiments the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ was determined (Figure 7). An exponential increase in this ratio occurred for both 5-LOX/MPO/GOD samples devoid of Cl⁻ (from 3.7:1 to 14.0:1) and 5-LOX/GOD samples (from 3.7:1 to 22.7:1) depending on the added glucose concentration, which is comparable to the inactivation of 5-LOX with H₂O₂ (see *Inactivation of human 5-LOX by H₂O₂*). The addition of Cl⁻ to 5-LOX, MPO, and the GOD/Glu system led in turn to a slower rise in the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ (from 3.7:1 to 14.0:1).

Figure 7: Recombinant human 5-LOX (6 U) was incubated with MPO, glucose oxidase, and various amounts of glucose (see Figure 6). Afterward, an activity assay of 5-LOX and chromatographic quantification of the product profile were performed. The ratio of 5-H(p)ETE to 6-trans-LTB₄ was related to the applied glucose concentration. GOD, glucose oxidase; Glu, glucose.

Discussion

5-Lipoxygenase is known to catalyze two successive reactions in the formation of potent bioactive metabolites derived from AA. First, this enzyme oxidizes AA into 5-HpETE and converts in a second step this hydroperoxide into LTA₄. Whereas the formation of 5-HpETE and 5-HETE, the reduced form of 5-HpETE, could be directly verified with our methods, the formation of the unstable LTA₄ was evaluated assessing its nonenzymatic hydrolysis product 6-*trans*-LTB₄.

The incubation of 5-LOX with the MPO-derived oxidants HOCl and HOBr significantly changed the product profile of 5-LOX in a concentration-dependent manner. There was a linear increase in the ratio of 5-H(p)ETE to 6-*trans*-LTB₄ with increasing concentrations of HOCl and HOBr. At low oxidant concentrations, there was a strong decrease in the yield of 6-*trans*-LTB₄, whereas 5-HpETE did not change or increased. 5-HpETE was decreased only at higher concentrations for HOCl and HOBr. Incubation of 5-LOX with H₂O₂ diminished all peaks of interest, with the highest impact on 6-*trans*-LTB₄. Therefore, also for the 5-H(p)ETE to 6-*trans*-LTB₄ ratio an increase was obvious with increasing H₂O₂ concentration, but not in a linear manner. Thus, whereas small MPO-related oxidants mostly disturb the conversion of 5-HpETE into LTA₄ by 5-LOX, H₂O₂ impaired the whole 5-LOX activity. A reaction of HOCl and HOBr with the AA-derived products itself can be ruled out. Previous studies showed that the double bonds are a target site for HOCl, but only in the absence of further reaction partners such as the thioether group of methionine [Zschaler and Arnhold 2014].

We applied two 5-LOX preparations from different suppliers. These preparations differed in their 5-H(p)ETE to 6-*trans*-LTB₄ ratio of the unperturbed sample. The initial ratios were 1.8 to 3.7 and 4.4 to 5.5 for supplier A and B, respectively (see Results). We can only speculate about the reasons for these differences. It is clear that the addition of HOCl or H₂O₂ to 5-LOX leads to an increased 5-H(p)ETE to 6-*trans*-LTB₄ ratio. Whether the increased ratio of the untreated 5-LOX is related to a preoxidized enzyme preparation could not be examined.

The 5-H(p)ETE to 6-*trans*-LTB₄ ratio increased also after incubation of 5-LOX with the MPO-H₂O₂-Cl⁻ system. Owing to the high impact of H₂O₂ on 5-LOX, glucose oxidase/glucose was applied as a source of H₂O₂. This reduced considerably the actual concentrations of hydrogen peroxide over a bolus addition. According to the results for H₂O₂, the product ratio of 5-H(p)ETE and 6-*trans*-LTB₄ increased also in a nonlinear manner using glucose oxidase/glucose alone or with MPO in the absence of chloride. However, the complete MPO system had a much lower impact on the product profile of 5-LOX.

A second observation was the increased formation of 8-HpETE and 12-HpETE in 5-LOX samples incubated with HOCl, HOBr, and the MPO-H₂O₂-Cl⁻ system, which could not be detected after incubation of 5-LOX with H₂O₂. Instead, H₂O₂ or the GOD/Glu system disturbed the primarily produced 8-HpETE and 12-HpETE formation. In addition to 5-HpETE these hydroperoxides are well known to be produced by 5-LOX with a lower yield [Walther *et al.* 2009], which could be verified by our results. We further observed an increased formation of these products with increasing HOCl and HOBr concentrations.

In two different studies human 5-LOX was converted into a 15-lipoxygenating enzyme by mutational introduction of a space-filling amino acid, reducing the volume of the substrate cavity, or by mimicking the Ser663 phosphorylation site [Gilbert *et al.* 2012; Schwarz *et al.*

2001]. We measured a slightly increasing peak for 15-HpETE in HOCl-influenced 5-LOX samples compared to the untreated sample. Even though 15-HpETE was already obvious in the AA stock solution, apparently owing to autoxidation, in untreated 5-LOX samples no 15-HpETE was present. Probably, 15-HpETE reacts during the enzymatic activity assay with ferrous 5-LOX during lipoxygenase activation, comparable to 13-HpODE [Riendeau *et al.* 1989; Schilstra *et al.* 1992]. Now, the slightly increased HOCl-dependent 15-HpETE peak could be due to an effect of HOCl on the general 5-LOX activity, leading to lower enzyme activation and thus a reduced consumption of 15-HpETE introduced via the AA stock solution. However, low concentrations of HOCl did not disturb the 5-HpETE-producing activity of 5-LOX, assuming that 5-LOX activation is comparable. Therefore, it is more possible that in addition to the changed oxidation specificity, leading to 12-HpETE and 8-HpETE, 15-HpETE is also produced by 5-LOX.

The influence of HOCl on the product profile of 5-LOX was also analyzed with LC-ESI-MS/MS, a sophisticated technique to measure a high number of oxidized metabolites in a relatively short time. In principal, a comparable HOCl effect was detected, leading to a linear increase in the 5-H(p)ETE to 6-*trans*-LTB₄ ratio. However, the quantitative results could not be compared. Here, for 5-HpETE and 6-*trans*-LTB₄, a 6.1- and 3.8-fold lower concentration was measured with LC-ESI-MS/MS compared to RP-HPLC, respectively, whereas for 5-HETE only a 1.2-fold lower concentration was obvious. This could be reasoned by the application of different precipitation procedures, the lack of the appropriate internal standard for 5-HpETE quantification, or the instability of 5-HpETE during electrospray ionization diminishing the effects of the HOCl-induced increase in 5-HpETE.

In addition to 6-*trans*-LTB₄ and 5,6-DiHETE as nonenzymatic hydrolysis products of LTA₄ [Gravel *et al.* 1993], 5-oxo-ETE was also measured in the LOX samples using the LC-ESI-MS/MS technique. 5-Oxo-ETE could be nonspecifically formed from the decomposition of 5-HpETE during the preparation or after injection onto the column, resulting in 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid. This phenomenon was reported for the injection of 12-HpETE or 15-HpETE onto an HPLC column without prior reduction [Fruteau de Laclos and Borgeat 1988]. However, the measured product might also be 5-oxo-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid, a further nonenzymatic hydrolysis product of LTA₄ [Gravel *et al.* 1993], differing only in the position of the double bonds. Gravel *et al.* described an MS/MS fragment with *m/z* 203 Da for 5-oxo-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid [Gravel *et al.* 1993], which is also used for the applied LC-ESI-MS/MS analysis of 5-oxo-ETE. Thus, the triene structure (5-oxo-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid) is thought to have the same retention time as the stereoisomer 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid in the LC-ESI-MS/MS analysis.

5-LOX possesses two functions during leukotriene biosynthesis, hydroperoxidation and epoxidation, certainly differing in their catalytic mechanism [Rouzer *et al.* 1986]. The hydroperoxidation is initiated by *pro*-S hydrogen abstraction at C7 of AA, whereas epoxidation proceeds after abstraction of the *pro*-R hydrogen at C10, suggesting a different position of the substrate relative to the ferric active center [Haeggstrom and Funk 2011]. Furthermore, the resulting radical reacts only in the hydroperoxidation step with molecular dioxygen to a peroxy radical species, which is subsequently reduced by ferrous iron. In contrast, during epoxidation the hydroperoxide group is homolytically cleaved by ferrous iron, yielding a hydroxide radical for epoxidation. Recently detailed kinetic investigations of

these two processes were performed, indicating that both the rate of substrate capture and the rate of product release are less efficient for epoxidation than for hydroperoxidation [Smyrniotis *et al.* 2014]. Furthermore, it was assumed that for 5-LOX it is more efficient to retain the 5-HpETE in its active site for sequential epoxidation, rather than binding free 5-HpETE [Smyrniotis *et al.* 2014]. Therefore, the impact of the MPO-derived oxidants on the product profile of 5-LOX could be related to the oxidation of critical amino acid residues in the substrate binding cavity disturbing mostly the less efficient epoxidation of 5-HpETE, whereas the hydroperoxidation of AA is unaffected. Further, 5-HpETE is more compact and more hydrophilic than AA. Thus, slight changes in the conformation of the substrate cavity due to amino acid oxidation could result in an impaired radical formation at C10 or the disturbance of a further step during epoxidation.

In addition, the crystal structure of human 5-LOX revealed a unique Phe, Tyr-cork feature blocking the possible entrance to the active site [Gilbert *et al.* 2011]. Modification of these two amino acids could result in a changed entrance mechanism, affecting the uptake of AA and 5-HpETE in a different way. However, at the moment, we can only speculate as to which fine mechanisms take place. To verify these differences, it is highly necessary to combine our measurements with proteomic analysis for any oxidant-mediated modifications in the protein structure of 5-LOX.

Hydrogen peroxide affected both reactions catalyzed by 5-LOX, hydroperoxidation and epoxidation, with a stronger impact on the formation of LTA₄. As explained above, this might be related to the less efficient epoxidation reaction. However, H₂O₂ clearly diminished the whole 5-LOX activity, assuming a direct impact on the active center of the 5-LOX. The inactivation mechanism could involve a reaction between H₂O₂ and ferrous iron [Percival *et al.* 1992]. Furthermore, the iron ligand histidines are potential target sites for metal-catalyzed oxidation reactions [Dean *et al.* 1989].

Changes in the product profile of 5-LOX were investigated in *in vitro* enzyme preparations. It is highly necessary to extend these measurements to neutrophil granulocytes containing both 5-LOX and MPO. Alternatively, eosinophils are also an interesting target as they contain two lipoxygenases, namely 5-LOX and 15-LOX, and eosinophil peroxidase producing HOBr. The activation of 5-LOX in granulocytes is an early event in inflammation and immune response. In neutrophils, LTB₄ is an important chemotactic agent, necessary for the directed movement of these cells to inflammatory loci. Enhanced oxidant formation by MPO is mostly related to phagocytosis of microorganisms and their digestion in phagosomes. Apparently, MPO-derived oxidants play a certain role in terminating signals for the formation of LTB₄.

Declaration of Interest

The authors have no conflicts of interest to declare. The authors alone are responsible for the content and writing of the paper.

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