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### The hydroperoxide moiety of aliphatic lipid hydroperoxides is not affected by hypochlorous acid



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### ABSTRACT

The oxidation of polyunsaturated fatty acids to the corresponding hydroperoxide by plant and animal lipoxygenases is an important step for the generation of bioactive lipid mediators. Thereby fatty acid hydroperoxide represent a common intermediate, also in human innate immune cells, like neutrophil granulocytes. In these cells a further key component is the heme protein myeloperoxidase producing HOCl as a reactive oxidant. On the basis of different investigation a reaction of the fatty acid hydroperoxide and hypochlorous acid (HOCl) could be assumed. Here, chromatographic and spectrometric analysis revealed that the hydroperoxide group due to a reaction acid (13-HpODE) is not affected by HOCl. No reduction of the hydroperoxide group due to a reaction with HOCl could be measured. It could be demonstrated that the double bonds of the fatty acid hydroperoxides are the major target of HOCl, present either as reagent or formed by the myeloperoxidase-hydrogen peroxide-chloride system.

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#### 1. Introduction

Animal and plant lipoxygenases are able to oxidize unsaturated fatty acids to aliphatic hydroperoxides, which can be further metabolized to an array of biologically active substances such as leukotrienes, lipoxins, and oxo-modified fatty acids (Stables and Gilroy, 2011). These fatty acid hydroperoxides can also undergo non-enzymatic homolytic decomposition into toxic electrophilic components in the presence of transition metal ions such as Fe<sup>2+</sup> and Cu<sup>+</sup> or ascorbic acid (Jian et al., 2009; Lee and Blair, 2000).

Hypochlorous acid (HOCl) is a reactive product of the heme protein myeloperoxidase found in neutrophilic granulocytes and to a lesser extent in monocytes (Arnhold and Flemmig, 2010). This species is involved in pathogen defense and regulation of immune reactions. HOCl is well known to react with hydrogen peroxide under formation of singlet oxygen as evidenced by infrared chemiluminescence at 1268 nm (monomol emission of singlet oxygen) or emission in the red spectral region (dimol emission of singlet oxygen) (Held et al., 1978; Kanofsky, 1989; Khan and Kasha,

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1970). In case of the reaction of linoleic acid hydroperoxide with HOCl, singlet oxygen was also generated, however, to a much lower yield as with hydrogen peroxide (Miyamoto et al., 2006). In contrast, no chemiluminescence of singlet oxygen was observed in the reaction of tert-butyl hydroperoxide with HOCl despite the detection of different reaction products (Arnhold et al., 1996; Miyamoto et al., 2006; Panasenko et al., 1997). Other indications for a possible involvement of lipid hydroperoxides in HOCl-driven reactions came from experiments where effects of previously accumulated lipid peroxidation products on HOCl-induced oxidation of unsaturated phospholipids were examined (Panasenko and Arnhold, 1999; Panasenko et al., 1997). In these investigations, a higher yield of peroxidation products was measured with increasing concentrations of hydroperoxides. In all experiments with lipid hydroperoxides as well as *tert*-butyl hydroperoxide the question raises, was really the hydroperoxide moiety involved in the initiating reaction with HOCl or were the observed changes in the product spectrum caused by other reactive groups present in the lipid material.

The confirmation of a direct reaction between a fatty acid hydroperoxide group and HOCl would be important for physiological reactions in neutrophilic granulocytes as during stimulation of these cells both the 5-lipoxygenase, which oxidizes arachidonic acid to 5S-hydroperoxy-6E,;8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE), and myeloperoxidase will be activated and therefore,

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reaction products of both enzymes would be present close together. In order to evaluate a potential reaction between fatty acid hydroperoxides and HOCl, 15S-hydroperoxy-5Z,8Z,11Z,13Eeicosatetraenoic acid (15-HpETE), the 15-hydroperoxide of arachidonic acid, was generated from soybean lipoxygenase and analyzed by an reverse-phase high performance liquid chromatography (RP-HPLC) technique with UV detection as well as by electrospray ionization mass spectrometry. Additionally, linoleic acid was applied as an alternative substrate for this enzyme. The HPLC technique allows by using appropriate standards a clear assignment of the corresponding lipid hydroperoxide molecules and to follow its changes upon reaction with a partner of interest. We could demonstrate that HOCl, present either as reagent or formed by the myeloperoxidase-hydrogen peroxide-chloride system, reacts with double bonds of lipid molecules, but not with the hydroperoxide moiety.

### 2. Material and methods

#### 2.1. Material

The chemicals used were obtained from the following sources: HPLC-standards 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13 (S)-HpODE), 15S-hydro(pero) xy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15(S)-H(p)ETE), 9S-hydroperoxy-10E,12Z-octadecadienoic acid (9(S)-HpODE), 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-oxo-ETE) from Cayman Chemical (distributed by Biomol, Hamburg, Germany), HPLC solvents from Carl Roth (Karlsruhe, Germany), arachidonic acid, linoleic acid and all other chemicals from Sigma (Taufkirchen, Germany). The enzymes used were obtained from the following sources: human neutrophil myeloperoxidase (MPO) from Planta (Vienna, Austria), lipoxidase (soybean) Type I-B (sLOX) and glutathione peroxidase from Sigma (Taufkirchen, Germany).

### 2.2. Synthesis of 15-HpETE with sLOX, chromatographic analysis and reaction with HOCl

The enzymatic synthesis of 15-HpETE was performed with soybean lipoxygenase (sLOX) in different buffer systems at pH 7.4 or pH9 (50 mM phosphate buffer at pH 7.4, 50 mM borate buffer at pH 9). Thereby, 500 µM of arachidonic acid was incubated with 3695 U sLOX at room temperature (20 °C) in a volume of 1 ml for 5 min. The reaction was stopped by separation of the enzyme by centrifugation through a filter (30 kDa) at 4 °C. The enzymatic product was assigned as AA-OOH, hydroperoxides of arachidonic acid. In some experiments linoleic acid was used and the product was assigned as La-OOH, hydroperoxides of linoleic acid. The amount of conjugated dienes was determined by UV-spectroscopy using an extinction coefficient of  $\varepsilon_{236} = 27,000 \text{ M}^{-1} \text{ cm}^{-1}$ , as specified from the manufacturer as coefficient for the synthetic standards of 15-HpETE. The identity of the synthesis product was confirmed by RP-HPLC after dilution in HPLC solvent. The separation was performed by using a C18 column (Supelcosil LC-18-DB, L.  $\times$  I.D. 25 cm  $\times$  4.6 mm, 5  $\mu$ m) isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/min and the eluate was monitored at 234 nm and 280 nm. 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 amount of 15-HpETE was quantified by comparison of the peak area with that of known amounts of synthetic standards. An average 15-HpETE concentration of  $370.26 \pm 21.77 \,\mu\text{M}$  (*n*=6, yield of 74.05%) and  $210.01 \pm 28.29 \,\mu$ M (*n*=6, yield of 42.00%) was determined for the synthesis in borate buffer at pH 9 and at neutral pH, respectively. This is in accordance with the activity optimum of sLOX at basic pH values. As minor product 15-HETE was measured in low concentrations at basic pH ( $5.3 \pm 3.37 \mu$ M 15-HETE, pH 9, n = 6, yield of 3.37%) and at neutral pH ( $1.37 \pm 0.77 \mu$ M 15-HETE, pH 7.4, n = 6, yield of 0.27%). Afterwards the known amount of hydroperoxy fatty acids was diluted in the appropriate buffer and incubated with a certain amount of HOCl at room temperature ( $20 \,^{\circ}$ C) for 5 min. The OCl<sup>-</sup> concentration was determined spectrophotometrically using  $\varepsilon_{292} = 350 \,^{-1} \, \text{cm}^{-1}$  at pH 12 in 1 N sodium hydroxide solution (Morris, 1966).

### 2.3. Mass spectrometric analysis of the reaction products from 15-HpETE and HOCl

Fatty acid hydroperoxides diluted in acetonitrile or acetonitrile/ water/acetic acid (60/40/0.2, v/v/v) were assessed with an ion trap mass spectrometer (amaZon SL, Bruker Daltonics, Bremen, Germany) via an ESI spray source in negative ionization mode with a flow rate of 2  $\mu$ l/min. A mass-range from 100 to 800 *m/z* was detected with enhanced resolution. Drying gas temperature was set to 180 °C, drying gas flow rate to 4 l/min, nebulizer gas to 7.3 psi, ionization voltage to 4500 V and ion charge control (ICC) to 35,000 counts. For MS/MS analysis isolation width was set to 4.0 m/z, fragmentation cutoff to 105 *m/z* and fragmentation amplitude to 0.4 V.

LC-ESI-MS/MS was performed according to Kortz et al. (Kortz et al., 2013). In brief, 200 µl of the sample solution ( $c_{15-HpETE} = 0.033-0.33$  ng/ml) were injected on a Strata-X column (L. × I.D. 20 × 2 mm, 25 µm, Phenomenex, Aschaffenburg, Germany) for on-line solid phase extraction and chromatographically separated on a Kinetex C18 column (L. × I.D. 100 × 2.1 mm, 2.6 µm, Phenomenex, Aschaffenburg, Germany). A 5500 QTrap mass spectrometer (AB Sciex, Darmstadt) in negative ionization mode was applied for MS/MS analysis with multiple reaction monitoring experiments for 15-HpETE (m/z 387.28/113.1) and monochlorohydrins of 15-HpETE (m/z 387.28/113.1).

# 2.4. Reduction of HOCI-treated 15-HpETE with the glutathione peroxidase system

Equimolar concentrations of 15-HpETE ( $250 \mu$ M) and HOCl were incubated at room temperature ( $20 \circ$ C) for 5 min in 50 mM borate buffer, pH 9. Afterwards, reduced L-glutathione ( $500 \mu$ M or 1 mM) and 2 U glutathione peroxidase was added and further incubated for 15 min. To stop the reaction, the enzyme was separated by centrifugation through a filter (30 kDa). The filter was previously equilibrated with the buffer system. Glutathione was dissolved shortly before usage in 10 mM EDTA. Control experiments were also performed without addition of EDTA, however, no differences occurred.

# 2.5. Modification of 15-HpETE by the myeloperoxidase-hydrogen peroxide-chloride system

15-HpETE (100 μM) was incubated in the presence of the peroxidase system composed of 200 nM or 400 nM MPO, 100 μM  $H_2O_2$  and 140 mM Cl<sup>-</sup>. The reaction was performed in phosphate buffer (50 mM, pH 7.4) at room temperature (20 °C) some experiments were also done at 37 °C. Hydrogen peroxide was added in small amounts to prevent the inactivation of MPO. The concentration of  $H_2O_2$  was measured spectrophotometrically using  $\varepsilon_{240}$  = 43.6  $M^{-1}$  cm<sup>-1</sup> (Beers and Sizer, 1952). During the 10 min incubation of 15-HpETE with the peroxidase  $H_2O_2$  was added in 20 steps every 30 s. Afterwards the samples were incubated for further 5 min. To stop the reaction of the

myeloperoxidase the sample was diluted four-fold in HPLC solvent (acetonitrile/water/acetic acid (60/40/0.2, v/v/v)) and centrifuged through a filter (30 kDa). The filter was previously equilibrated with the HPLC solvent.

### 3. Results

## 3.1. Reaction of 15-HpETE or 13-HpODE with HOCl as assessed by RP-HPLC

The enzymatic synthesis of hydroperoxides from arachidonic acid (AA) and linoleic acid (LA) was performed with soybean lipoxygenase (sLOX). Thereby sLOX selectively produce 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HpETE) and 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13-HpODE), respectively (Brash et al., 1987; Hamberg and Samuelsson, 1967). The identity of the synthesis product was confirmed by reverse-phase high performance liquid chromatography (RP-HPLC). Under our conditions, intense product peaks at 234 nm were found at a retention time ( $t_r$ ) of 14.6 min for 15-HpETE and 14.0 min for 13-HpODE as revealed by the corresponding hydroperoxide standards. A less intense peak was detected at 13.5 min corresponding for 15-HETE. With the utilized chromatographic method the separation of

fatty acids with a hydroxyl or a hydroperoxy moiety was possible. This was important to assess the ability of the fatty acid hydroperoxide as reaction partner of HOCI.

Afterwards, the enzymatic product of 15-HpETE synthesis was incubated with different amounts of HOCl at pH 9 (50 mM borate buffer) for 5 min and was examined by analytic RP-HPLC with UVdetection at 234 nm (Fig. 1A). The 15-HpETE peak decreased with increasing HOCl concentration. Equimolar ratio of 15-HpETE and HOCl resulted in a strong decrease of 15-HpETE by 84% compared to the untreated 15-HpETE sample. Instead two new peaks emerged with lower  $t_r$  at 7.4 and 7.9 min. The proportion of the two peaks relative to 15-HpETE depended on the amount of HOCI. Further peaks were not obvious. Importantly, any changes in 15-HETE ( $t_r = 13.5 \text{ min}$ ) were not measured. Changes in all peak intensities are related to the applied HOCl concentration (Fig. 1B). It is obvious that the 15-HpETE concentration was reduced to a larger degree in comparison to the formation of the two new peaks. Apparently further products have to be formed that are not detected by this HPLC approach.

15-HpETE was also incubated with HOCl at pH 7. Here the reduction of the 15-HpETE content after reaction with HOCl was with 66% lower at an equimolar 15-HpETE: HOCl ratio as compared to the reaction at pH 9. Also at pH 7 two peaks with lower retention



**Fig. 1.** Reaction of 15-HpETE with HOCl. Hydroperoxides or arachidonic acid (AA-OOH) was synthesized using arachidonic acid and sLOX in 50 mM borate buffer at pH 9 and quantified by RP-HPLC. (A) 15-HpETE (380  $\mu$ M) was incubated with 380  $\mu$ M, 190  $\mu$ M, 95  $\mu$ M, 63  $\mu$ M and 48  $\mu$ M HOCl at room temperature (20 °C) for 5 min (50 mM borate buffer, pH 9). For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/ min. Peaks: 15-HETE ( $t_r$  = 13.5 min), 15-HPETE ( $t_r$  = 14.6 min). The peak changes are related to the used HOCl concentration (B). Similar experiments were also performed at pH 7 (C). Thereby, the pH shift was done after 15-HPETE synthesis by addition of 1 N HCl. The chromatograms are representative examples of three independent experiments of 15-HPETE incubated with HOCl.



**Fig. 2.** Reaction of 13-HpODE with HOCl. Hydroperoxides of linoleic acid (La-OOH) was synthesized using 500  $\mu$ M linoleic acid and 3695 U sLOX in 50 mM borate buffer at pH 9 and quantified by RP-HPLC. (A) 400  $\mu$ M of 13-HpODE was incubated with HOCl in different ratios at room temperature (20 °C) for 5 min. For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/min. Peaks: 13-HpODE ( $t_r$  = 14.0 min), 9-HpODE ( $t_r$  = 14.4 min). The peak changes are related to the used HOCl concentration (B).

time ( $t_r$  of 7.4 and 7.9 min) emerged in dependence on the used HOCl amount. However, the relative proportion of these peaks relative to 15-HpETE was lower compared to the experiment at pH 9. When these peak changes are related to the applied HOCl concentration (Fig. 1C) it can be seen that also at pH 7 the changes of the 15-HpETE peak area are more intense than the increase of the peaks at 7.4 and 7.9 min.

The two novel peaks have apparently an intact conjugated diene structure due to their absorption at 234 nm. Because of the absence of any formation of 15-HETE it can be excluded that HOCl converts the hydroperoxide of 15-HpETE to 15-HETE. Apparently, the detected changes in the product spectrum are caused by interaction of HOCl with double bonds in 15-HpETE.

To specify this conclusion, HOCl was also incubated with 13-HpODE. This hydroperoxide of linoleic acid contains only two double bonds that form a diene conjugate. 13-HpODE was incubated with HOCl at pH 9 (50 mM borate buffer) and examined by analytic RP-HPLC with UV-detection at 234 nm (Fig. 2A). Without HOCl a double peak of 13-HpODE and the synthesis by-product 9-HpODE is present with a  $t_r$  of 14.0 and 14.4 min, respectively. After addition of HOCl a drastic decrease of this double peak was monitored. In particular this becomes obvious in relation of the 13-HpODE peak area to the used HOCl concentration (Fig. 2 B). No further peaks emerged. Thus, the decrease of the 13-HpODE peak is probably due to a reaction of HOCl with a double bond resulting in the loss of the conjugated diene structure and in disappearance of the absorption at 234 nm.

Summarizing, the measured decrease of 15-HpETE and 13-HpODE is most likely due to a reaction of HOCl with the double bonds, but not by a reaction with the hydroperoxide moiety.



**Fig. 3.** Reaction of 15-HpETE with HOCl as assessed by ESI-MS. Ethanol of the synthetic available 15-HpETE solution was evaporated by vacuum and the remaining neat oil was dissolved in borate buffer (50 mM, pH 9). 300  $\mu$ M of 15-HpETE was incubated in the absence (A) and presence of 300  $\mu$ M (B) or 600  $\mu$ M (C) HOCl at room temperature (20 °C) for 5 min. For spectrometric analysis the samples were diluted in acetonitrile and measured in the negative ion mode with an ion trap ESI-MS. The spectra are representative examples of three independent experiments.

Additional experiments were also performed in the presence of further HOCl reaction partners, in particular methionine and taurine (data not shown). Interestingly, at equimolar concentration of methionine and taurine the intensity of the HOCl-derived 15-HpETE peaks ( $t_r$  of 7.4 and 7.9 min) were clearly decreased. That means HOCl shows a stronger affinity to the thioether group of methionine or the amino group of taurine than to 15-HpETE. This is in accordance with the assumption that the double bonds of 15-HpETE are target sites of HOCl. The reaction of HOCl with aliphatic double bonds is well described and leads to the formation of chlorohydrins (Spalteholz et al., 2004; Winterbourn et al., 1992). To what extent the reaction of 15-HpETE with HOCl is accompanied by the formation of chlorohydrins and whether the hydroperoxide moiety participates was examined in the following experiments.

### 3.2. Reaction of synthetic and enzymatically synthesized 15-HpETE with HOCl as assessed by ESI-MS and LC-ESI-MS/MS

The formation of chlorohydrins can be easily detected by the mass changes of the fatty acids. Therefore the incubation products of HOCl with the synthetic standards 15-HpETE was measured with ion trap mass spectrometry (MS) (Fig. 3). The electrospray ionization (ESI) was chosen as soft ionization technique due to the instability of the hydroperoxide moiety.

The fatty acid hydroperoxide 15-HpETE has a monoisotopic mass of 336.5 Da (Fig. 3). Consequently, in the negative ion mode a peak at m/z 335.3 Da can be found as negative ion of 15-HpETE, and a more prominent peak with a mass of m/z 317.3 Da was obvious due to the elimination of a water molecule apparently from the hydroperoxide moiety of 15-HpETE vielding a keto-acid or an epoxy-acid (MacMillan and Murphy, 1995). Applying equimolar concentrations of HOCl the peaks at m/z 335.3 Da and m/z 317.3 Da decreased considerably, whereas several new peaks with higher masses were formed. The intense peak couple with m/z 387.3 and 389.3 Da can be assigned to monochlorohydrins of 15-HpETE due to mass shift of 52 Da. Additionally, the intensity ratio of 3 to 1 between these mass peaks corresponds well to the isotope ratio of <sup>35</sup>Cl and <sup>37</sup>Cl. An excess of HOCl in relation of the 15-HpETE concentration resulted also in a dichlorohydrin formation of 15-HpETE with peaks at m/z 439.2, 441.2 and 443.2 Da. The intensity ratio of these three peaks corresponds to 9:6:1 as expected from the natural abundance of chlorine isotopes. Further minor peaks around m/z 369.3 Da and 421.2 Da can be assigned to mono- and dichlorohydrins of 15-HpETE, which eliminated a water molecule. The less intense peaks at m/z 351.3 Da and 403.3 Da are apparently attributed to the formation of epoxide intermediates through elimination of HCl from chlorohydrins under slightly basic conditions (Spalteholz et al., 2004; Winterbourn et al., 1992). Furthermore, the reaction of 15-HETE and HOCl was also measured with ESI-MS leading to a comparable chlorohydrin formation of 15-HETE as for 15-HpETE (data not shown).

To get a clear connection between the chromatographic analyzed peaks with lower retention time and the formation of chlorohydrins of 15-HpETE, the analytic RP-HPLC was coupled with ESI-MS measurements. On the one hand, this was performed by collection of aliquots from the eluate after RP-HPLC analysis corresponding to retention time of 7.4 and 7.9 min. These aliquots were subsequently analyzed with an ESI quadrupole ion trap (Fig. 4A). On the other hand, the reaction mixture was analyzed with a further instrument, a triple quadrupole linear ion trap mass spectrometer (Fig. 4B).

The mass spectrometric analysis of the two HPLC peaks ( $t_r$  of 7.4 and 7.9 min) clearly assigns them as monochlorohydrins of 15-HpETE due to an intense peak couple at m/z 387.2 and 389.2 Da (Fig. 4 A and inset). Furthermore, a less intense peak at m/z 351.3 Da can be apparently attributed to the formation of epoxide

intermediates through elimination of HCl from chlorohydrins as in Fig. 3. Evidences for the occurrence of dichlorohydrins of 15-HpETE were not detected in the measured eluates. The chlorohydrin formation was further evaluated by a direct LC-ESI-MS/MS analysis. Thereby the mass transitions for 15-HpETE and 15-HpETE + HOCl were monitored. Interestingly, the LC separation resulted in a comparable chromatogram with lower retention peaks for 15-HpETE + HOCl ( $t_r$  of 4.8 and 4.9 min) compared to 15-HpETE ( $t_r$  of 6.0 min). For 15-HpETE the characteristic fragment ion was measured with m/z 113.1 Da, which was also applied for 15-HpETE + HOCl. Apparently this fragment arise from cleavage of the double bond allylic to the hydroperoxide as shown in a comprehensive analysis of fragment ions of lipid hydroperoxides from MacMillan and Murphy (MacMillan and Murphy, 1995).

Based on these results the chlorohydrin peaks and the 15-HpETE peak eluted by RP-HPLC were further evaluated by MS/MS analysis. The resulting fragment spectra are shown in Fig. 5.

The collision induced fragmentation of 15-HpETE (Fig. 5A) and of the corresponding monochlorohydrin peaks (Fig. 5B and C) resulted in a characteristic fragmentation pattern. For 15-HpETE the isolation mass was set to 317 m/z because the dehydrated form of 15-HpETE occurred in higher abundance. For the monochlorohydrin species of 15-HpETE the isolation mass was set to 388 m/z(isolation width 4 m/z) to ensure an equal isolation of both isotopes species.

For 15-HpETE different product ions were observed formed by loss of  $H_2O$  and  $CO_2$  from the carboxylate anion (273 *m*/*z*), by loss of



**Fig. 4.** Reaction of 15-HpETE and HOCl as assessed by coupling of liquid chromatography and ESI-MS. 15-HpETE was synthesized using arachidonic acid and sLOX in 50 mM borate buffer at pH 9 and quantified by RP-HPLC. 15-HpETE (225  $\mu$ M) was incubated with 225  $\mu$ M HOCl at room temperature (20 °C) in 100  $\mu$ I for 5 min (50 mM borate buffer, pH 9). (A) For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/min. Aliquots of the eluate were collected and measured in negative ionization mode with ESI-MS. Left and right inset: characteristic MS spectra of the two peaks at 7.4 and 7.9 min are shown respectively. (B) For direct online-SPE-LC-ESI-MS/MS analysis 15-HpETE +HOCl and 15-HpETE were diluted 1:1000 and 1:10000 in methanol/water (62/38, v/v), respectively, and measured in negative ionization mode. Chromatograms for 15-HpETE +52 Da (HOCl) are shown.



**Fig. 5.** Reaction of 15-HpETE and HOCl as assessed by ESI-MS/MS. 15-HpETE was synthesized using arachidonic acid and sLOX in 50 mM borate buffer at pH 9 and quantified by RP-HPLC. 15-HpETE (250  $\mu$ M) was incubated with 250  $\mu$ M HOCl at room temperature (20 °C) in 100  $\mu$ l for 5 min (50 mM borate buffer, pH 9). For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/min. Aliquots of the eluate were collected and measured in negative ionization mode with ESI-MS/MS. The isolation width was set to 4.0 m/z, fragmentation cutoff to 105 m/z and fragmentation amplitude to 0.4 V. (A) MS/MS spectra of 317 m/z (15-HpETE-H<sub>2</sub>O as isolation mass). (B) MS/MS spectra of 388 m/z (isolation mass) of the sample corresponding to the peak at 7.9 min. (D) Possible fragmentation pattern of 15-HpETE and 15-HpETE-epoxide intermediates yielding characteristic ions shown in the corresponding spectra and marked with \*.

two H<sub>2</sub>O (299*m*/*z*) or by loss of two water molecules and CO<sub>2</sub> (255*m*/*z*). Furthermore, two characteristic fragment ions were detected also previously described (MacMillan and Murphy, 1995). The ion at 113 *m*/*z* results apparently from cleavage of the double bond -C13=C14- adjacent to the point of attachment of the hydroperoxide group and loss of water. This fragment was also detected in the LC-ESI-MS/MS analysis. Another fragment ion at 219*m*/*z* results from charge driven allylic fragmentation of dehydrated 15-HpETE after 1,5-sigmatropic proton rearrangement resulting in an intermediate with a triene structure between C8 and C13. The cleavage occurs between C14 and C15.

The collision induced fragmentation of the corresponding monochlorohydrin peak (387 m/z) resulted in an abundant formation of the product ion 351 m/z due to elimination of HCl and formation of an epoxide intermediate. In addition, product ions arising from 351 m/z by loss of H<sub>2</sub>O (333 m/z), by loss of H<sub>2</sub>O and CO<sub>2</sub> from the carboxylate anion (289 m/z), by loss of two H<sub>2</sub>O

(315 m/z) or by loss of two water molecules and  $CO_2(271 m/z)$  were measured comparable to the 15-HpETE fragmentation spectra. In both monochlorohydrin forms of 15-HpETE ( $t_r$  of 7.4 and 7.9 min) the ion with a mass of 113 m/z was detected. The common appearance of this fragment in 15-HpETE and both monochlorohydrin products indicates the preservation of the structure between C13 and C20 in 15-HpETE and the investigated chlorohydrin samples. This is in accordance with the assumption that the monochlorohydrin forms of 15-HpETE detected by RP-HPLC have an intact conjugated diene group.

In the MS/MS spectra of the peak at 7.4 and 7.9 min two further characteristic ions at 205 m/z (more abundant for the peak at 7.4 min) and 217 m/z (more abundant for the peak at 7.9 min) were detected (Fig. 5B and C). Apparently these fragments arise from a similar rearrangement and cleavage of the corresponding mono-chlorohydrin structure as for 15-HpETE. The fragment with a mass of 205 m/z may be formed by cleavage of the -C13=C14-



**Fig. 6.** Reaction of 15-HPETE or 15-HETE with HOCI. Ethanol of the synthetic available 15-HPETE or 15-HETE solution was evaporated by vacuum and the remaining neat oil was dissolved in PBS at pH 7.3. 100  $\mu$ M HOCI was incubated with equimolar concentration of 15-HPETE (A) or 15-HETE (B) at room temperature (20 °C) for 5 min, respectively. For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (50/50/0.2, v/v/v) at a flow rate of 1 ml/min. Peaks: 31.1 min 15-HETE/36.6 min 15-HPETE.

combined with release of water. In this fragment the epoxide could reside at position -C5 = C6— or -C8 = C9— assuming that both possible monochlorohydrin structures of 15-HpETE with an intact conjugated diene group yielding this fragment. The ion at 217 m/z seems to be formed by a cleavage of the bond between C14 and C15 of the epoxide intermediate under H<sub>2</sub>O release. This fragmentation pattern requires a 1,5-sigmatropic proton rearrangement as described for 15-HpETE. This could only be expected in a monochlorohydrin species of 15-HpETE, where the chlorohydrin is formed at the double bond at -C5 = C6—.

Therefore it may be hypothesized that the peak at 7.4 min represent the monochlorohydrin of 15-HpETE, where the chlorohydrin is formed at the double bond at -C8 = C9— yielding a fragmentation pattern with a prominent fragment at 205 m/z. The peak at 7.9 min could represent the monochlorohydrin of 15-HpETE, where the chlorohydrin is formed at the double bond at -C5 = C6— yielding a fragmentation pattern with both ions at 205 m/z and 217 m/z.

### 3.3. Reaction of synthetic 15-HpETE in comparison to 15-HETE with HOCl as assessed by RP-HPLC

Whether the hydroperoxy moiety is involved in the reaction of 15-HpETE with HOCl, was further investigated by a direct comparison of the reaction of HOCl with synthetic standards 15-HpETE and 15-HETE, respectively. Therefore the ethanol solvent of 15-HpETE and 15-HETE was evaporated by vacuum and the remaining neat oil was dissolved in PBS (pH 7.3). Then equimolar concentrations of HOCl and 15-HpETE or 15-HETE were incubated for 5 min and afterwards analyzed by RP-HPLC (Fig. 6).

To improve the resolution of the chromatogram, especially in that part where the HOCl dependent fatty acid peaks are located, a lower amount of acetonitrile was used in the solvent. As a consequence the retention times of 15-H(p)ETE (15-HETE  $t_r$  = 31.1 min and 15-HpETE  $t_r$  = 36.6 min) and of the HOCl modified fatty acid peaks (Fig. 6) were increased. Both fatty acids show a reaction with HOCl. For 15-HpETE the simultaneous decrease of the 15-HpETE content and the appearance of two new peaks ( $t_r$  of 14.1 and 15.5 min) reappeared (Fig. 6A). This was also the case for the 15-HETE/HOCl incubation (Fig. 6B). A comparable decrease of the 15-HETE content was measured and two new peaks with a lower retention time emerged. Whereby one of them is a double peak  $(t_r = 11.8 \text{ and } 12.2 \text{ min})$  the other one is a single peak  $(t_r = 13.4 \text{ min})$ . The HOCl modified peaks of 15-HpETE and 15-HETE can be clearly distinguished according their retention time. This is a further indication that the products of the reaction of HOCl with 15-HpETE differ clearly from 15-HETE in their chemical identity. Additionally it shows, that during the reaction of 15-HpETE with HOCl the hydroperoxide moiety does not participate. Therefore it can be assumed that only the double bonds of 15-HpETE and 15-HETE are the target for HOCl.

In a further experiment 15-HpETE and 15-HETE was incubated together with HOCl, whereby HOCl was added in equimolar concentration to the sum of 15-H(p)ETE (Fig. 7).

After incubation of 15-H(p)ETE with HOCl a drastic decrease of the 15-H(p)ETE peaks occurred. Furthermore, four peaks with lower retention times were observed with comparable  $t_r$  to the single incubation of 15-H(p)ETE with HOCl. That means also in the presence of both fatty acids their appropriate HOCl dependent peaks can be identified.

## 3.4. Reduction of HOCI-treated 15-HpETE with the glutathione peroxidase system

Glutathione peroxidase (GpX) is able to reduce the hydroperoxy moiety of 15-HpETE to a hydroxyl group (15-HETE). This characteristic of GpX was utilized to reduce the products of the 15-HpETE/HOCl reaction. Therefore 15-HpETE was incubated with HOCl and further treated with a mixture GpX and glutathione (GSH) or GSH alone (Fig. 8). As already shown in Fig. 1, the incubation of 15-HpETE with HOCl yielded two new peaks with  $t_r$ at 7.4 min and 7.9 min. Upon application of GpX and GSH, these peaks shifted to lower retention ( $t_r$  = 6.9/7.3 min). Applying only GSH, three peaks with  $t_r$  at 6.9 min, 7.3 min and 7.9 min emerged. The peak around  $t_r$  of 7.3 min is apparently composed of two



**Fig. 7.** Reaction of 15-H(p)ETE with HOCl. A mixture of the two standards 15-HpETE and 15-HETE containing ethanol solutions was evaporated by vacuum and the remaining neat oil was dissolved in PBS at pH 7.3. 200  $\mu$ M HOCl was incubated with 100  $\mu$ M of 15-HpETE and 100  $\mu$ M of 15-HETE at room temperature (20 °C) for 5 min. For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (50/50/0.2, v/v/v) at a flow rate of 1 ml/ min. Peaks: 31.0 min 15-HETE/36.5 min 15-HETE.



**Fig. 8.** Reaction of 15-HpETE with HOCl und further reduction using GpX and GSH. Hydroperoxides or arachidonic acid (AA-OOH) was synthesized using arachidonic acid and sLOX in 50 mM borate buffer at pH 9 and quantified by RP-HPLC. 250  $\mu$ M of 15-HpETE was incubated with equimolar concentration of HOCl at room temperature (20 °C) for 5 min and then mixed with 2 U GpX and 1 mM GSH or 500  $\mu$ M/1 mM GSH alone. These samples were incubated for further 15 min. The reaction was stopped by separation of the enzyme by centrifugation through a filter. For better comparison also the samples without GpX were centrifuged through a filter. For chromatographic analysis the samples diluted in solvent were isocratically eluted with acetonitrile/water/acetic acid (60/40/0.2, v/v/v) at a flow rate of 1 ml/ min. Peaks: 13.4 min 15-HETE/14.6 min 15-HpETE. The chromatogram is a representative example of three independent experiments.

closely overlapping peaks. The third peak ( $t_r$  = 7.9 min) could be comparable to the second peak of the HOCl derived 15-HpETE signal.

Taking into account that GpX and GSH convert the hydroperoxy group into a hydroxyl group, which can also be seen in the increase of the 15-HETE signal (Fig. 8), it is apparent that under mild oxidizing conditions a stepwise transformation of the HOCI modified 15-HPETE signal into the HOCI modified 15-HETE signal occurred. This is also supported by the fact that the HOCI modified 15-HETE peaks have a lower retention time than the corresponding 15-HPETE peaks. Glutathione alone mediates also the conversion, however, with a lower efficiency as the GpX/GSH system.

### 3.5. Modification of 15-HpETE by myeloperoxidase

The heme-containing myeloperoxidase (MPO) is an important enzyme of neutrophil granulocytes producing HOCl under inflammatory conditions. The MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system was now

#### Table 1

Reaction of 15-HpETE with the MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system. 100  $\mu$ M of 15-HpETE, synthesized using arachidonic acid and soybean lipoxygenase and quantified by RP-HPLC, was incubated with 200 nM or 400 nM MPO with or without the addition of final 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, supplied in small amounts, in the presence or absence of 140 mM Cl<sup>-</sup>. The reaction was performed in phosphate buffer (50 mM, pH 7.4) at room temperature (20 °C). After 10 min incubation the reaction was stopped by four-fold dilution in HPLC solvent (acetonitrile/water/acetic acid (60/40/0.2, v/v/v)). Afterwards the samples were centrifuged through a filter (30 kDa) and were analyzed by HPLC. The mean values and standard deviations were calculated from three to five independent experiments. A statistical hypothesis test was performed using a two-tailed *t*-test (unequal sample sizes, unequal variances,  $p \le 0.001 = ^{***}$ ).

	Chlorohydrins related to original 15-HpETE (area %)		15-HpETE (in μM)
	<i>t</i> <sub>r</sub> = 7.4 min	<i>t</i> <sub>r</sub> = 7.8 min	<i>t</i> <sub>r</sub> = 14.5 min
15-HpETE	-	-	87.53 ± 2.85 🗕
15-HpETE + MPO + H <sub>2</sub> O <sub>2</sub> + Cl <sup>-</sup>	9.07 ± 3.95%	9.55 ± 3.87%	41.45 ± 2.07 — ***
15-HpETE + MPO + H <sub>2</sub> O <sub>2</sub>	-	-	59.03 ± 4.23 — ***
15-HpETE + MPO	-	-	69.20 ± 5.94 — ***
15-HpETE + MPO + Cl <sup>-</sup>	-	-	70.38 ± 4.87 — ***
15-HpETE + H <sub>2</sub> O <sub>2</sub>	-	-	81.23 ± 5.77 🔟 -

applied at physiological pH (pH 7.4) (Table 1). As control sample 15-HpETE was equally treated like the enzyme samples.

A small decrease of the original used 100 µM 15-HpETE to 88 µM was apparently related to the additional centrifugation step through a 30 kDa filter. When MPO, H<sub>2</sub>O<sub>2</sub> and Cl<sup>-</sup> were present together a reduction of the 15-HpETE content from previous  $87.5 \,\mu\text{M}$  to  $41 \,\mu\text{M}$  and the appearance of two new peaks with lower retention time ( $t_r$  = 7.4 and 7.8 min) was obvious. Thus, the whole MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system caused the same alterations on 15-HpETE as the reactant HOCl. The incubation of 15-HpETE with H<sub>2</sub>O<sub>2</sub> alone did not induce any significant changes in the 15-HpETE content, only a slight reduction to 81 µM was detected. Interestingly, in the presence of MPO, either alone or together with Cl<sup>-</sup> or H<sub>2</sub>O<sub>2</sub>, the amount of 15-HpETE was significantly diminished, but to a lower extent than in the presence of the complete MPO system. The recovery of 15-HpETE was about 70 µM by MPO and MPO + Cl<sup>-</sup> and only 59  $\mu$ M by MPO + H<sub>2</sub>O<sub>2</sub>. However, no new peaks appeared neither for chlorohydrins nor 15-HETE in the chromatograms. Control experiments of the reaction of 15-HpETE with the MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> were also performed at 37 °C, however no temperature influence could be determined (data not shown).

#### 4. Discussion

The reaction of HOCl with aliphatic fatty acid hydroperoxides was investigated by RP-HPLC and ESI-MS. We could not detect any products originating from a direct interaction of the hydroperoxide moiety with HOCl. Upon incubation of 15-HpETE with HOCl. no increase in the amount of the corresponding hydroxy fatty acid (15-HETE) could be detected. However, the reaction of HOCl with unsaturated fatty acid hydroperoxides, like 15-HpETE, resulted in formation of chlorohydrins. The chromatographic analysis of 15-HpETE products using RP-HPLC revealed two new chlorohydrins  $(t_r = 7.4 \text{ and } 7.8 \text{ min})$  with an intact conjugated diene configuration. These new products have still the hydroperoxide group because the reaction of synthetic 15-HETE with HOCl resulted in products with different retention times. Furthermore, the hydroperoxide group of the chlorohydrin products could be reduced into the hydroxyl form using the glutathione peroxidase system. Different reaction conditions were tested, the chlorohydrin formation takes place both in phosphate buffer (pH 7.4) and borate buffer (pH 9) also no differences were determined performing the reaction at 37 °C (data not shown). During the incubation of linoleic acid hydroperoxide with HOCl singlet oxygen has been detected (Miyamoto et al., 2006). Apparently, singlet oxygen should

originate not from the direct reaction between both partners. Another candidate reaction for the formation of singlet oxygen is the interaction of two peroxyl radicals according to the Russell mechanism (Russell, 1957).

The formation of chlorohydrins upon the reaction of HOCl with unsaturated fatty acids or phospholipids containing double bonds in their acyl chains is well described (Arnhold et al., 2001; Spalteholz et al., 2004; Winterbourn et al., 1992). Possibly all four double bonds of 15-HpETE are the target site for HOCl. In case of chlorohydrin formation on double bonds at -C5=C6- or -C8 = C9 - ,the diene configuration at -C11=C12-C13=C14- remains intact and these chlorohydrin can be detected by HPLC at 234 nm. Chlorohydrin formation on double bonds at -C11=C12- or -C13=C14- disrupts the diene conjugate structure and these chlorohydrins are not detectable at 234 nm. This is also reflected by the fact that the decrease of the 15-HpETE peak at 14.6 min was about twice the increase of the summary peak area of both chlorohydrin peaks at 7.4 and 7.9 min. To further assess the position of the chlorohydrin group after reaction of 15-HpETE with HOCl fragmentation spectra were obtained of the monochlorohydrin species of 15-HpETE eluted at lower retention time by RP-HPLC. Fragmentation pattern analysis revealed that apparently the peaks at  $t_r$  of 7.4 and 7.9 min represent the monochlorohydrins of 15-HpETE, where the chlorohydrin is formed at the double bond at -C8=C9- and -C5=C6-, respectively. The equimolar incubation of synthetic 15-HpETE with HOCl leads to the formation of monochlorohydrins of 15-HpETE. Only a surplus of HOCl leads to the detection of dichlorohydrins, although in a lower extent. In the presence of further reaction partners for HOCl, like the thioether group of methionine or the amino group of taurine, the chlorohydrinforming reaction can be suppressed. Therefore, the double bonds of 15-HpETE are a minor attractive target site of HOCl in comparison to other HOCl targets. Rate constants for the reaction of HOCl with methionine  $(3.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}, \text{ pH 7.4} (\text{Pattison and Davies, 2001}))$ or taurine  $(3.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}, \text{ pH 4.7} (\text{Marquez and Dunford}, 1994))$ are much higher than rate constants for the reaction of HOCl with aliphatic double bonds in phospholipids. Values for the later constants are reported to be  $9 M^{-1} s^{-1}$  (Pattison et al., 2003) or 0.5 M<sup>-1</sup> s<sup>-1</sup> (Panasenko et al., 1997).

The modification of 15-HpETE was also possible through the complete MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system. Thereby, the formation of the characteristic two peaks with lower retention time was detected similar to the incubation of 15-HpETE with HOCl. That means that MPO system is also able to form chlorohydrins of 15-HpETE via the production of HOCI. Interestingly, further control reactions of 15-HpETE with several components of the MPO-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system revealed that already the presence of MPO or MPO+Cl<sup>-</sup> diminished the yield of 15-HpETE. However, there was no formation of chlorohydrins or 15-HETE. Thus, any two-electronic reduction of 15-HpETE to 15-HETE by MPO can be excluded. Ferric MPO is well known to reduce different organic hydroperoxides to the corresponding hydroxides (Furtmuller et al., 2000). However, the interaction of hydroperoxides of arachidonic acid with MPO was not investigated by these authors. Maybe some 15-HpETE was non-specifically bound to the enzyme and removed during the centrifugation step. The further decrease in the 15-HpETE yield in the presence of MPO +  $H_2O_2$  remains puzzling. This indicates either an oxidation of 15-HpETE by compound I of MPO under formation of the corresponding peroxyl radical or a reaction of 15-HpETE with superoxide anion radicals which can result from the oxidation of H<sub>2</sub>O<sub>2</sub> by compound I of MPO.

A direct reaction between HOCl and the hydroperoxide moiety in unsaturated lipids is unlikely. Thus, an enhancement of lipid peroxidation reactions in lipid systems by HOCl (Panasenko and Arnhold, 1999; Panasenko et al., 1997) should be related to other targets in lipid samples. Oxidized lipid material contains also different carbonyls, carbohydrates, cyclic endoperoxides, radical species and other oxidant products (Frankel, 1987). Some of these products can be targeted by HOCl.

Primarily chlorohydrin formation of fatty acids in phospholipids are implicated in pathophysiological activities due to the alteration of membrane integrity and the enhanced formation of lysophospholipids from unsaturated phosphatidylcholines under the influence of hypochlorous acid (Arnhold et al., 2002). Products of MPO and eosinophil peroxidase (EPO) are also known to inactivate the formation of leukotrienes and prostaglandins in neutrophil and eosinophil granulocytes (Goetzl, 1982; Henderson et al., 1982; Paredes and Weiss, 1982). Whether the formation of chlorohydrin derivatives of the eicosanoids or direct interaction of MPO metabolites with 5-lipoxygenase or prostaglandin H synthase is the reason for this inactivation, cannot be clarified at the moment. However, a reaction of the hydroperoxide group of arachidonic acid metabolites with HOCI can be excluded.

### 5. Conclusion

A possible reaction of fatty acid hydroperoxides with HOCl was assumed in different studies. Thereby the occurrence of fatty acid radicals and the subsequent reduction of the hydroperoxide group were hypothesized. But these assumptions have been disproved for a reaction of 15-HpETE with HOCl. Both the chromatographic and spectrometric analysis of the reaction products revealed that no reduction of the hydroperoxide moiety occurs. Instead, chlorohydrin of 15-HpETE were formed in consequence of the incubation of 15-HpETE and HOCl. Therefore double bonds of 15-HpETE are a more preferred target during the reaction of 15-HpETE and HOCl.

### **Conflict of interest**

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

#### **Transparency document**

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

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