The Fluorescein-derived Dye Aminophenyl Fluorescein Is a Suitable Tool to Detect Hypobromous Acid (HOBr)-producing Activity in Eosinophils

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Background: Aminophenyl fluorescein was developed to detect the halogenating activity of myeloperoxidase.

Results: Aminophenyl fluorescein can also be used to quantify the formation of HOBr by eosinophil peroxidase.

Conclusion: Aminophenyl fluorescein is suitable to evaluate the halogenating activity of eosinophil peroxidase and peroxidase activity modulators.

Significance: Eosinophil peroxidase, a mammal heme peroxidases family member, may contribute to the regulation of inflammation.

The specific detection of peroxidase activity in human granulocytes is essential to elucidate their role in innate immune responses, immune regulation, and inflammatory diseases. The halogenating activity of myeloperoxidase in neutrophils can be determined by the novel fluorescent probe aminophenyl fluorescein (APF). Thereby non-fluorescent APF is oxidized by HOCl to form fluorescein. We successfully verified that APF equally detects the hypobromous acid (HOBr)-producing activity of eosinophil granulocytes. This was revealed by three different approaches. First, we investigated the conversion of non-fluorescent APF into fluorescein by HOCl and HOBr by means of fluorescence and mass spectrometry approaches. Thereby comparable chemical mechanisms were observed for both acids. Furthermore in vitro kinetic studies were used to detect the halogenating activity of myeloperoxidase and eosinophil peroxidase by using APF. Here the dye well reflected the different substrate specificities of myeloperoxidase and eosinophil peroxidase regarding chloride and bromide. Finally, peroxidase activities were successfully detected in phorbol ester-stimulated neutrophils and eosinophils using flow cytometry. Thereby inhibitory studies confirmed the peroxidase-dependent oxidation of APF. To sum up, APF is a promising tool for further evaluation of the halogenating activity of peroxidases in both neutrophils and eosinophils.

Eosinophil granulocytes, also called eosinophils, are a multifaceted protagonist of the innate immune system. In addition to their task as a cytotoxic effector cell against parasitic pathogens, eosinophils contribute to epithelial barrier functions, regulation of inflammation, and tissue remodeling (1). However, dysregulations regarding these cells have physiological relevance. The delayed apoptosis of eosinophils is a key feature of the pathogenesis of asthma (2–4). Other airway diseases such as chronic obstructive pulmonary disease as well as allergic diseases like allergic rhinitis are also accompanied by an impaired eosinophil apoptosis (5–7). Granule proteins, proinflammatory mediators, and reactive oxygen species from these cells are regarded as important mediators for chronic proinflammatory and autoimmune conditions (2, 3, 5).

Among the specific constituents of eosinophil granules is the heme-containing protein eosinophil peroxidase (EPO)3 that is structurally related to myeloperoxidase (MPO) from neutrophil granulocytes (neutrophils) (8–10). Upon activation by hydrogen peroxide, both enzymes are able to oxidize several (pseudo)halides to the corresponding hypo(pseudo)halous acids by abstracting two electrons. Bromide and thiocyanate are oxidized by both peroxidases under the formation of hypohalous acid (HOBr) and hypothiocyanate (OSCN), respectively. The formation of hypochlorous acid (HOCl) from chloride is well reported for MPO, but in the case of EPO it takes place only under strong acidic conditions (11, 12). Considering physiological concentrations and neutral pH values, MPO mainly forms HOCl and OSCN, whereas EPO is presumably involved in the generation of HOBr and OSCN heme proteins (13, 14).

Besides their halogenating activity, these heme proteins also exhibit a peroxidase activity by oxidizing numerous substrates in two consecutive one-electron reactions (15). Derivatives such as 2',7'-dihydrofluorescein or dihydrorhodamine123 are commonly used to detect reactive oxygen species like H₂O₂, NO, OH⁻, HOCl as well as a variety of other oxidizing agents

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3 The abbreviations used are: EPO, eosinophil peroxidase; HOBr, hypobromous acid; MPO, myeloperoxidase; 4-ABAH, 4-aminobenzoic acid hydrazide; a.u., arbitrary units; APF, aminophenyl fluorescein; DPI, diphenyleneiodonium chloride; HPF, hydroxyphenyl fluorescein; iNOS inducible nitric oxide synthase; L-NMMA, N⁶-monomethyl L-arginine; PMA, phorbol 12-myristate 13-acetate; HOX, HOCl and HOBr.

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(16–18). Yet the lack of specificity of these detection systems toward human peroxidases resulted in the development of new substances suitable to detect more specifically the formation of HOCl (16, 19–21). Probably the most promising candidates for a sensitive and specific detection of HOCl in granulocytes are the non-fluorescent fluorescein derivatives (2-(6-(4’-amino-phenoxo)-3-oxo-xanthen-9-yl)-benzoic acid (aminophenyl fluorescein (APF)) and (2-(6-(4’-hydroxy-phenoxo)-3-oxo-xanthen-9-yl) benzoic acid (hydroxyphenyl fluorescein (HPF)) (22). Upon oxidation of APF and HPF, an O-dearylation leads to the formation of fluorescein (16). Yet, these substances are only oxidized by hydroxyl radicals (OH·), peroxynitrite (ONOO−), and HOCl (18). As the latter oxidizes APF much better than HPF, the application of both fluorescein derivatives can be used to specifically detect HOCl formation (22).

To date, APF was successfully used to quantify MPO activity in porcine neutrophils and myeloid leukemic cells by means of flow cytometry and confocal microscopy (22, 23). This dye was already applied for the detection of HOBr in vitro (24). Yet to date it is totally unknown whether the APF system is also suitable for the detection of HOBr production in eosinophils. Here we addressed the question of whether HOBr, as a sign for EPO activity in human eosinophils, can also be detected via APF staining.

Therefore, we investigated the ability of both HOCl and HOBr to convert APF and HPF into fluorescent species by combined fluorescence and mass spectrometry approaches. The kinetics of chlorinating and brominating activity of isolated MPO and EPO was also successfully monitored by APF. Finally we were able to detect these enzyme activities in phorbol ester-stimulated neutrophils and eosinophils. Thus, APF also detects the production of HOBr in granulocytes.

EXPERIMENTAL PROCEDURES

Materials—Human neutrophil MPO (EC 1.11.1.22) and eosinophil peroxidase (EPO, EC 1.11.1.7) were obtained from Planta GmbH, Vienna, Austria. APF and HPF were purchased from Biomol GmbH, Hamburg, Germany. Magnetic beads (microbeads conjugated with monoclonal mouse anti-human-CD16 antibodies) for the isolation of eosinophils were supplied by Miltenyi Biotec GmbH, Bergisch Gladbach, Germany. Antibodies for the evaluation of the purified eosinophils were supplied from eBioscience, Frankfurt, Germany. These include monoclonal mouse anti-human CCR3 antibodies conjugated with allopheophycin and monoclonal mouse anti-human-CD16 antibodies conjugated with fluorescein isothiocyanate. All other chemicals were obtained from Sigma.

Working solutions of HOCl and H2O2 were prepared by dilution of the corresponding stock solutions. Their concentrations were tested by using ε290 = 350 M−1 cm−1 for −OCI (25) at pH 12 and ε290 = 43.6 M−1 cm−1 for H2O2 (26), respectively. HOBr was obtained from HOCl by mixing it with a 2-fold excess of NaNb (27). The concentration of −OBr was checked at pH 12 using ε329 = 332 M−1 s−1 for −OBr (28). The solutions were essentially stable within 1 h and were used in this time. HOSCN was produced by adding 20 mM HOCl in 0.1 M NaOH dropwise to an 8 M NaSCN solution in 0.1 M NaOH at 4 °C under turbulent mixing. The concentration of HOSCN was checked using ε376 = 26.5 M−1 cm−1 (29).

Fluorescence of APF and HPF Modified by Hypohalous Acids—The dyes APF or HPF (each 1 μM final concentration) in phosphate-buffered saline (PBS), pH 7.4, were mixed with 0.1–20 mM of HOCl, HOBr, HOSCN, or H2O2. Afterward the samples were stored in the dark until measurement. Fluorescence spectra were obtained from a Spex Fluoromax-2 spectrophotometer, HORIBA Jobin Yvon GmbH, Bensheim, Germany. An excitation wavelength of 488 nm was chosen corresponding well to the flow cytometry measurement conditions. The emission spectrum was recorded from 495 to 600 nm with an increment of 1 nm. Excitation and emission slit width were set to 1 nm. Control measurements with fluorescein were performed using final concentrations between 1 nM and 1 μM in PBS, pH 7.4.

Mass Spectrometry of Hypohalous Acid-modified APF and HPF—The modification of APF/HPF by HOCl or HOBr was investigated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Bruker Autoflex, Bruker Daltonics GmbH, Leipzig, Germany, supplied with a 337-nm nitrogen laser. The spectra were obtained in the positive ion mode without matrix suppression at 20% laser intensity for the HOCl samples and 40% laser intensity for the HOBr samples.

Samples were prepared by incubating final concentrations of 100 μM APF or HPF in PBS, pH 7.4, with or without 10, 100, or 1000 μM HOCl or HOBr for 5 min. Afterward equal volumes of the probe and the matrix solution were mixed. As a matrix, 10 mg/ml α-cyano 4-hydroxyccinnamic acid in 50% v/v acetonitrile/water supplied with 0.2% trifluoroacetic acid was used. This matrix was chosen because of its ability to detect small molecules with low fragmentation (30).

Kinetic Studies of the Modification of APF and HPF by Heme Peroxidases—The ability of APF or HPF to detect the halogenating activity of MPO or EPO was verified by enzymatic measurements. APF or HPF (each 10 μM) was preincubated with 10 nM MPO or EPO in phosphate buffer, pH 7.4, at 37 °C in the presence of either 140 mM chloride or 100 μM bromide or both of these ions. The halogenating activity of the peroxidases was started by injection of 20 μM H2O2. All indicated concentrations are final ones.

Fluorescence was monitored at 525 nm (with excitation at 488 nm) using a fluorescence microplate reader Tecan Infinite 200 PRO, Männedorf, Switzerland. As a peroxidase inhibitor, 500 μM 4-aminobenzoic acid hydrazide (4-ABAH) was included in some experiments. Control experiments were also carried out in the absence of enzyme, H2O2, the fluorescein derivatives, or halogenides.

Isolation of Human Neutrophils and Eosinophils—Neutrophils were isolated from heparinized peripheral human blood from healthy volunteers using a well-established protocol (31). Briefly, dextran-enhanced sedimentation, Ficol Histopaque density centrifugation, and hypotonic lysis of remaining erythrocytes were applied as purification steps. Eosinophils were isolated from the blood using the same protocol followed by a negative immunomagnetic selection of eosinophils (4). Briefly, CD16 antibodies linked to magnetic beads were used to retain
neutrophils on a column with a ferromagnetic matrix (2, 32). Purity of the cells was tested by staining with CD16-FITC and CCR3-allophycocyanin antibodies and flow cytometry. Eosinophils strongly express CCR3, but no CD16, whereas neutrophils are CD16-positive and CCR3-negative (2, 33).

Cells were cultivated at 37 °C, 95% humidity, and 5% CO₂ content in RPMI 1640 medium in the presence of 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 ng/ml streptomycin. Eosinophils were incubated in the additional presence of 100 μM bromide. Only granulocytes with purity as well as vitality values higher than 90% were used for further experiments.

**Flow Cytometry of APF-stained Cells** —For the experiment 10⁶ cells per ml in HBSS with calcium, pH 7.4, were first preincubated in the presence or absence of enzyme inhibitors for 15 min. Briefly, 500 μM 4-ABAH (peroxidase inhibitor), 50 μM diphenyleneiodonium chloride (DPI, NADPH oxidase inhibitor), or 200 μM N⁵-monomethyl L-arginine (L-NMMA, inhibitor of the inducible nitrite oxide synthase (iNOS)) was used (17, 34, 35). Afterward, 5 μM APF or HPF was added to the samples followed by 30 min of incubation. Then cells were stimulated with 1 nM phorbol 12-myristate 13-acetate (PMA) and incubated for a further 60 min (22, 23, 36). All incubation steps took place at 37 °C. In the case of eosinophils, 100 μM bromide was included in all used buffer solutions as a substrate for EPO.

Thereafter the cells were washed by centrifugation (400 × g, 5 min), and the samples were analyzed by flow cytometry using the Fl-1 channel (band pass, 530 ± 15 nm). For all flow cytometry measurements, a FACSCalibur (BD Biosciences) supplied with a 488-nm argon laser was used. 10⁶ events were detected per measurement, and results were analyzed using the software Flowing Software 2.3.3 by Perttu Terho.

**RESULTS**

**Both HOCl and HOBr Induce Fluorescence in APF and to a Minor Degree in HPF** —The non-fluorescent dye APF was converted into a fluorescent species upon incubation with both HOCl (Fig. 1A) and HOBr (Fig. 1B). The spectrum of 1 μM APF alone did only show a small maximum of about 2.6 × 10⁴ arbitrary units (a.u.) at 509 nm. Yet, upon the addition of small amounts of HOX (HOX stands collectively for HOCl and HOBr), the maximum fluorescence strongly increased. The strongest fluorescence signals were observed using equimolar amounts of hypohalous acids or a 2-fold excess of them (Fig. 1C). With further increasing amounts of HOCl or HOBr, the fluorescence yield decreased continuously. In addition, there was a shift in the position of the fluorescence maximum from about 509 to 535 nm with increasing concentrations of HOCl and HOBr (Fig. 1D). At a molar excess of 10 or higher between HOX and APF, the fluorescence yield decreased continuously. In addition, there was a shift in the position of the fluorescence maximum from about 509 to 535 nm with increasing concentrations of HOCl and HOBr.

Both hypohalous acids differed only in the obtained fluorescence intensity after the incubation with APF. At equimolar reactant ratios HOCl-treated APF showed about 1.8-fold higher values (6.3 × 10⁴ a.u.) as compared with the HOBr samples (3.5 × 10⁴ a.u.) (Table 1). Thus, HOBr is also able to oxidize APF. Apparently, the mechanism of HOBr-induced conversion of APF is comparable with HOCl.

Next, we performed a similar set of experiments with HPF instead of APF. HPF is also converted into a fluorescent species by HOCl and HOBr (supplemental Fig. 1). However, although using molar ratios up to 2 between the hypohalous acid and HPF, the fluorescence intensity was considerably lower in con-
fluorescence was observed by applying 50 nM fluorescein (3.5 fluorescence maximum on the applied concentration. The strongest dependence of both the fluorescence intensity (supplemental Fig. 2, A) and the position (supplemental Fig. 2, B) of the fluorescence maximum on the applied concentration. The strongest fluorescence was observed by applying 50 nM fluorescein (3.5 × 10^8 a.u. at 518 nm). With increasing dye concentrations the position of the fluorescence maximum continuously shifted from 511 to 558 nm. Thus, the observed similar effects of higher acid concentrations on the APF/HPF fluorescence may be simply attributed to the release of increasing amounts of fluorescein. To test the direct effect of HOCI and HOBr on the dye, we incubated 50 nM fluorescein with increasing amounts of HOCl (supplemental Fig. 2, C and D). Only by applying a 1000-fold excess of either hypohalous acid did a significant decrease in the fluorescence intensity of fluorescein (27% for HOCI, 45% for HOBr) occur. Moreover for both acids a shift of the maximum from 518 to 526 nm was observed.

Free Fluorescein Was Formed after Reaction of APF with Both HOCI and HOBr—To gain insights into the mechanism of the APF oxidation by HOCI and HOBr, reaction products were investigated by mass spectrometry. In each figure the displayed spectra were referenced to the highest peak of the monitored m/z range.

The dye APF has a monoisotopic mass of 423.1 Da. An intense peak at m/z 424.1 Da can be found in the positive ion MALDI-TOF mass spectrum for the monoprotonated APF (Fig. 2A). A further minor peak at m/z 393.4 Da may correspond to either a byproduct of the APF synthesis or to a laser-induced fragmentation product as it was also observed in the presence of HPF. All other peaks in the mass spectrum of Fig. 2A at m/z values of 379.2, 445.2, and 461.2 Da can be assigned to the (α-cyano 4-hydroxycinnamic acid matrix. The spectra are representative examples of three independent experiments.

FIGURE 2. MALDI-TOF mass spectra of APF after the incubation with HOCI. 100 μM APF were incubated with up to 1000 μM HOCI. In the absence of HOCI (A), only peaks from protonated APF (m/z 424.1 Da) and a byproduct of APF (m/z 393.4 Da) were observed. After incubation with 10 μM HOCI (B) the formation of fluorescein (m/z 333.1 Da) was observed. In the presence of 100 μM HOCI (C), the formation of monochlorinated fluorescein (m/z 367.1 and 369.1 Da) and, using 1000 μM HOCI (D) dichlorinated fluorescein (m/z 401.1 to 405.1 Da), was detectable. The peaks at m/z 379.2, 445.2, and 461.2 Da can be attributed to the (α-cyano 4-hydroxycinnamic acid matrix. The spectra are representative examples of three independent experiments.

After incubating 100 μM APF with increasing amounts of HOCI, several new peaks were formed that can be attributed to fluorescein and chlorinated fluorescein derivatives (Fig. 2, B and D). The peak at m/z 333.1 Da for monoprotonated fluorescein was already seen at low concentrations of HOCI (Fig. 2B). It increased at an equimolar HOCI/APF ratio (Fig. 2C) and disappeared almost at excess of HOCI (Fig. 2D). Applying equimolar or higher HOCI concentrations, a less intense peak group developed at m/z 367.1 and 369.1 Da that can be attributed to monochlorinated fluorescein. The first peak of this group is more intense, reflecting the isotope ratio between 35Cl and 37Cl of 3 to 1. In addition, after incubating APF with excess HOCI, a prominent couple of new peaks was observed for dichlorinated fluorescein at m/z 401.1, 403.1, and 405.1 Da. The intensity ratio of these three peaks corresponds to 9:6:1 as expected from the natural abundance of chlorine isotopes. Under these conditions the peak at m/z 424.1 Da for unmodified APF was hardly observable.

Comparable experiments were also performed using HOBr instead of HOCI (Fig. 3). Again in the absence of HOBr (Fig. 3A), a peak at m/z ratio 424.1 Da was observed corresponding to protonated APF. The peaks at m/z ratios 335.3, 379.2, and 441.2 Da can be assigned to the matrix. In the presence of 10 μM HOBr (Fig. 3B), only a small peak at m/z ratio 333.1 Da was observed corresponding to fluorescein. Yet by applying equimolar amounts of HOBr, a significant formation of fluorescein was detectable (Fig. 3C). In addition, several new peak groups arose starting at m/z ratios 411.1,
489.1, and 502.1 Da. The double peak at \( m/z \) ratios 411.1 and 413.1 Da can be attributed to monobrominated fluorescein. The intensity ratio of these two peaks corresponds to 1:1 as expected from the equal natural abundance of bromine isotopes \(^{79}\text{Br} \) and \(^{81}\text{Br} \). The same holds for monobrominated APF, which can be seen at \( m/z \) ratios 502.1 and 504.1 Da. The peak group at \( m/z \) ratios 489.1, 491.1, and 493.1 Da showing a ratio of about 1:2:1 reflects the formation of dibrominated fluorescein. Upon incubation of APF with a 10-fold excess of HOBr (Fig. 3D), the APF peak at \( m/z \) 424.1 Da nearly vanished, and the general peak intensity decreased.

In further experiments HPF was incubated with HOCl. This dye provides an intense peak at \( m/z \) 425.1 Da for the monoprotonated form (supplemental Fig. 3). Again the less intense peak at 393.4 Da can be attributed to a byproduct of the synthesis or to a fragmentation product of the fluorescein derivatives (22). In the presence of 10 \( \mu \text{M} \) HOCI (supplemental Fig. 3B) no HPF modification was observed at all. Yet upon incubation of equimolar amounts of HPF and HOCI (supplemental Fig. 3C) two less intense new peak groups appeared starting at \( m/z \) ratios of 459.1 and 493.1 Da. These peak groups can be attributed to mono- and dichlorinated HPF. Only by applying a higher excess of HOCI (supplemental Fig. 3D), the formation of the dichlorinated fluorescein derivative was observed in the \( m/z \) range from 401.1 to 405.1 Da.

Upon incubation of HPF with 10 \( \mu \text{M} \) HOBr, only unmodified HPF (\( m/z \) ratio 425.1 Da) and two small peaks corresponding to monobrominated HPF (\( m/z \) ratios 503.1 and 505.1 Da) were detected (not shown). Using equimolar concentrations of HOBr, these peaks became more prominent, whereas the peak intensity of unmodified protonated HPF significantly decreased. In addition, new peak groups were observed starting at \( m/z \) ratios 489.1 and 567.1 Da. These peaks can be assigned as di- and tribrominated fluorescein. Upon incubation of HPF with a 10-fold excess of HOBr, none of the described brominated HPF/fluorescein products was detected, and the general peak intensity strongly decreased.

To sum up, the oxidation of APF by HOCl or HOBr is well reflected in the spectra of the MALDI-TOF measurements, as significant amounts of fluorescein were formed by both hypohalous acids. This suggests comparable chemical mechanisms. Especially in the presence of excess HOX, mono- and dihalogenated fluorescein derivatives appeared as well. This may be an additional explanation for the lower fluorescence intensities and the shift in the position of the fluorescence maximum observed under these conditions. In contrast to APF, the addition of HOX to HPF led to a primary halogenation of the dye. By applying excess HOCI or HOBr, halogenated fluorescein species were also detected.

**APF Detects the Enzymatic Activity of Both MPO and EPO**—In the next series of experiments we applied APF to study the halogenating activity of myeloperoxidase and eosinophil peroxidase. Both enzymes were preincubated with APF in phosphate buffer, pH 7.4, supplemented with either 140 mM chloride or 100 \( \mu \text{M} \) bromide or both of these ions. The reaction was started by injecting 20 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). The resulting increase in fluorescence at 525 nm was plotted logarithmically against the time (Fig. 4).

Regarding MPO (Fig. 4A) in the presence of 140 mM chloride, a fast increase in the fluorescence intensity at 525 nm up to a value of about 26,600 a.u. was observed within the first 3 min. This indicates a strong formation of HOCI that in turn readily oxidizes APF under the formation of fluorescein. Control measurements in the presence of the enzyme inhibitor 4-ABAH confirmed that the formation of fluorescein is driven by MPO.

In the sole presence of 100 \( \mu \text{M} \) bromide a much slower increase in the fluorescence intensity was observed that reaches a value of about 350 a.u. after 4 min. Long term measurements revealed that after 70 min a maximum of about 6100 a.u. was achieved. Although bromide is readily oxidized by MPO, this slow kinetics reflects the lower concentration of HOBr as compared with HOCI and the resulting minor HOX formation.

Interestingly, in the presence of both ions (140 mM \( \text{Cl}^- \) and 100 \( \mu \text{M} \) \( \text{Br}^- \)) the initial APF oxidation slightly exceeded the kinetics observed in the sole presence of HOCI, reflecting a kind of co-operative effect of the ions on the enzyme activity. Yet an \(~30\%\) less intensive fluorescence maximum (18,200 a.u.) was reached than in the sole presence of chloride and slightly decreased during longer incubation times. The lower fluorescence intensities may reflect the formation of certain amounts of HOBr, which oxidizes APF worse than HOCI.

In the case of EPO (Fig. 4B) in the sole presence of 140 mM chloride, only a slow increase in the fluorescence intensity at 525 nm was observed. After 4 min, an intensity of about 200 a.u. was reached. Yet, longer measurements revealed a maximum fluorescence intensity of 11,300 a.u. after about 2 h. In fact, EPO is also able to oxidize chloride even though this reaction is very slow at neutral pH (12).
In the presence of 100 μM bromide a much faster kinetics of the APF oxidation by EPO was observed with a fluorescence maximum of about 8400 a.u., reached after 4 min. This reflects well both that bromide is the main substrate of EPO and that HOBr also oxidizes APF but leads to lower final fluorescence intensities as compared with the APF oxidation by HOCl.

Again, the fastest kinetics were observed in the presence of both 140 mM chloride and 100 μM bromide, thus confirming the co-operative effect mentioned above. Moreover, under these conditions the highest fluorescence intensities were observed with values of about 12,000 a.u. after 1 min. This may reflect the formation of small amounts of HOCl, which oxidizes APF much better than HOBr. Again, control measurements in the absence of enzyme or H₂O₂ resulted in no APF/HPF oxidation.

In Table 2 the results obtained with APF are summarized and compared with the results from kinetic measurements with MPO and EPO in the presence of HPF. Experiments in halide-free buffer only led to a very slow formation of minor fluorescence signals, suggesting that chloride or bromide is necessary for the formation of fluorescein. Further control experiments in the absence of enzyme or H₂O₂ resulted in no APF/HPF oxidation at all.

Using APF, the Peroxidase Activity in Both Neutrophils and Eosinophils Can Be Observed—We next performed experiments to test whether APF can also specifically detect the halogenating activity of heme peroxidases in living cells. Therefore, we stained granulocytes with APF to detect MPO or EPO activity in PMA-stimulated cells by means of flow cytometry. For neutrophils the incubation with 1 nM PMA for 60 min led to a multipeaked distribution. In the case of the stimulated eosinophils a 5.3 compared with the unstimulated cells. In the case of eosinophils, fluorescence intensities as compared with the APF oxidation by HOCl.

In the presence of 100 μM concentrations of enzyme in phosphate buffer (pH 7.4) were incubated with either 140 mM chloride or 100 μM bromide or both ions or without (w/o) halides. Thereafter the reaction was started by injecting 20 μM H₂O₂, and the oxidation of APF or HPF was monitored at 525 nm. The given intensities and times refer to 95% of the maximum fluorescence observed in the experiments. The R² values reflect the accuracy of the applied non-linear curve fit (Boltzmann equation). Fluorescence intensities are given in arbitrary units (a.u.). All data are means from three measurements.

| Sample   | APF fluorescence | HPF fluorescence |
|----------|------------------|------------------|
|          | Intensity | Time | R² | Intensity | Time | R² |
| MPO      |           |      |    |           |      |    |
| Cl⁻      | 26663     | 2.70 | 1.00 | 1419      | 16.44 | 1.00 |
| Cl⁻ + Br⁻| 18212     | 1.42 | 1.00 | 2108      | 5.70  | 1.00 |
| Cl⁻ w/o halides | 648     | 96.14 | 0.99 | -         | -     | -   |
| EPO      |           |      |    |           |      |    |
| Cl⁻      | 11280     | 111.67 | 1.00 | 1722      | 32.90 | 0.98 |
| Br⁻      | 8415      | 4.05  | 1.00 | 2803      | 19.65 | 1.00 |
| Cl⁻ + Br⁻| 11996     | 1.13  | 0.98 | 2804      | 1.40  | 0.99 |
| w/o halides | 993     | 70.95 | 0.99 | -         | -     | -   |

Oxidation was slightly inhibited by the NADPH oxidase inhibitor DPI and the peroxidase inhibitor 4-ABAH, reflecting a basal peroxidase activity in these cells. Comparable effects can be seen for the unstimulated eosinophils (Fig. 5B). In both granulocytes the presence of the iNOS inhibitor L-NMMA led to slightly higher fluorescence values.

Upon stimulation of the granulocytes with PMA, a higher fluorescence intensity and, in the case of eosinophils, also a multipeaked distribution was observed. This divergent fluorescence distribution may be attributed to the stronger effect of the PMA stimulation on eosinophils as compared with neutrophils. In fact, the plot of forward and sideward scattering of the cells also confirmed a higher necrosis rate in the former cell type (not shown). Therefore, necrotic cells were always excluded using a scatter-gated fluorescence analysis.

In the presence of 4-ABAH or DPI, the fluorescence intensity of PMA-stimulated neutrophils (Fig. 5C) was strongly diminished, confirming an MPO-dependent enhanced HOCl production in these cells. In the case of the stimulated eosinophils (Fig. 5D), the application of 4-ABAH or DPI caused a similar
significant reduction of the enhanced EPO-dependent HOBr production. For both granulocytes, L-NMMA did not show any influence concerning the fluorescence signal.

For statistical analysis the fluorescence intensity (geometric mean) of the stimulated cells was always compared with the stimulated cells in the presence of inhibitors. The analysis of the data from five independent experiments with stimulated neutrophils (E) and eosinophils (F) confirmed that for both granulocytes 4-ABAH and DPI led to a highly significant (***, p < 0.001) reduction of the enhanced peroxidase activity, whereas L-NMMA had no significant effects.

Experiments with APF-stained eosinophils were always performed in the presence of 100 μM bromide. For unstimulated cells control experiments without additional bromide (not shown) showed about 1.8 ± 0.5 times lower fluorescence intensities confirming the oxidation of APF by EPO-derived HOBr.

Further experiments with granulocytes and HPF (not shown) also resulted in certain fluorescence values in the flow cytometry analysis. Yet no dependences on 4-ABAH were observed, confirming that HPF is unsuitable for the detection of peroxidase activity in granulocytes. Also, L-NMMA showed no effect on the HPF-stained probes. Only by applying the NADPH oxi-
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dase inhibitor DPI, a small decrease in the fluorescence intensity of HPF was observed in PMA-stimulated neutrophils.

**DISCUSSION**

Non-fluorescent APF originally developed to detect HOCl is also converted by HOBr leading to fluorescein. Thereby comparable concentration dependences regarding the intensity and position of the fluorescence maximum indicate similar chemical mechanisms. By applying equimolar amounts of HOBr, the maximal fluorescence from APF oxidation was merely 1.8-fold lower as compared with HOCl. Measurements with HPF confirmed that this dye is much harder oxidizable by HOCl than APF (22). This conclusion is also valid for the oxidation of these dyes by HOBr.

In APF and HPF, the fluorescence is quenched by protection of the hydroxy group at the 6'-position of fluorescein by a p-benzoquinone monoamine (APF) or a p-benzoquinone hydroxyl moiety (HPF) (18, 22). The mechanism of this quenching is a photo-induced electron transfer between the xanthene and the benzoic acid moiety that diminishes the ability of the xanthene part to emit light upon excitation (38, 39).

The fluorescence quenching can be overcome by strong oxidants that act presumably on the benzoic acid moiety followed by changes in the electron density and the weakening and disruption of the bond between both molecule parts (40, 41). Hydroxyl radicals and peroxynitrite are able to convert both APF and HPF to their high one-electron reduction potentials (at pH 7.0: 2.31 V for the redox couple OH/HPF due to their high one-electron reduction potentials (40, 41). The mechanism of this quenching is a photo-induced electron transfer between the xanthene and the benzoic acid moiety that diminishes the ability of the xanthene part to emit light upon excitation (38, 39). The fluorescence quenching can be overcome by strong oxidants that act presumably on the benzoic acid moiety followed by changes in the electron density and the weakening and disruption of the bond between both molecule parts (40, 41).

Hydroxyl radicals and peroxynitrite are able to convert both APF and HPF due to their high one-electron reduction potentials (at pH 7.0: 2.31 V for the redox couple OH/H2O (42) and 1.6–1.7 V for ONOOH, H+/NO2−, H2O (43, 44)). The strong two-electron oxidants HOCl and HOBr target presumably the amino group in APF. Attacks of both hypohalous acids toward components bearing amino groups are well described (45). The standard reduction potential of the couples HOCl/Cl−, H2O and HOBr/Br−, H2O are 1.29 and 1.13 V, respectively, at pH 7.0 (46). The weaker oxidant HOSCN (0.72 V for HOSSCN/SCN−, H2O at pH 7.0 (46)) was unable to oxidize APF. Hydrogen peroxide, having a reduction potential equal to 1.32 V for the couple H2O2/H2O at pH 7.0 (42), also failed to oxidize APF. This is a further indication that the amino group in APF is the primary target for HOCl and HOBr.

The performed fluorescence measurements with HOCl or HOBr showed that the fluorescence yield decreased upon application of higher HOX/APF ratios. In fact, control measurements with fluorescein revealed comparable dependences of the fluorescence maximum intensity and position as in the APF experiments. Both effects can be explained by aggregation of fluorescein (47–49). Moreover, fluorescein shows pH-dependent fluorescence properties due to changes in the ratio of the mono- and dianionic form (50, 51). Therefore, minor changes in the fluorescence intensity observed at a fixed fluorescein concentration in the presence of very high HOX concentrations may also be attributed to slightly higher pH values (50).

The formation of fluorescein upon APF oxidation by hypohalous acids was also proved by mass spectrometry measurements (18). Additionally, we addressed the question of whether the described effect in the APF-derived fluorescence can be solely explained by the properties of fluorescein or whether any chemical modifications of APF and/or fluorescein also take place. In fact, in the presence of HOCl or HOBr, mono- and dihalogenated fluorescein derivatives were observed. Most probably 4'- or 2'-halogen fluorescein and 4',5'- or 2',7'-dihalogen fluorescein are formed (52). These modifications may contribute to the lower fluorescence values observed in the presence of large amounts of HOX (52). In fact it is known that any halogenation of aromatic rings in fluorescein quenches the fluorescence due to an increased probability of intersystem crossing (53, 54). For brominated fluorescein (eosin Y) but not for chlorinated fluorescein, a significantly lower quantum yield as compared with fluorescein was reported (53). In both derivatives a bathochromic shift occurs in the excitation optimum, leading to lower fluorescence values upon excitation at 488 nm (53, 54). This is nicely reflected by our experiments with fluorescein and HOCl or HOBr (see supplemental Fig. 2).

Although in the MALDI measurements a bromination of fluorescein was observed after incubation of APF with equimolar amounts of HOBr, excess HOCl was necessary for a significant fluorescein-chlorination. This is well in line with the literature, as the reactivity of HOBr with aromatics is much higher as compared with HOCl (55, 56).

It has to be stated that for the detection of APF reaction products in the presence of HOBr, a higher laser intensity was needed as compared with the experiments with HOCl. In addition, in the presence of high amounts of HOBr, the general peak intensity strongly decreased. This may be attributed to a disturbed sample desorption under these conditions. In fact, the cationic surfactant cetyltrimethylammonium bromide used for matrix ion suppression in mass spectrometry with α-cyano-4-hydroxycinnamic acid was reported to strongly influence also the analyte signal intensity (57). It can be hypothesized that bromide ions, which are formed upon oxidation of APF by HOBr, may have similar effects.

Regarding the MALDI-TOF experiments with HPF, we observed a halogenation of the native molecule by HOX instead of the primary formation of fluorescein. Accordingly, in the presence of higher acid concentrations, halogenated fluorescein was formed. These data may suggest an additional explanation for the inadequate ability of HPF to detect hypohalous acids as compared with APF. In the experiments with isolated MPO and EPO, the fluorescence values obtained from HPF were about 3–10 times lower as compared with APF. Moreover, during the enzyme kinetics measurements the fluorescence maximum was reached much later, confirming that HPF is worse oxidized by HOCl/HOBr than APF.

Using APF, the measurements with the isolated enzymes showed the expected results. In the sole presence of either halide, MPO exhibited the fastest kinetics with chloride, whereas EPO more efficiently oxidized bromide. Thus the oxidation of APF by both HOCl and HOBr reflects well the substrate preference of both peroxidases under physiological conditions (58–60). Either of the enzymes displayed the fastest kinetics in the presence of both 140 mM chloride and 100 μM bromide, suggesting a kind of cooperative effect. Control experiments revealed the dependence of the APF oxidation on the peroxidase/H2O2/halide system. Thereby the commonly used
suicidal MPO inhibitor 4-ABAH (35) emerged also as a suitable inhibitor for EPO.

As approved by fluorescence measurements with MPO or EPO and H2O2 in the absence of chloride and bromide, only a very slow formation of minor fluorescein was observed. Therefore, a possible oxidation of APF in the peroxidase cycle either does not take place or does not interfere with the detection of halogenation products HOCl and HOBr by APF.

In conclusion, APF is able to detect both the HOCl formation by MPO and the HOBr formation by EPO. The lower fluorescence values obtained in the latter system reflect the slightly poorer ability of HOBr to oxidize APF, already observed in the nonenzymatic fluorescence measurements and the MALDI-TOF experiments.

We finally tested whether APF can also be applied for the peroxidase activity determination in granulocytes. In contrast to others, we used blood-derived native human cells instead of porcine cells or cell lines (22, 23). Although the usage of leukemic cell lines like HL-60 allows comprehensive and reproducible experiments, these cells show strong differences to native immune cells and are transferable to in vivo conditions only to a limited extent (3).

Both the MPO and EPO activity in human peripheral blood neutrophils and eosinophils, respectively, can be detected by APF staining and flow cytometry analysis. Thereby inhibitory studies confirmed that the oxidation of APF mainly depends on the peroxidases activity and not on the formation of peroxynitrite. A further proof for the peroxidase-specific oxidation of the dye is the bromide-dependent APF-derived fluorescence in eosinophils. The slightly enhanced APF-derived fluorescence observed with 1-NMMA regarding unstimulated cells may be due to metabolic responses to the iNOS inhibition. It cannot be excluded that the formation of hydroxyl radicals also contributes to a minor degree to the observed fluorescence signals.

In our experiments the stimulation of eosinophils always resulted in a more divergent, multipeaked fluorescence as compared with neutrophils. This may be a result of a stronger pre-stimulation of the former cells due to their longer isolation procedure. In addition, the strong excess of neutrophils during cell isolation may lead to the formation of specific eosinophil activators such as the arachidonic acid metabolite 5-oxo-eicosatetraenoate (61). Furthermore, eosinophils possess a larger quantity and, upon activation, a better translocation of the NADPH oxidase components as compared with neutrophils (62).

Control experiments with HPF also led to higher fluorescence values as compared with unstained cells, suggesting a significant uptake and a certain oxidation of the dye (23). Yet no significant effect of the peroxidase inhibitor 4-ABAH was observed. The reported small effect of DPI may be attributed to a minor oxidation of the dye by OH·. The peroxidase-independent higher fluorescence of HPF-stained cells may be attributed to the better uptake of this dye as compared with APF.

In contrast to other groups, in our cellular experiments we only used 1 nM PMA but a longer incubation time to achieve mild cell stimulation (22, 63). For moderately stimulated myeloid leukemic cells, MPO was already identified as a key mediator during the apoptosis by producing reactive oxygen species (23). In these experiments the polyphenol (−)-epigallocatechin-3-gallate was used as an apoptosis inducer (23). In vitro studies by our group revealed that (−)-epicatechin as well as related substances including (−)-epigallocatechin-3-gallate are strong MPO activators as they overcome the accumulation of compound II of the enzyme (64, 65). Thus, studies using APF to determine the halogenating activity of MPO in neutrophils confirmed in vivo our hypothesis on the importance of the enzymatic activity of heme peroxidases during termination of inflammation (66). The apoptosis of granulocytes is one important prerequisite during cellular completion of immunological reactions.

Besides APF/HPF, different other fluorescent probes have been tested to specifically detect the halogenation activity of MPO, including sulfonaphthoaminophenyl fluorescein, tetramethylrhodamine, and p-methoxyphenol-coupled boron dipyrromethene (67–69). However some of these dyes are only partially applicable to flow cytometry and/or are not commercially available. Other HOCl detection systems need rather high pH values and/or unphysiologic buffer additives and are, therefore, also not suitable to detect HOCl formation in living cells (16, 70–72).

Currently a new APF-derived fluorescent probe, namely the acetoxyethyl ester of aminophenoxycalcein, was tested (16). This probe has better cell loading properties and a reduced dye leakage compared with APF. Accordingly, it showed a higher sensitivity regarding the detection of OH·, ONOO−, and HOCl in HL-60 cells (16, 19). Still, in our experiments the detected APF-derived fluorescence intensity was sufficient for further analysis of peroxidase activity in human peripheral blood neutrophils and eosinophils.

Regarding the specific detection of HOCl, the substituted tetramethylfluorescein derivative HySO3 was successfully applied in phagosomes (16, 20). The rhodamine-based probe R19-S was also reported to specifically detect HOCl (21). Again both dyes, HySO3 and R19-S, have not yet been applied on HOBr. Currently we are testing the ability of the latter to detect the halogenating activity of eosinophils.

To sum up, in this paper it was shown for the first time that the fluorescein-based dye APF, used for the detection of the HOCl producing activity of MPO, can also be used to quantify the halogenating activity of eosinophils by detecting HOBr. This method may contribute to investigations regarding the role of eosinophil peroxidase in innate immunity, immune regulation, and eosinophil-derived pathologies.

REFERENCES
1. Rothenberg, M. E., and Hogan, S. P. (2006) The eosinophil. Annu. Rev. Immunol. 24, 147–174
2. Ilmarinen, P., Hasala, H., Sareila, O., Moilanen, E., and Kankaanranta, H. (2009) Bacterial DNA delays human eosinophil apoptosis. Palm. Pharmacol. Ther. 22, 167–176
3. Kankaanranta, H., Moilanen, E., and Zhang, X. (2005) Pharmacological regulation of human eosinophil apoptosis. Curr. Drug Targets Inflamm. Allergy 4, 433–445
4. Kankaanranta, H., Lindsay, M. A., Giembycz, M. A., Zhang, X., Moilanen, E., and Barnes, P. J. (2000) Delayed eosinophil apoptosis in asthma. J. Allergy Clin. Immunol. 106, 77–83
5. Ilmarinen-Salo, P., Moilanen, E., and Kankaanranta, H. (2010) Nitric oxide induces apoptosis in GM-CSF-treated eosinophils via caspase-6-dependent lamin and DNA fragmentation. Palm. Pharmacol. Ther. 23, 365–371
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6. Kankaanranta, H., Janka-Junttila, M., Imlarinen-Salo, P., Ito, K., Jalonen, U., Ito, M., Adcock, I. M., Mollanen, E., and Zhang, X. (2010) Histone deacetylase inhibitors induce apoptosis in human eosinophils and neutrophils. J. Inherited Metab. Dis. 7, 1–15

7. Chihara, J., Kurachi, D., Yamamoto, T., Yamada, H., Wada, T., Yasukawa, A., and Nakajima, S. (1995) A comparative study of eosinophil isolation by different procedures of CD16-negative depletion. Allergy 50, 11–14

8. Loughran, N. B., O’Connor, B., O’Fágáin, C., and O’Connell, M. J. (2008) The phylogeny of the mammalian heme peroxidases and the evolution of their diverse functions. BMC Evol. Biol. 8, 101–115

9. Spaltelholz, H., Panasenko, O. M., and Arnold, J. (2006) Formation of reactive halide species by myeloperoxidase and eosinophil peroxidase. Arch. Biochem. Biophys. 445, 225–234

10. Malle, E., Furtmüller, P. G., Sattler, W., and Obinger, C. (2007) Myeloperoxidase. A target for new drug development? Br. J. Pharmacol. 152, 838–854

11. Kooter, I. M., Moguilevsky, N., Bollen, A., van der Veen, L. A., Otto, C., Dekker, H. L., and Wever, R. (1999) The sulfonium ion linkage in myeloperoxidase. Direct spectroscopic detection by isotopic labeling and effect of mutation. J. Biol. Chem. 274, 26794–26802

12. Wever, R., Plat, H., and Hamers, M. N. (1981) Human eosinophil peroxidase. A novel isolation procedure, spectral properties, and chlorinating activity. FEBS Lett. 123, 327–331

13. Galajasevic, S., Saed, G. M., Hazen, S. L., and Abu-Soud, H. M. (2006) Myeloperoxidase metabolizes thiocyanate in a reaction driven by nitric oxide. Biochemistry 45, 1255–1262

14. van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J. (1997) Thiocyanate and chloride as competing substrates for myeloperoxidase. Biochem. J. 327, 487–492

15. Arnhold, J., Furtmüller, P. G., and Obinger, C. (2003) Redox properties of myeloperoxidase. Redox Rep. 8, 179–186

16. Nagano, T. (2009) Bioimaging probes for reactive oxygen species and active nitrogen species. J. Clin. Biochem. Nutr. 45, 111–124

17. Azadniv, M., Torres, A., Boscia, J., Speers, D. M., Frasier, L. M., Utell, M. J., and Frampton, M. W. (2001) Neutrophils in lung inflammation. Which oxidative stress-mediated apoptosis in myeloid leukemic cells. J. Biol. Chem. 276, 17877–17882

18. Fierro, I. M., Nascimento-DaSilva, V., Arruda, M. A., Freitas, M. S., Plotkowski, M. C., Cunha, F. Q., and Barja-Fidalgo, C. (1999) Induction of NOS in rat blood PMN in vivo and in vitro. Modulation by tyrosine kinase and involvement in bactericidal activity. J. Leukoc. Biol. 65, 508–514

19. Kettle, A. J., Gedey, C. A., and Winterbourn, C. C. (1997) Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. Biochem. J. 321, 503–508

20. Nagano, T., Kankaanranta, H., Shimazawa, M., Mishima, S., and Ha, E. M. (2003) Rational design principle for modulating fluorescence properties of myeloperoxidase-based probes by photoinduced electron transfer. J. Am. Chem. Soc. 125, 8666–8671

21. Persike, M., Zimmermann, M., Klein, J., and Karas, M. (2010) Quantitative determination of acetylcholine and choline in microdialysis samples by MALDI-TOF MS. Anal. Chem. 82, 922–929

22. Ueno, T., Ueno, Y., Satsukiina, K., Takakusa, H., Kojima, H., Kikuchi, K., and Obinger, C. (2007) Rational design of fluorescein-based fluorescence probes for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45–80

23. Setsukinai, K., Urano, Y., Hanaoka, K., Terai, T., and Nagano, T. (2009) A simple and effective strategy to increase the sensitivity of fluorescence probes in living cells. J. Am. Chem. Soc. 131, 10189–10200

24. Eno, T., Urano, Y., Satsukiina, K., Takakusa, H., Kojima, H., Kikuchi, K., and Obinger, C. (2004) Rational principles for modulating fluorescence properties of fluorescein-based probes by photoinduced electron transfer. J. Am. Chem. Soc. 126, 14079–14085

25. Koppelen, W. H. (1987) Thermodynamics of reactions involving oxiradicals and hydrogen peroxide. Bioelectrochem. Bioenerg. 18, 3–11

26. Merényi, G., and Lind, J. (1997) Thermodynamics of peroxyxinite and its CO2 adduct. Chem. Res. Toxicol. 10, 1216–1220

27. Senthilmohan, R., and Kettle, A. J. (2006) Bromination and chlorination reactions of myeloperoxidase at physiological concentrations of bromide and chloride. Arch. Biochem. Biophys. 445, 235–244

28. Arnhold, J., Monzani, E., Furtmüller, P. G., Zederbauer, M., Casella, L., and Obinger, C. (2006) Kinetics and thermodynamics of halide and nitrite oxidation by mammalian heme peroxidases. Eur. J. Inorg. Chem. 3801–3811

29. Doughty, M. J. (2010) pH-dependent spectral properties of sodium fluorescein ophthalmal solutions revisited. Ophthalmic Physiol. Opt. 30, 167–174

30. Schauenstein, K., Schauenstein, E., and Wick, G. (1978) Fluorescence properties of free and protein bound fluorescein dyes. I. Macrospectrofluorometric measurements. J. Histochem. Cytochem. 26, 277–283
49. Machwe, M. K. (1970) Effect of concentration on fluorescence spectrum of fluorescein. *Curr. Sci.* 18, 412–413

50. Sjöback, R., Nygren, I., and Kubista, M. (1995) Absorption and fluorescence properties of fluorescein. *Spectrochim. Acta A* 51, L7–L21

51. Romanchuk, K. G. (1982) Fluorescein: Physiochemical factors affecting its fluorescence. *Surv. Ophthalmol.* 26, 269–283

52. Hurst, J. K., Albrich, J. M., Green, T. R., Rosen, H., and Klebanoff, S. (1984) Myeloperoxidase-dependent fluorescein chlorination by stimulated neutrophils. *J. Biol. Chem.* 259, 4812–4821

53. Zhang, X. F., Zhang, L., and Liu, L. (2010) Photophysics of halogenated fluoresceins. *J. Fluoresc.* 18, 741–747

54. Ge, F. Y., and Chen, L. G. (2008) pH Fluorescent probes. Chlorinated fluoresceins. *J. Fluoresc.* 18, 492–498

55. Davies, M. J. (2011) Myeloperoxidase-derived oxidation. Mechanisms of biological damage and its prevention. *J. Clin. Biochem. Nutr.* 48, 8–19

56. Pattison, D. I., and Davies, M. J. (2006) Reactions of myeloperoxidase-derived oxidants with biological substrates. Gaining chemical insight into human inflammatory diseases. *Curr. Med. Chem.* 13, 3271–3290

57. Grant, D. C., and Helleur, R. J. (2007) Surfactant-mediated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of small molecules. *Rapid Commun. Mass Spectrom.* 21, 837–845

58. Furtmüller, P. G., Burner, U., and Obinger, C. (1998) Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. *Biochim. Biophys. Acta* 1392, 17923–17930

59. Furtmüller, P. G., Burner, U., and Obinger, C. (1998) Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. *Biochim. Biophys. Acta* 1392, 17923–17930

60. Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D., and Regiani, S. (1986) Brominating oxidants generated by human eosinophils. *Science* 234, 200–203

61. O’Flaherty, J. T., Kuroki, M., Nixon, A. B., Wijkander, J., Yee, E., Lee, S. L., Smitherman, P. K., Wykle, R. L., and Daniel, L. W. (1996) 5-Oxo-eicosatetraenoate is a broadly active, eosinophil-selective stimulus for human granulocytes. *J. Immunol.* 157, 336–342

62. Someya, A., Nishijima, K., Nuno, H., Irie, S., and Nagaoka, I. (1997) Study on the superoxide-producing enzyme of eosinophils and neutrophils. Comparison of the NADPH oxidase components. *Arch. Biochem. Biophys.* 345, 207–213

63. Franch, T., Kohnen, S., de la Rebière, G., Deby-Dupont, G., Deby, C., Niesten, A., and Serteyn, D. (2009) Activation of equine neutrophils by phorbol myristate acetate or N-formyl-methionyl-leucyl-phenylalanine induces a different response in reactive oxygen species production and release of active myeloperoxidase. *Vet. Immunol. Immunopathol.* 130, 243–250

64. Kirchner, T., Flemmig, J., Furtmüller, P. G., Obinger, C., and Arnhold, J. (2010) (−)-Epicatechin enhances the chlorinating activity of human myeloperoxidase. *Arch. Biochem. Biophys.* 495, 21–27

65. Spalteholz, H., Furtmüller, P. G., Jakopitsch, C., Obinger, C., Schewe, T., Sies, H., and Arnhold, J. (2008) Kinetic evidence for rapid oxidation of (−)-epicatechin by human myeloperoxidase. *Biochem. Biophys. Res. Commun.* 371, 810–813

66. Arnhold, J., and Flemmig, J. (2010) Human myeloperoxidase in innate and acquired immunity. *Arch. Biochem. Biophys.* 500, 92–106

67. Kenmoku, S., Urano, Y., Kojima, H., and Nagano, T. (2007) Development of a highly specific rhodamine-based fluorescence probe for hypochlorous acid and its application to real-time imaging of phagocytosis. *J. Am. Chem. Soc.* 129, 7313–7318

68. Shepherd, J., Hilderbrand, S. A., Waterman, P., Heinecke, J. W., Weissleder, R., and Libby, P. (2007) A fluorescent probe for the detection of myeloperoxidase activity in atherosclerosis-associated macrophages. *Chem. Biol.* 14, 1221–1231

69. Sun, Z.-N., Liu, F.-Q., Chen, Y., Tam, P. K. H., and Yang, D. (2008) A highly specific BODIPY-based fluorescent probe for the detection of hypochlorous acid. *J. Am. Chem. Soc.* 130, 7313–7318

70. Lou, X., Zhang, Y., Li, Q., Qin, J., and Li, Z. (2011) A highly specific rhodamine-based colorimetric probe for hypochlorites. A new sensing strategy for the detection of hypochlorous acid. *Chem. Commun.* 47, 3189–3191

71. Chen, X., Wang, X., Wang, S., Shi, W., Wang, K., and Ma, H. (2008) A highly selective and sensitive fluorescence probe for the hypochlorite anion. *Chem. Commun.* 14, 4719–4724

72. Yang, Y. K., Cho, H. J., Lee, J., Shin, I., and Tae, J. (2009) A rhodamine-hydroxamic acid-based fluorescent probe for hypochlorous acid and its application to biological imagings. *Org. Lett.* 11, 859–861